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Pesticides

The Impacts of Pesticides Exposure

Edited by Margarita Stoytcheva

PESTICIDES - THE IMPACTS OF PESTICIDE EXPOSURE

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Notice

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Meet the editor

Margarita Stoytcheva is born in Bulgaria. She graduated from the University of Chemical Technology and Metallurgy of Sofia, Bulgaria, with titles of Chemical Engineer and Master of Electrochemical Technologies. She has Ph.D. and D.Sc. degrees in chemistry and technical sciences. She has acted in research and teaching in several Universities in Bulgaria, Algeria and France. From 2006 to the present she has participated in activities of scientific research, technological development and teaching in Mexico at the University of Baja California (UABC), Institute of Engineering, Mexicali, as a full time researcher. She has given courses of general chemistry, analytical chemistry and physical chemistry. Her interests and areas of research are biosensors, electrochemistry and analytical chemistry. Her publications, including articles, books and professional congress proceedings (altogether 150), have been cited more than 300 times in the scientific literature. She has been a member of the following scientific organizations: New York Academy of Sciences, Association of Scientists (Bulgaria) and Association of Chemists (Bulgaria). Since 2008 she has been a member of the National System of Researchers (SNI) of Mexico.

Contents

Preface

> "Then a strange blight crept over the area and everything began to change. Some evil spell had settled on the community: mysterious maladies swept the flocks of chickens; the cattle and sheep sickened and died. The farmers spoke of much illness among their families."
>
> Rachel Carson
> "Silent Spring"

The World Health Organization and the UN Environment Program report that yearly 3 millions of agricultural workers in the developing world experience acute poisoning from pesticides and about 18000 die. This book is dedicated to the various aspects of pesticides exposure and its adverse effects in humans and the environment.

Chapter 1 furnishes numerous statistic data on pesticides pollution in farming environments and the caused adverse health effects. The invisibility of pesticides external costs to farm families of regular pesticides-related ill health is extensively commented in Chapter 2 and illustrated by case studies on endosulfan exposure in West Africa. Chapter 3 provides an overview of the dietary intake of environmentally persistent pesticides by the European population and the potential adverse consequences of this exposure to human health. The available environmental mixture toxicity assessment methods are summarized in Chapter 4.

The assessment of the exposure of the agricultural population to organophosphorus and carbamates pesticides in Putumayo, Colombia is the objective of Chapter 5. Chapter 6 comments on the association between pesticide exposure and Parkinson's disease and reviews the status of the currently available pesticide-induced animal models of the disease. Chapter 7 is a study highlighting the role of the pesticides and other endocrine disruptors' exposure in the increased rates of hypospadias. Chapter 8 demonstrates the prevailing situation of excessive pesticides exposure and its health effects on different systems in the agricultural and industrial workers of developing countries. Chapter 9 introduces the Health Risk Analyses as a complementary methodology to microbial studies for evaluating the health risks caused by chlorinated pesticides in water bodies used for recreational bathing in Argentina. The determination of the concentration of polyaromatic hydrocarbons and pesticides in oysters from Caloosahatchee estuary, Florida, as an indicator of the water quality and environmental health is the objective of Chapter 10.

The results of the investigation on the possible relation between the neurotoxic pesticides exposure and the occurrence of congenital and acquired diseases are presented in Chapter 11. The mechanisms of organophosphorus pesticides toxicity are discussed in details in Chapter 12. The toxic effects of a range of organophosphorus pesticides and their degradation products are quantitatively evaluated using Daphnia Magna, Lepidium Sativum and Vibrio Fischeri as test organisms. Chapter 13 summarizes the results obtained.

Chapter 14 gives details demonstrating the efficacy of the Fluorescent in Situ Hybridization Technique (FISH) in risk assessment due to pesticides exposure, particularly in the evaluation of their carcinogenic potential.

The aim of Chapter 15 is to evaluate the genotoxic effect produced by pesticides mixtures on workers occupationally exposed in Mexico states: Morelos, Sinaloa and Guerrero.

Chapter 16 demonstrates the relevance of the models applied in this study: CGCs and cortical neuronal primary cultures and gene expression, and neuronal electrical activity measurements for neurodevelopment toxicity evaluation induced by pesticides.

Chapter 17 estimates and discusses the factors contributing to the generation of oxidative and genotoxic damages in rural population exposed to pesticides in the area of Santa Fe, Argentina. The basic methods and the interpretation of data for monitoring of in vivo oxidative and genotoxic stress of various pesticides on DNA structure are reported in Chapter 18. The spectroscopic, voltammetric and molecular modeling techniques which are routine for in vitro studies of pesticides-DNA interactions are described, too.

Chapter 19 revises and explains the contribution of the molecular modeling to the knowledge of pesticides. Selected reports cover various techniques: from quantum and semi-empirical calculations to QSAR/QSPR models.

The book provides significant information on the diverse impacts of the pesticides exposure, discussing basic concepts and case studies collected from around the world. The multi-faceted approach and the multi-authored character of the edition enrich it and make it compelling and accessible to a wide range of specialists interested in pesticide issues.

Special thanks are extended to all chapter authors for their contribution to the volume, sharing their time and expertise.

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1

Pesticide Pollution, Resistance and Health Hazards

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1. Introduction

The origin and concept, known exactly, of pesticides is not known. Pesticides or insecticides (insect killers) are among the most extensively used chemicals in the world today and they are also among the most hazardous compounds to the human being as well. Though, some pesticides can be beneficial in decreasing the populations of harmful or destructive insects, while others can be damaging to the environment and can cause serious disturbances. In this connection, studies have shown that pesticides can be extremely unsafe, particularly when they run off into water ways or if used indiscriminately can cause both short term and long term damage to the people and the environment. Humans can also be adversely affected by pesticides and this can cause many people to change their life style according to the situation. Synthetic pesticides are behind many people's decision to switch to organic products and practices, especially where diet is concerned. The number of people demanding pesticide – free organic food has increased sharply in recent years as more information has been uncovered about the health risks associated with pesticides. Additionally, some pesticides have a resistance to breaking down over time, which means that their effects can continue over a long period of time.

All pesticides may be effective against the pests, when they are used for control. They must be biologically active or toxic. As pesticides are toxic by nature, they are also potentially hazardous to human, animals, other organisms as well as the environment. Therefore, people who use pesticides or regularly come in contact with them must understand the relative toxicity and preventive measures to reduce exposure to the products they use. A report from World Health Organization (WHO) indicated that over 200,000 people are killed due to the toxicity of these dangerous chemicals every year. The casualty figure in fact do not confirm the real picture of poisoning caused by the frequent use of pesticides but about over three million of poisoning cases have been reported annually. Exposure to these pesticides or hazardous chemicals therefore leads to several health problems such as asthma attacks, skin rashes as well as chronic disorders like emphysema and cancer. Therefore, what steps or needs to be done immediately is to reduce the adverse health effects caused by the pesticides and if they are found to be dangerous beyond a maximum level, restrictions should be imposed on their use as well as exposure to human health. Some organochlorine pesticides have been banned in this connection. (e.g. DDT, dieldirin, endrin etc.).

2. Important

It is important to note that pesticides and pollution are linked together. Pesticides are a cause of pollution affecting land and water in particular. Water pollution is one of the leading cause of death. At present, only a small portion of waste water is treated. The rest is discharged into our water bodies. Due to this pollutants enter in ground water, rivers and other water bodies. Such water which ultimately ends up in our households is often highly contaminated and produces diseases. Agricultural run off or the water from the fields that drains into rivers is another major pollutant as it contains pesticides and fertilizers. Pesticides when used properly are of tremendous benefits to human beings but their indiscriminate use however may cause considerable hazards to health and environment.

Pointing out the effects of pesticides on human body, there is a wide range of health problem caused by the continuous exposure of pesticides like still births, neonatal deaths, congenital birth defects, paralysis, depressed respiration, cardiovascular dysfunctions, cancer and tumor etc. Problems with the use of pesticides are usually worse in developing countries where many products of the WHO category I are still in use. As these products are highly toxic in nature (WHO, 1962), their continuous exposure ultimately result in poisoning.

Keeping the environmental and health problem in priority, there is a strong public pressure to reduce their use as they are costly and causing various problems e.g., pesticide pollution, resistance in pests and accumulation of residues of pesticides in the body of animals and human beings.

On this basis, the developed countries are preferring the use of phytopesticides and hormonal pesticides under IPM program to avoid any risk to human health. In this chapter, discussion is focused on the environmental impact of pesticide exposure particularly in relation to pollution, resistance and hazardous effects on human health. Through this work, we strongly recommend suggestions to the pesticide users and regulatory bodies in providing extensive awareness programs for the safe use of pesticides before spraying and handling. Furthermore, effective health monitoring policy should be taken into consideration with in the community for minimizing human exposure to pesticides.

3. Information

Pesticides

Any substance or mixture of substances intended for preventing, destroying, repelling or mitigating any insects, rodents, nematodes, fungi or weeds or any other forms of life declared to be pests are called pesticides. Pests are those organisms which harm us and our belongings. Pesticides are used globally and extensively for the control of pests. Plant growth regulators, which are used to influence particular growth process in plants, are also regulated as pesticides.

Classification of pesticides

a. Pesticides is a general term which may be classified according to the pests e.g.,
   - Insecticides which kill or destroy insects
   - Acaricides which kill ticksand mites cause seabiasis (Acarines)
   - Rodenticides which kill rodents (Rats etc)
   - Fungicides which kill fungi
   - Algaecides which kill algae

- Weedicides which kill weeds
- Bactericides which kill bacteria
- Molluscicides which control slugs or snails
- Nematicides which control nematodes
- Virucides which control viruses

b. Pesticides can also be grouped on the basis synthetic organic chemical compounds.

i. *Chlorirated hydrocarbons* (*OC*):

They include DDT, BHC (Lindane), Heptachlor, Aldrin, Dieldrin, Endrin etc. In addition PCB's are also hydrocarbons but not used as insecticides. This group came in general use in 1940. It is persistent and have long half life (i.e. 15-30 years). Some of them are very toxic e.g. endrin (LD_{50} 10 - 15 mg / kg).

ii. *Chlorophenoxy acids* (*2,4-D*):

They are being used since 1930 as herbicides and include 2, 4, dichlorophenoxy acetic acid. They are toxic for plants especially broad leaf plants or weeds and mimic plant hormones. They are less toxic to animals but may produce eye inritation and GIT disorder. They are degraded in about two weeks but regular exposure may be teratogenic in animals.

iii. *Organo-phosphates (OP)*:

Organo-phosphates came in general use as pesticides in 1950 as a result of the development of resistance against OC, DDT etc. They are more toxic to mammals but they are degraded in 2-4 weeks. The common insecticides are malathion (comparatively more safe for mammals), parathion, methyl parathion, dimecron, diazinon, dimethoate, chlorpyriphos, DDVP or dichlorovos, TTEP etc. They are compound of phosphorus and sulphur. Basically, they are acetylcholine esterase inhibitors. Among them TEPP is the most toxic i.e. LD_{50} 1 mg / kg while malathion is least toxic i.e. LD_{50} 1500 mg / kg.

In Pakistan they were being frequently used (50%) for plant protection till 1991. Some of the components of this series are used as nerve gases for military purpose e.g. Agents GB

They have shorter half life and quickly degrade, but their first metabolite is more toxic e.g. paraoxon, malaoxon.

iv. *Carbamates (CB)*:

They are esters of carbonic acid and first developed in Nigeria by U.K. scientists. They include propoxur (baygon), carbaryl (sevin), Temik and Zectran. They are also less peristant and degrade in about 4 weeks. They are also acetylcholinesterase inhibitors. Some are less toxic while some are more toxic e.g. propoxur LD_{50} 30 mg / kg.

v. *Synthetic pyrethroids* (*SP*):

They are being mostly used these days because they are less toxic for mammals but sufficiently toxic for insects. They also inhibit cholinesterase. They have been developed on the basis of natural pyrethroids (pyrethrins I-IV) found in chrysanthemum flower. The natural pyrethrins have immediate knockdown effect but are less toxic and less persistant. The synthetic pyrethroids have more toxic and persistant effect. The examples of these are permethnin, bioallethrin, resmethrin, cypermetrhin etc. Synthetic pyrethroids also have a half life of 2 – 4 weeks. They are also general esterase inhibitors and have effects on other enzymes as well.

Different groups of pesticides remained popular in different periods. In early days inorganic and plant extracts were popular, but in 40's 50's organochlorine (OC) became popular especially DDT was reported as **"Wonder drug"** during 2nd World War. For this great work

of achievement Muller was awarded a noble prize. In 60's and 70's Organophosphates (OP) and Carbamates (CB) became popular and OP are still being used. However, in 80's synthetic pyrethroids (SP) dominated the sense and still the maximum import and use of pyrethroids is being done by government and private sectors because pyrethroids are comparatively safer and more effective as well. It is belived that in the next century, plant products will dominate due to their safe use and less polluting effect. Moreover, they also remain active physiologically by nature.

At present following pesticides are in major use:

Cypermethrin, Permethrin, Bioallithrin, Fenvalerate, Methamidophos, Monocrotophos, Sumithion, Dimethoate, Endosulphan, Triazophos, Acephate etc.

Mode of Action

Pesticides are basically poisons and therefore toxic to living organism at particular dose. They are inhibitors of enzymes and disturb the normal biochemical reactions necessary for metabolism. Some of them are neurotoxins while others are cytotoxins. They affect the action of enzymes in the body. However, the body has the ability to degrade and detoxify them by the help of various enzymes for example:

a. Organochlorines:
   Organochlorines (OC) are degraded by dehydrochlorinases.
b. Organophosphates:
   Organophosphates (OP) are degraded by general esterases such as cholinesterases (ChEase), Phosphomonoesterases (ACP, AKP), Carboxylesterases and Oxidases.
c. Carbamates:
d. Carbamates (CB) and synthetic pyrethroids (SP) are also degraded by the above enzymes. Most of them are cholinesterase inhibitors that disturb the nerve transmission system. However, the body has the power to synthesize more enzymes and degrade these poisons by increasing the enzyme levels such as the development of resistance in case of pests.

A. Pesticide Pollution:

Although pesticides are intended to harm only the target pest, if not used correctly, they can also harm to the people or the environment. Pesticide intoxication may be caused either by swallowing accidently, or by inhalation of fumes or by skin contact or accidental eye exposure.

In case of eye exposure through washing, it is necessary to wash the eyes with running water for at least 15 minutes. If there is still burning effect use eye drops or rose water several times. In severe cases consult eye specialist and a toxicologist.

In case of skin contact, immediately remove the clothing and wash the effected clothings in washing machines separetly, If there is still some effect consult skin specialist and toxicologist.

In case of Inhalation take the patient to open and uncontaminated area. During difficulty in breathing, use proper respiratory equipment and if there is severe difficulty in breathing use oxygen. Do not leave unconscious person unattended. Consult a physician and toxicologist for proper guidance.

In case of swallowing, immediately induce vomiting. (never induce vomiting to an unconscious person). After vomiting advice the patient to take milk, butter and egg, so that pesticide may bind with them and excreted. Consult a physician and toxicologist for advice.

Some pesticides evaporate more easily and quickly than others so these pesticides are more likely to be inhaled. Some pesticides degrade more quickly on surfaces where as others last longer. Therefore, the more a person is exposed to a particular pesticide or substance the greater is the risk or chance of harm. So, the degree of harm depends on the chemical nature of chemical compounds, the situation of environment and the person life style. This means that very small amounts of pesticides or even the most toxic material may either cause nothing or can cause little harm to the person. On the other hand less toxic materials taken in large amount can cause greater harm.

Assessment of human exposure with pesticides

Basically pesticides are potentially dangerous and harmful to human and other living organisms. They not only pollute the environment but also produce pathological effects in various organs. In human beings, the pesticide residual level is an index of exposure. Presence of different pesticide residual level in the blood of human being clearly indicate that up to which extent or degree to which the person is exposed and this has been done through high performance liquid chromatographic (HPLC) technique and gas chromatography (GC) to quantify the level of pesticide residues in their blood using chromatogram. The incidence of exposure to pesticides may either be acute, occupational or incidental. In general population the residual level is a measure of the incidental exposure and the levels of persistent pesticides remain in the soil and tissues for year together and their bioaccumulation takes place via food chain. Unfortunately, the body has the ability to biotransformation and excretes part of these compounds in urine, fecal matter, bile and air. However when the rate of absorption and deposition in fat exceeds to the rate of elimination, their higher concentration results in toxic and pathological effects.

In view of this, in the developed countries EPA keeps a constant watch on the pesticidal pollution by regular check up and do not hesitate in destroying the large food stocks. Extensive literature in these countries are available on this issue (Heath 1961; Cohen and Oostarbaan 1963; O'Brien 1967, 1976; Casarette *et al*. 1968; Krauthacker *et al*. 1980; Mercedes and Theil 1986; Anna *et al*. 1988; Alawi *et al.* 1992; Cantor *et al.* 1992; Ferrer *et al.* 1992; Matuo *et al*. 1993; Saddy *et al*. 1993; Swaen *et al*. 1994). However, in Pakistan little work has been done by Mughal and Rehman 1973; Naqvi and Jahan, 1996. Some work has also been done by Pakistan Agriculture Research Center (PARC) but mostly on crops. Therefore there is urgent need of investigating pesticide residues in human blood and their pathological effects.

Pesticide Residues

Pesticide residues are very small amounts of pesticides that can remain in the blood, tissues or on a crop for years and their bioaccumulation takes place via food chain. Not all foods contain pesticide residues and whenever they occur they are found typically at low levels. Pesticide residues also include any breakdown products from the pesticides.

Pesticide residues in human blood

Studies have been done extensively all over the world pertaining to the presence of pesticides in human biological material. In this connection several reports have been published by Krauthacker *et al*. 1980; Saxena *et al*. 1980; Mercedes and Thiel 1986; Sabbah *et al*. 1987; Krawinkel *et al. 1989;* Greve and Zoonen 1990; Chikuni *et al*. 1991; and Kanja *et al*. 1992. However, this type of information in Pakistan is scanty and the only report available

in early 70's is that of Mughal and Rahman (1973), Therefore, a study was conducted by Naqvi and Jahan, (1995) in which thirty random samples of blood were taken for pesticide determination from three different laboratories of Karachi. Almost all the samples reported here were found contaminated with organochlorine (OC) compounds. However, samples obtained from two centres were found to have more pesticides. Workers deputed to chlorination plants (i.e., third centre) have greater quantity of tenakil and other OC compounds. Among all the samples analyzed, only a few were found to have deltamethrin (pyrethroids) and malathion (organophosphate) possibly because of fresh exposure. The detail has been given in tables 1, 2 and 3 for information.

Samples	DDT	DDE	Dieldrin	Aldrin	Deltamethrin	Malathion
1.	-	-	0.033	-	-	-
2.	-	0.022	-	-	-	2.0
3.	-	0.423	-	-	-	-
4.	-	-	0.02	-	-	2.0
5.	-	0.004	-	-	-	30.0*
6.	-	-	-	-	-	4.0
7.	-	0.008	-	18.0*	-	-
8.	0.006	0.008	-	-	-	-
9.	-	0.008	-	-	-	0.2
10.	-	-	0.035	-	-	-

Table 1. Contents of pesticides (µg / ml) in sera samples obtained from Aziz Laboratory, Karachi. (*Above maximum residue limit).

In this centre, samples of blood were taken from persons whose age varied from 6 to 36 years. Only two pesticides viz., malathion (30.0 µg / ml) and Aldrin (18.0 µg /ml) were found in highest concentration. Sample 9 of this centre indicates the presence of one OC and one OP compound. However, sample 10 showed a very peculiar case. This sample was obtained from a girl who was only 6 years old but the serum sample had 0.035 µg / ml of DDE. It is possible that she received this parent compound of DDE i.e., DDT through her mother which then degraded to DDE. This is in accordance with the reports of Chikuni *et al*. (1991) and Kanja *et al.* (1992). Therefore, it is clear from the data summarized in these tables

Samples	DDT	DDE	Aldrin		Deltamethrin
			P1	P2	
1.	0.026	0.57	36.0	0.131	12.22*
2.	0.063	0.627	24.7*	0.019*	3.40
3.	-	0.640	-	-	-
4.	-	0.373	10.0*	0.057	2.23
5.	-	0.113	-	-	-
6.	-	0.069	-	-	-
7.	0.004	0.069	-	-	-
8.	0.009	-	-	-	-
9.	0.008	-	-	-	-
10.	-	-	-	-	1.03

Table 2. Content of pesticides (µg / ml) in sera samples from Fatimid Foundation (*Above maximum residue limit).

Samples	DDT	DDE	Dieldrin	Tenakil
1.	-	-	0.09	1.336
2.	-	-	0.23	4.456*
3.	0.005	0.028	-	7.278*
4.	-	-	0.09	1.336
5.	-	-	0.23	1.336
6.	-	-	0.27	1.188
7.	-	-	0.08	7.278*
8.	-	-	0.51	13.369
9.	0.004	-	0.51	43.673*
10.	-	-	5.73*	11.580*

Table 3. Contents of pesticides (µg / ml) in sera samples from chlorination plant of PCSIR (*Blood sample taken with in an hour after exposure to tenakil).

that dieldrin and DDE were the main pollutants in all the anaylyzed samples (Table 2 and Table 3). However, tenakil and dieldrin were indentified in higher amounts in persons at the chlorimation plant and Aziz laboratory.

The same type of study was also conducted by Azmi and Naqvi (2005) in which blood samples were collected from the persons (especially farm workers and spray man) engaged in different fruit and vegetable farm stations located at Gadap (rural area) Karachi – Pakistan. Total 287 blood samples were taken from exposed and control persons and 81 samples were taken for residual analysis by HPLC and 55 samples were found positive. Analysis of all the samples by HPLC revealed the presence of Polytrin – C only in one sample, while deltamethrin and diazinon were detected in 4 and 7 samples, respectively. However, the frequently detected pesticides were cypermethrin, monocrotophos and DDE (one of the metabolite of DDT). Therefore, it is concluded that 29.6% cases of organophosphates, 29.6% cases of organochlorines and 48.1% cases of pyrethriods were detected.

We are presenting here the data related to the pesticide residues only of such persons from Gadap (rural area) of Karachi and different regions of Sindh province of Pakistan who are highly exposed with one or more pesticides. The total number of samples (city wise in asterisk superscript) analyzed from each centre and the highest / lowest value are given in the Table 4 (as the data is quite extensive) for information.

Thus, the data presented regarding the detection of pesticide residues in the blood samples of the farm workers and general population belonging to different cities of Sindh divisions of Pakistan clearly indicate that the persons engaged in the field work have greater quantity of pesticides such as cypermethrin, deltamethrin profenofos, diazinon, monocrotphos, DDT, DDE and permethrin because of greater exposure of these pesticides during spraying time in these areas. In this connection highest quantity of pesticides such as 12.22 mg / ml by Jahan (1995) and Bissacot and Vassilieff (1997-b) was reported in case of deltamethrin; 100 – 200 mg / ml by Abu-Qare and Abu-Donia (2001-a), 80 - 200 mg / ml by Abu-Qare and Abu-Donia (2001-b); slightly high value 0.025 – 5.0 µg / ml by Musshoff *et al*. (2002) in case of diazinon. In case of monocrotophos; slightly high level 0.025 – 50 mg / g by Musshoff *et al*. (2002) 0.27 mg / ml by Kocan *et al*. (1994), 0.9 mg / L by Guardino *et al*. (1996), 4.71 mg / L and 38.13 mg / L by Dua *et al*. (1996), 1.9 ppb by Luo *et al*. (1997), 0.78 mg / kg by Waliszewski *et al*. (2000) was reported in the blood samples. Low level of DDT 0.22 mg / L, 0.25 mg / L and 0.30 mg / L was also reported by Heudorf *et al*. (2003). In case of DDE, high

City	Pesticide Residues (µg / ml)									
	Cyper	Delta	Polytrin-C		Dia	Mono	DDT	DDE	Mal	Perm
			Cyper	Prof						
Gadap[81] Karachi	31* ↑32.40	4* ↑40.00	-	1* ↑65.50	9* ↑28.80	16* ↑43.50	12* ↑21.60	13* ↑42.50	-	-
Thatta[17]	4* ↑10.53	5* ↑5.49	-	-	-	-	1* ↑2.45	1* ↑1.71	-	1* ↑ 3.58
Hyderabad[7]	2* ↑5.61	2* ↑11.12	-	↑16.51	-	-	-	-	-	2* ↑ 8.82
Rohri[3]	-	-	-	-	-	-	-	-	-	1* ↑ 6.66
Shadadpur[3]	-	1* ↑ 3.52	-	-	-	-	-	-	-	-
Tando M. Khan[3]	1* ↑ 9.36	-	-	-	-	-	-	-	-	-
Sukkur[4]	-	1* ↑ 4.39	-	-	-	-	-	-	-	1* ↑24.05
Dadu[4]	1* ↓ 2.65	-	-	-	-	-	-	1* ↓ 1.10	-	-
Nawab Shah[2]	-	-	-	-	-	-	-	-	-	1* ↑ 3.94
Larkana[3]	-	2* ↑22.42 ↓0.34	-	1* ↑30.76	-	-	-	-	-	2* ↑ 6.15 ↑ 4.01
Sanghar[4]	1* ↑ 11.17	1* ↑ 3.98	-	-	-	-	-	1* ↑ 3.06	-	-

Superscript number in cities = Total No. of Samples, Cyper = Cypermethrin, Delta = Deltamethrin
*number = Number of detection of pesticides, Prof = Profenofos, Mala = Malathion, Perm = Permethrin, Dia = Diazinon, Mono = Monoerotophos, ↑ = Highest value, ↓ = Low value, - = Not detecrted.

Table 4. Quantitative analysis of pesticide residues in the blood of effected persons from different arm stations of Gadap (rural area).

quantity 8.0 mg / L by Guardina *et al*. (1996), 9.10 mg / ml by Rubin *et al*. (2001) and 380 mg / kg by Ntow *et al*. (2001) was reported in the exposed persons. Residues of DDE were also detected such as 5.2, 6.2 and 2.5 ng / g by Ahmed *et al*. (2002), 3.99 mg / ml and 1.42 mg / ml by Butler *et al*. (2003). High level of DDE was also reported by Van Ooastdam *et al*. (2004).

Pesticide residues in glandular tissue

Thyroid gland tissues were tested for the presence of pesticides (i.e., DDT, DDE, aldrin, dieldrin, malathion and deltamethrin). This study was based on the glandular tissue samples that were collected from Jinnah Postgraduate Medical Centre (JPMC) during surgical operations. The data collected from this centre for the detection of pesticide residue is summarized as below:

Sample (20)	Pesticide residues (μg / gm)					
	DDT	DDE	Dieldrin	Aldrin	Malathion	Deltamethrin
I	0.011	-	-	-	-	-
II	0.044	-	-	20.0	-	-

Table 5. Content of pesticide residues in glandular tissue samples from Jinnah Hospital, Karachi-Pakistan.

The table indicates the presence of DDT and Aldrin only in two positive samples out of total 20 samples. The amount of DDT detected in both samples is much less than MPL (Maximum Residual Limit). However, the amount of Aldrin is more than MRL which seems to be dangerous for the health point of view because the organochlorine pesticides are lipophilic. If thyroid gland can accumulate this much quantity of aldrin, then large quantity of this pesticide might have been deposited in the fatty tissues of that patient. In case of prolonged illness the fatty tissue may release lethal dose of aldrin (LD_{50} for mammals is 10 mg / kg). So, there is a probable risk for this patient. Moreover effect of pesticides on hormones and endocrine glands (adrenal cortex) have been reported by Vilar and Tullner (1959); Kuservitsky *et al*. (1970) on thyroid gland and by De Sola *et al*. (1998); Jarrer *et al*. (1998); You *et al*. (1998) and Padungtod *et al*. (1998) on hormones. Effect of dieldrin (Cyclodiene) has been reported by Wakeling *et al*. (1972) on 5-dihydrotestosterone binding with specific protein receptors, as 33% inhibition. This indicates that pesticides affect the hormones and endocrine glands.

Pesticide residues in fruits and vegetables

Pesticide residues were also detected in fruits and vegetables samples collected from Karachi markets. Only 45 commodities were tested (out of 145 samples) for residual analysis. The data obtained are given as under:

Karachi Fruit Market		Karachi Vegetable Market	
Fruits	**Pesticides**	**Vegetables**	**Pesticides**
Mango Peel	Heptachlos P'P' DDT	Cauliflower	Alpha-BHC Aldrin
Peach Peel	Heptachlor	Potato	gamma-BHC Heptachlor
Pear	P'P' DDT	Lady Fingers	Heptachlor Aldrin
Banana Peel	Beta-BHC	Cucumber	Heptachlor
Orange Peel	Alpha-BHC	Turnip	gamma-BHC
Blackberry	gamma-BHC Heptachlor	Lady Fingers	gamma-BHC Aldrin

Table 6. Organochlorine pesticide residues in fruits and vegetables of Karachi market.

The table indicates that mostly the fruits and vegetables contain organochlorine pesticide residues such as Heptachlor, BHC and DDT which is quite dangerous and alarming for the health of general people as these food items are the basic need of human being. It is therefore, suggested that the fuits and vegetables used by human should be washed thoroughly for any hazardous incident.

Pesticide residues in tissues, fresh water lakes and fishes

Data on pesticide residues related to fresh water lakes of Sindh and the fishes present in these lakes as well as different tissues are representing in tables.

Sample	Pesticide residues (µg / g)				
	Dimethoate	**DDT**	**DDE**	**Cypermethrin**	**Dieldrin**
Water	ND	ND	ND	ND	ND
Fat	4.06 ± 0.67	2.38 ± 0.26	1.54 ± 0.19	0.2 ± 0.01	0.12 ± 0.12
Muscles	0.12 ± 0.66	0.68 ± 0.29	0.56 ± 0.26	ND	ND
Liver	0.01 ± 0.01	0.28 ± 0.17	0.56 ± 0.19	ND	ND

Table 7. Content of pesticide residues in lake water, fat, muscles and liver samples from *Egretta* sp. found in Kalri lake. (ND = Not detected).

Sample	Pesticide residues (µg / g)				
	Dimethoate	**DDT**	**DDE**	**Cypermethrin**	**Dieldrin**
Water	2.2 ± 0.18	2.8 ± 0.65	4.4 ± 0.76**	2.1 ± 0.17	0.05 ± 0.03
Fat	17.44 ± 1.49**	7.62 ± 0.48*	73.3 ± 0.16***	7.08 ± 0.16*	6.92 ± 0.59*
Muscles	11.48 ± 1.92**	5.56 ± 0.81*	31.88 ± 3.63***	5.34 ± 0.38*	1.24 ± 0.08
Liver	2.28 ± 0.51	2.7 ± 0.51	6.86 ± 0.36*	5.52 ± 0.24*	0.41 ± 0.09

Table 8. Content of pesticide residues in lake water, fat, muscles and liver samples from *Egretta* sp., found in Haleji lake. (ND = Not detected)

The present data indicates that in the birds of Kalri lake only the fat samples contain dimethoate to some extent. This may be due to the constant use of this pesticide by the farmers and that too by bioaccumulation. The water samples however from this lake had no pesticide in detectable quantity. In Haleji lake, the water samples had DDE in slightly high quantity. However, the birds had high level of dimethoate, very high level of DDE and slightly high level of DDT, cypermethrin and dieldrin. Although, use of DDT has been banned long ago, but is very persistent and metabolized slowly this is evident from the very high level of its metabolite DDE. In muscles also the high level of DDE was found which supports the findings found in fat samples. In case of muscles, DDT and cypermethrin was found in slightly high quantity. This means that birds bioaccumulate pesticides in fats and muscles which is dangerous for human beings, as they reach via food chain.

Residues in Adipose tissues

By the present report it may be conluded that adipose tissues of Karachi people have OC, OP and pyrethroid insecticide residues. As far as their concentration is concerned, the higher concentration was of pyrethroid group i.e., deltamethrin. Adipose tissues had no OP compound instead they had OC pesticides among which dieldrin and DDT are dominating. Aldrin was found in higher concentration, possibly because of its extensive use in termite control. As the pesticides interfere with Ca^{++} metabolism and act as enzyme inhibitors also, their indiscriminate use poses great human health risk as evident from the present data.

Hospitals	Pesticide residues (µg / g Fat)					
	DDT	DDE	Dieldrin	Aldrin	Deltamethrin	Malathion
Jinnah[19]	12* 7-ND	2* 17-ND	4* 15-ND	2* ↑31.000 17-ND	2* ↑ 7.990 ↑ 6.290 17-ND	19-ND
Baqai [10]	10-ND	1* 9-ND	10*	10-ND	10-ND	10-ND

Superscript number in hospital = Total no. of samples, * number = Number of detection,
ND = Not detected, ↑ = High value.

Table 9. Content of pesticide residues in Adipose tissue samples of Jinnah hospital.

Residues in fish tissues

Detection of pesticide residues DDT, DDE, aldrin, dieldrin and deltamethrin in fat, muscles and liver of three Labeo species of fish found in Kalri and Haleji lakes were also done by Saqib *et al*. (2005). In this study total 45 samples were taken and out of which 18 samples were found positive. The data indicates that DDT was found in small quantities while DDE was found higher in most of the samples. In few samples deltamethrin was also detected. This means slightly high level of residues were found in Kalri lake samples. However quantity of pesticides were higher in Haleji lake due to polluted nature of water while number of pesticides was more in Kalri lake water possibly due to the surrounding adjacent agricultural farms. It is then concluded that use of pesticides should be done with great

Fish Tissue Sample	KALRI LAKE			HALEJI LAKE		
	Pesticide residues (µg/gm)			Pesticide residues (µg/gm)		
	L. rohita	L. calabasu	L. sindensis	L. rohita	L. calabasu	L. sindensis
Fat [6]	Deltamethrin 0.35 DDT (Traces) DDE ↑ 11.3 Aldrin 0.135	DDT 0.40 DDE ↑ 9.30	Deltamethrin 2.2 DDT 0.14 DDE ↑ 5.78	Dieldrin ↑ 3.5 Aldrin 0.15	DDT 0.41 DDE ↑ 142.2	DDT 0.75 DDE ↑ 11.0
Muscles [6]	Deltamethrin 0.40 DDE 3.0 Aldrin 0.46	Deltamethrin 1.8	Deltamethrin 1.66 DDT 0.09 DDE 3.3	DDE 4.7 Aldrin 0.56	DDT 1.74 DDE ↑ 70.67	DDT 1.35 DDE ↑ 13.88 Dieldrin 1.84
Liver [6]	Deltamethrin 1.7 DDT (Traces) DDE 0.70 Aldrin 0.03	Aldrin 0.31	Deltamethrin 2.7	Not detected	DDT 0.33 DDE ↑ 15.83	Deltamethrin 0.14

Superscript number in hospital = Total number of fish tissue samples, ↑ = High value

Table 10. Quantity of pesticide residues in different tissues of three fish species from Kalri and Haleji lakes.

care, otherwise it will end up in hazardous effects as evident from the presence of pesticide residues in fishes in the present data. This is especially dangerous because Kalri lake water is being used as a source of water supply for Karachi population (16 millions).

Pesticide degradation

Degradation of pesticides is defined as the breakdown of toxic chemicals into nontoxic compounds and in some cases they return back into their original elements. The degradation or breakdown of pesticides can occur in plants, animals, soil and water. It can also occur upon exposure of ultra-violet radiation. The most common type of degradation that occurs is through the activity of microorganisms particularly the fungi and bacteria. According to the nature of degradation in the environment, the pesticides are grouped as persistent pesticides (i.e., DDT, aldrin, dieldrin and cadmium compounds) and non-persistent pesticides (i.e., malathion, lindance, paraquat, mancozeb etc) were indentified.
We are presenting here the pathway of degradation of some of the pesticides e.g.

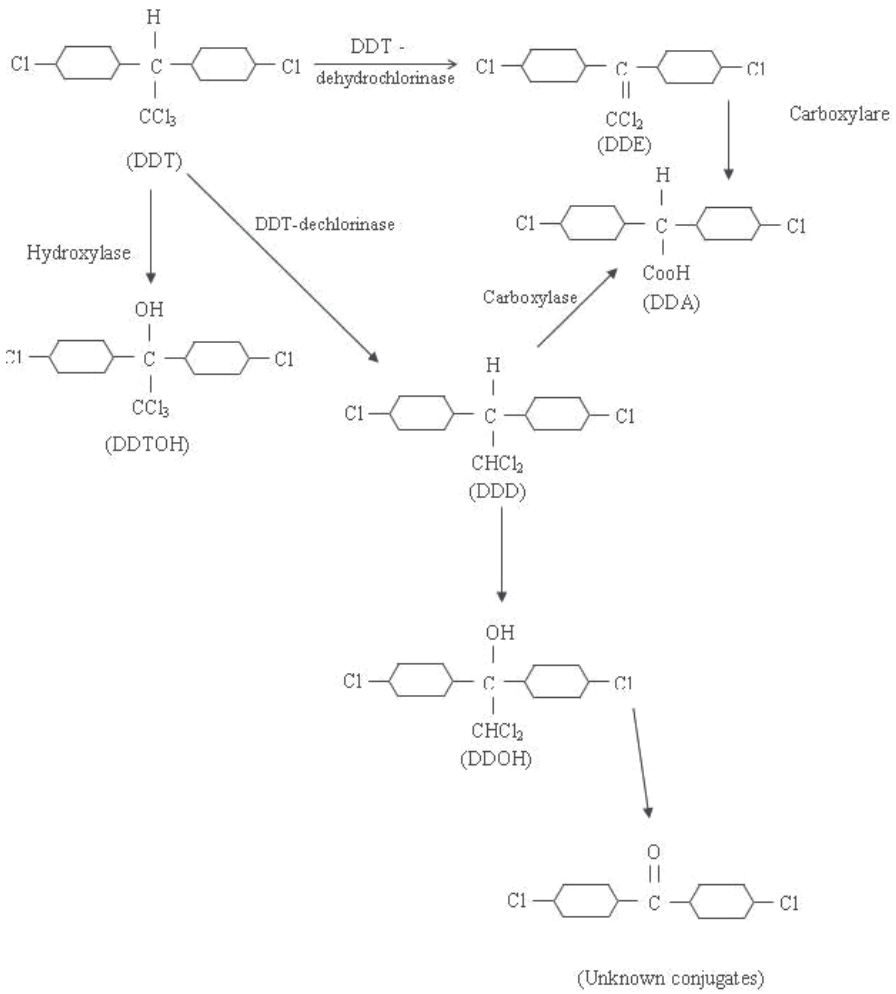

Fig. 1. Degradation and metabolism of DDT

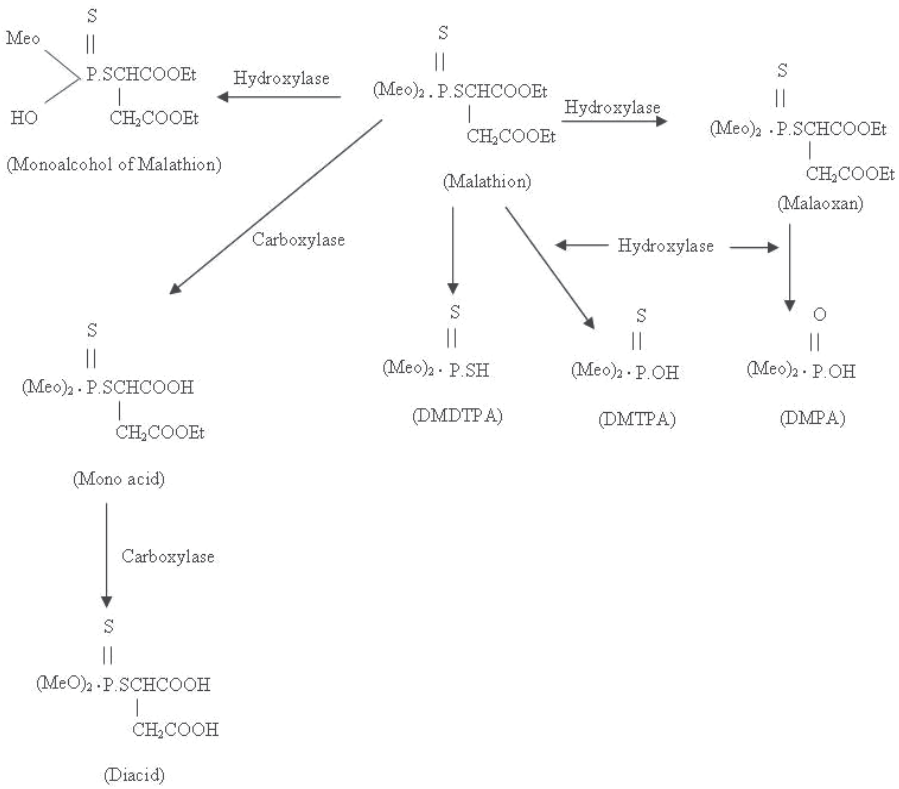

Fig. 2. Degradation and metabolism of Malathion

B. Pesticide resistance:

Today, possibly no one can ignore from the word "Pesticides" whether living in villages or in cities. This is due to the fact that everyone is exposed to pesticides directly or indirectly or comes in contact with them to a lesser or greater degree, in an attempt to kill our common enemy the "Pest" (generally insects) either in the field or at home. This struggle is going on since the man stepped on this earth. Various methods of controlling pests have been used by man from the very beginning. At present the use of conventional pesticides is the most common, economical and easiest method. However, there are certain problems associated with it like bioaccumulation, pollution, residues and resistance. In this section, we will briefly discuss the last one i.e., resistance.

Pesticide resistance is the adaptation of pest species targeted by a pesticide resulting in decreased susceptibility to that chemical. In other words, pests develop a resistance to a chemical through selection; after they are exposed to a pesticide for a prolonged period it no longer kills them as effectively. These factors increase the level of tolerance in the particular pest strain to a great limit. The phenomenon is biochemical and genetically. It may be determined by simple bioassay method or by determination of enzyme levels and marking of particular genes located on various chromosomes. Here we will not discuss the methods of determination of resistance but we will provide the information regarding the survey positions of resistance to pesticides in and outside of Pakistan. Hundreds of species and strains have been reported to be resistant to various pesticides or groups of pesticides. Sometimes cross resistance also develops. Only among mosquitoes 96 species have been

reported resistant to one or more groups of pesticides as described by Georghiou and Mellon (1983). Among these 36 were resistant to one group, 32 to two groups, 18 to three groups, 8 to four groups and only one to all five groups of pesticides. One of the earliest report in this connection is that of Chattoraj and Brown (1960).

Factors responsible for resistance

As mentioned earlier resistance may develop due to the indiscriminate use or constant use or selection pressure but the factors identified in case of various groups are genetical as reported by Wood (1983). Resistance against organophosphates (OP) and Carbonates (CB) in case of mosquitoes has been reported due to increased level of detoxifying esterases (Georghious and Pasteur 1978, 1980; Wood *et al*. 1984; Wachendorf – Neumann 1984; Wang *et al*. 1984; Yasotomi, 1970). An increase in detoxifying phorsphomono-estereases was reported by Naqvi *et al*. (1969) as well. Hemigngway (1982) reported similar increase in the level of Carboxylesterase in a resistant strain. Resistance to pyrethroids in *Aedes aegypti* was reported by Chadwick *et al*. (1977). Higher resistance against permethrin was obtained by selection method and has been reported by Malcolm (1983). Due to this, attempts are being made to overcome this by using alternate methods or control or using mixture of pesticides with negative correlation of resistance or by rotating the pesticides. For this purpose computer stimulation technique and recombinant DNA technique is being tried now.

Position in Pakistan

Very little work on pesticide resistance is being done in Pakistan and that too only in health sector. Several studies have been conducted to support the resistant phenomenon against different pesticides. In this connection a study was carried out by Naqvi *et al*. (1987) for the resistance development in mosquitoes and houseflies in Karachi against DDT and malathion. In this study pesticides (DDT and malathion) were tested against different strains of mosquito species to determine the resistance phenomenon. The details are as under:

Strain	DDT (µg)	Malathioin (µg)
Musca domestica DDT resistant strain (PCSIR Strain)	8.8	2.2
Musca domestica (PCSIR Strain)	2.0	2.4
Musca domestica (Malir Strain)	19.0	8.8
Aedes aegypti (Susceptible London strain)	0.020%	0.8%
Aedes aegypti (PCSIR Strain)	0.019%	0.16%
Culex pipiens (Karachi University strain)	0.27%	0.19%

Table 11. Comparison of LC_{50} of DDT and Malathion against various strains of mosquitoes and houseflies.

Similar type of studies have been investigated by other researchers (Azmi *et al*. 1988, 1990, 1991; Naqvi and Tabassum 1992; Naqvi *et al*. 1993; Azmi *et al*. 1993; Rahila *et al*. 1993; Khan *et al*. 1993; Rahila *et al*. 1994; Azmi *et al*. 1995; Naqvi *et al*. 1995; Khan *et al*. 1996; Kahkashan and Naqvi, 1997; Naqvi *et al*. 1999; Kahkashan *et al*. 2001; Azmi *et al*. 2002).

Keeping in view excessive use of pesticides, the reports of Baig (1982), Khan *et al*. (1987), Naqvi *et al*. (1987) and Azmi *et al*. (2001) about increased resistance against certain organochlorine and organophosphate compounds in houseflies, vinegar flies and various species of mosquitoes have been reported that better alternative methods should be adopted to overcome the situation. Recently, the similar type of study has been conducted by Zafar *et al*. (2009) about the residual analysis of biopesticide by HPLC and resistance determination in different populations of *Sitophilus oryzae* against Biosal, Cypermethrin and Phosphine on the basis of enzyme activity. Thus, it may be concluded that neem fractions may successfully be used as pesticides, although the dose may be higher but not lethal. Availability of neem in Agro-Asian countries is more and this will help in developing a cheaper, safer and easier method to apply which will not produce the problem of resistance and pollution in comparison to imported conventional pesticides.

C. Pesticidal hazards affecting health and environment:

Pesticides when used properly are of tremendous benefits to human being, their indiscriminate use, however may cause considerable hazards to health and environment. Investigators feel that some workers did not report illness due to pesticide exposure; therefore, including un-reported cases the total numbers might be significantly higher Howitt and Moore (1975).

Problems associated with the indiscriminate use or misuse of pesticides

Among many problems, we have been facing in Pakistan is the problem of pollution, because convential pesticides are chemicals which act as poisons or toxic agents. So they pollute the atmosphere. By general spraying not only the target species but non-target animals and even human beings are affected. Therefore, there is a general tendency in the West towards the use of 4th generation pesticides. A greater part of pesticides used for killing pests persist in the environment and may be accumulated in human body by many ways such as through drinking, water, vegetables and fruits etc. The meager quantity of such insecticides gradually increase in the body which become the cause of many human diseases like gastric cancer, cytogenetic damage, kidney infections and others (Anna *et al.* 1988; Sandra *et al.* 1992). Chlorinated, organophosphates and carbamates pesticides have also been reported in various human matrices (Saltatas and Gagliardi, 1990; Alwai *et al.* 1992; Ferrer *et al.* 1992; Saad *et al*. 1992).

Pesticide poisoning:

Pesticides are poison, and their acute exposure or continuous exposure result in several neurotic and pathological effects. Acute and severe exposure results in the inhibition of enzymes and physiological disorder. Continuous exposure results in malfunctioning of liver, kidney and heart. They are cytotoxic and damaging the cells and therefore these organs are affected. Certain pesticides e.g. ethyl selenac, mirex, penfluron are carcinogenic. Pesticide inhalation for a longer period affect the alveoli of lungs and so person develops into respiratory diseases. If tissues of the kidney is damaged then kidney function is affected. If the cholinesterase level is low continuously, it results in muscular twitching and

trembling of arms and legs. Most of the pesticides are teratogenic at high dose. They also disturb the metabolism (Ca++, Mg++). Cummulative effect of lipophilic pesticides may result in instantaneous death which is not detectable or diagnosable.

Different group of pesticides such as organchlorines have been correlated with dehydrogenases while organophosphates and carbamates have been correlated with cholinesterase, phosphomonoesterase, aliesterase, carboxylesterases, transaminases, monoxygenases, P-450 etc. Large number of publications is available in this respect. However, some recent reports are referred here e.g., Akgur *et al*. (1999) reported the effect of OP's on cholinesterase and paroxonase activities in human exposed persons. Husin *et al*. (1995) reported the effect of pesticide exposure on cholinesterase in farm workers. Krieger and Dim-off (2000) also reported the effect of malathion on cholinesterase in California date palm spray men. Regarding pesticidal effect on blood parameters scanty literature is available such as Dunstan *et al*. (1996) examined the effect of chtorinated hydrocarbons on various blood parameters in exposed persons.El-saeed and Hassan (2000) reported the relationship between chronic lymphocytic leukemia and pesticide exposure among Egyptian farm workers.

In Pakistan, Khan *et al*. (2000) reported the effect of pesticides in cotton field workers. Naqvi *et al*. (1970), Naqvi and Jahan (1999) and Rehman *et al*. (2002) reported the inhibition of acid and alkaline phosphatase in various cases. Khan *et al*. (2003) also investigated the inhibitory effect of pesticides in liver and kidney enzymes of Agama lizard. Similarly Azmi *et al*. (2006) examined the enzymatic activities of GOT, GPT and ALP in pesticide exposed farm workers of Gadap area, Karachi. During investigation they found that most of the workers showed elevated levels of enzymes except in few cases. This shows that these persons are highly exposed with pesticides during spraying time and their case history clearly indicate the degree of exposure due to which they get suffered with hepatitis, liver dysfunction and also complained about the clinical effects of liver. Similarly some workers who were exposed to cypermethrin, diazinon, monocrotophos simultaneously showed high level of of GOT and ALP. These persons due to the cumulative effect of these pesticides ultimately suffered with dyspnea, cyanosis, vomiting, backache and burning sensation in urine.

In another study which is carried out by Azmi *et al*. 2007 in the rural area of Gadap, Karachi almost all the persons showed higher level of cholinesterase except two persons that showed low level of cholinesterase probably due to the receptor binding by the molecule of pesticides or poisoning effect of pesticides used in these areas by the workers. Similar type of study is also carried out by Shahida *et al*. (2008) to observe the enzyme activities of GOT, GPT, ALP, cholinesterase and gamma-GT in the general population belonging to the different division of Sindh province, Pakistan.

Various blood components are also affected by the exposure of pesticides e.g., Bhalla and Agrawal (1998) reported the alteration in RBC membrane in rats by HCH exposure. Bailey and Jenkins (2000) reported the development of chronic leukemia in Egyptian farm workers. They also reported hight lymphocyte, WBC and platelet counts. The same findings have also been noticed by Azmi *et al*. (2009) in which 90% of exposed farm workers had high lymphocyte count thus confirming the report of El-Saeed and Hassan (2000) about the relationship between chronic lymphocytic leukemia and pesticide exposure.

Symptoms and treatment of pesticide poisoning

Different group of pesticides produce characteristic adverse effects on human health and so alter the normal functioning of human life.

Organochlorines (OC)

This group mainly affects the CNS which appears as headache, fever, and convulsion or muscular twitching, unconsciousness in extreme condition. Inhalation of carbogonium (5-10% CO_2 in O_2), then give phenobarbital (0.25 -0.5 gm). Injection of calcium gluconate may also be given. Restore dehydrochlorine activity.

Organophosphate (OP)

OP is inhibitors of cholinesterase and other enzymes like GOT, GPT and general esterases. Symptoms of poisoning are headache, giddiness, blurred vision, weakness, nausea, cramps, convulsions and discomfort in breathing. Immediately ChEase activity in blood serum should be checked (50-100% activity normal 20-50% mild effect, 10-20%, moderate effect and 0-10% severe effect). Artificial respiration should be given. Administer atropine sulphate 25 mg and repeat every 10 minutes till tachycardia as high as 120-140 per minute. Give 2-PAM 1 gram for adult and 0.25 gram for infant. Observe the patient for 24-48 hrs. (Do not give large amount of fluids by any route and in case of egamotic condition first restore respiration and then give atropine sulphate).

Carbamates (CB) and Synthetic Pyrethroids(SP):

They are also reversible cholinesterase – inhibitors. If used constantly produce different symptoms which are: salivation, constriction of pupil, profuse sweating, vomiting, pulmonary difficulty and muscular in coordination. Artificial respiration should be given and then administer atropine sulphate. Administration of 2-PAM and other oximes may not be useful and sometime harmful so it should be given under the advice of specialist or toxicologist.

This data which is a fragment of the whole literature on this topic is a significant proof of the hazardous effects of the over use and misuse of pesticides. We believe that this is a clear health risk to human health.

Conclusion and recommendations:

Being potentially dangerous and harmful to human health little attention was paid to the long-term impact of chemical pesticides on our environment as well as on the health hazards.

Poisoning due to the use of chemical pesticides sometimes do not readily show up. The signs of affected health become visible only after prolonged exposure to the poisons. Persistent insecticides such as DDT and other organochlorine insecticides which retain their toxicity even after a lapse of many years and pesticides which degrade into toxic residues are of great danger to human and animals. Several of the organophosphates (OP) and synthetic pyrethroids (SP), cause inhibition and elevation of cholinesterase due to the cumulative effect of pesticides and show the symptoms of neurotoxicity, hepato-toxicity, RTI and kidney dysfunctions etc.

There are two extremes if we see the policy of pest management in the west and our farmers. In west they have switched over to IPM and not only this but as a rule they have made it compulsory that atleast 50% agro-chemicals should be phytopesticides, where as in Pakistan the farmers want to use only synthetic, highly toxic pesticides and that too they will spray without proper clothing, gloves, googles and masks, on the pretext that the weather is too hot. We know that our farmers hesitate in using phytopesticides, because their effects can be observed after 72 hours and not in few minutes as seen in case of

Groups	Pesticides	Antidotes
Group-I Organophosphates	Azodrin, trithiion, dasanit, DDVP, demeton, dimethoate, durstban, ethion, fncthion, metasytox, methyl parathion, monitor, phorate & phosphamidon,	• Atropine Sulphate is used. Injections should be repeated as symptoms recure. • Prontopam chloride (2-PM) should be injected intravenously.
Group-II Carbamates	Aldicarb, Carbofuran, Propoac, Methomyl & Carbaryl.	• Artophine sulphate. • Protopam chloride (2-PM) must not be used.
Group-III Chlorinated	Endrin, dieldrin, aldrin, Lindane, endosulfan, BHC, DDT & toxaphane	• Barbiturates for convulsions or restlessness • Calcium gulconate given intravenously. • Epinephrine (adrenalin) should not be used.
Group-IV: Inorganic arsenicals	Sodium arsenite & Paris green.	• BAL (dimereaprol) is specific for arsenic poisoning, intramuscular injection.
Group-V: Cyanides	HCN or Cyanogas	• Amylnitrite through inhalation. • Sodium nitrite given intravenously. • Sodium thiosulphate given intravenously.
Group-VI: Anticoagulants	Warfarin, Valene, Pival & Dephacin	• Vitamin K by mouth, intravenously or intramuscularly. • Vitamin C is useful adjunct.
Group-VII: Fluoracetates	Sodium fluroacetate	• Monacetin (Glyceral monacetate) intramuscularly.
Group-VIII Dinitrophenols	DNOC & Dinoseb	• Atropine sulphate must not be used • Life support should be maintained. • Sodium methyl thiouracil may be used to reduce basal metabolic rate.
Group-IX: Bromides	FDB & MB or mixture of EDB+MB	• BAL (dimercaprol) may be given before symptoms appear. • Barbiturates for convulsions.
Group-X: Chlorophenoxy Herbicides, Urea	2-4-D, 2,4,5-T Momiron, Diuron, Bromacil, Paraquat & Diaquat.	• None available. • Life support should be maintained.

Table 12. Antidotes for pesticide positioning

conventional pesticides. So, one should think that is it not better to get slightly lesser kill of pests in the long run and using a safe method of control, so that your health and health of your family as well as your animals are saved". We wish to end this manuscript with the following phrase:

"Pesticides are double edged sword, if used intelligently will kill the enemy – and if carelessly will kill you".

THEREFORE, BETTER TO ADOPT SAFETY MEASURES TO SAVE YOUR HEALTH AND ENVIRONMENT FROM POLLUTION.

4. References

Abu-Qare, A.W. & Abu-Donia, M.B.,(2001-a). Determination of diazinon, chlorpyrifos and their metabolites in rat plasma and urine by high performance liquid chromatography. *J. Chromatogr.* 39: 200 – 204.

Abu-Qare, A.W. & Abu-Donia, M.B., (2001-b). Performance of liquid chromatographic determination of diazinon, permethrin. DEET (N, N-diethyl-m-toluamide), and their metabolites in rat plasma and urine. *J. Anal. Chem.* 370: 403 – 407.

Ahmed, M.T., Loutfy. N. & El-Sheikh, E. (2002). Residue level of DDE and PCB's in the blood serum of women in the Port Said region of Egypt. *J. Hazard. Mater.* 89: 41 -48.

Akgur, S.A., Ozturk, P., sozimen, E.Y., Delen, Y., Tanyalcin, T. & Ege, B. (1999). Paraoxonase and achtylcholinesterase activities in human exposed to organophosphorus compounds. *J. Toxicol. Environ. Health A.* 58: 469 -474.

Alawai, M.A., Ammari, N. & Yousuf. A.S. (1992). Organochlorine pesticide contamination in human with samples from women living in Amman, Jordan. *Toxicol*. 23: 235 – 239.

Anna, P., Nandoor, P. & IIdiko, F.(1988). Pesticide use related to cancer incidence as studied in a rural district of hungary. *Sci. Total. Environ.* 73: 229 – 244.

Azmi, M.A. Naqvi, S.N.H., Akhtar, K. & Ahmad, I. (1988). Determination of cholinesterase in standard and wild culicine mosquito larvae of Karachi region after treatment with DDT and Malathion. *Pak. J. Ent.* 9: 115 – 124.

Azmi, M.A., Naqvi, S.N.H. & Akhtar, K. (1990). Determination of esterases in DDT susceptible and resistant strains of culicine mosquitoes. *J. Sci. Kar. Univ.* 18: 137 – 151.

Azmi, M.A., Naqvi, S.N.H. & Akhtar, K. (1991). Determination of resistance in culicine mosquitoe larvae of Karachi by WHO method. *J. Sci. Kar. Univ.* 19: 139 – 144.

Azmi, M.A., Naqvi, S.N.H. & Akhtar, K. (1993). Comparative study of toxicity of DDT and Malathion and solfac against various strains of *Culex Fatigans* by WHO method. *Proc. Pak. Congr. Zool.*, 13, pp. 359 – 366.

Azmi, M.A., Naqvi, S.N.H. & Akhtar, K. (1995). Correlation of resistance with phosphomonoesterases in susceptible and resistant strains of mosquitoes. *Pak. J. Entomol*. 10: 19 -24.

Azmi, M.A., Naqvi, S.N.H. & Akhtar, K., Ahmed, I., Rizvi, S.A. & Rais, S. (2001). Determination of toxicity of DDVP and cypermethrin on *Collosbruchus analis.* F and

their effect on alkaline phosphatase and choinesterace activity. *Proc. Pak. Congr. Zool.* 21: 289 -293.

Azmi, M.A., Ahmed, I., Naqvi, S.N.H. & Akhtar, K. (2002). Level of lactate dehydrogenase (LDH) in resistant and surceptible strains of culicine mosquitoes of the Karachi region after treatment with DDT, malathion and cyfluthrin. Turk. J. Zool. 26: 97 - 100.

Azmi, M.A., Naqvi, S.N.H., Azmi, M.A. Jr. & Aslam, M. (2006). Effect of pesticide residues on health and different enzyme levels in the blood of farm worker from Gadap (rural area) Karachi-Pakistan. *Chemosphere.* 64: 1739 – 1744.

Azmi, M.A., Naqvi S.N.H., Moinuddin., Azmi, M.A. Jr., Aslam, M. & Rehana, P. (2007). Effect of pesticide residues on health and enzymes in the blood of farm workers around Karachi-Pakistan *J. Exp. Zool. India.* 10: 43 - 54.

Azmi M.A., Naqvi S.N.H., Kehkashan, A., Moinuddin., Shahida, P., Rehana, P. & Aslam, M. (2009). Effect of pesticide residues on health and blood parameters of farm workers from rural Gadap, Karachi-Pakistan. *J. Environ. Biol. Ind.* 30: 747 – 756.

Baig, M.M.H. (1982). Resistance of pesticide use in public health in Pakistan. FAO *International workshop on resistance.* Srilanka.

Bailely, B.J. & Jenkins, J.J. (2000). Association of azinphos – menthyl with rat erythrocytes and hemoglobin. *Arch. Toxicol.* 74: 322 – 328.

Bhalla, P. & Agarwal, D. (1988). Alterations in rat erythrocyte membrane due to hexachlorocycle-hexane (technical) exposure. *Hum. Expt. Toxicol.* 17: 638 -642.

Bissacot, D.Z. & Vassilieff, I. (1997-b). HPLC determination of flumethrin, deltamethrin, cypermethrin and cyhalothrin residues in the milk and blood of lactating dairy cows. *J. Anal. Toxicol.* 21: 397 – 402.

Butler, W.J., Saddon, L., McMullen, E., Houseman, J., Tofflemire, K., Corriveau, A., Weber, J.P., Mills, C., Smith, S. & Van Oastdam, J. (2003). Organochlorine levels in maternal and umbilical cord blood plasma in Arctic Canada. *Sci. Total. Environ.* 2: 27 – 52.

Cantor, K.P., Blair, A., Everett, G., Gilsor, R., Brumeister, L.F., Brown, L.M. *et al.*(1992). Pesticides and other agricultural risk factors for non – Hodgkin's lymphoma among men in Jowa and Minnesota. *Cancel Res.* 52: 2447 – 2455.

Casarette, J.J., Freyer, G.C., Yauger, W.L. & Klemner, H.W. (1968). Organochlorine pesticides residues in human tissue. *Hawaii Arch. Environ, Health.* 17: 306 – 311.

Chadwick, P.R., Invest, F.J. & Brown, M.J. 1977. Pyrethroid reistance in *Aedes aegypti* in a Bangkok population Pestic. *Sci.* 8: 618 -624.

Chattraj, A.W. & Brown, A.W.A. (1960). Internal DDE production by normal and DDT resistant larvae of *Aedes aegypti. J. Econ, Ent.* 53: 443 - 455.

Chikuni, O., Skare, U., Nyazema, N. & Poider, A. (1991). Residues of organochlorine pesticides in human milk from mothers living in greater Harare area of Zimbabwe. *Cent. Afr. J. Med.* 37: 136 -141.

Cohen, J.A. & Oosterbaan, R.A. (1963). The active site of acetylcholinesterase and related esterases and its reactivity towards substrates and inhibitor Springer-Verlag, Berlin.

Desola, S.R., Bisshop, C.A., Van der Kiraak, G.J. & Brook, R.J. (1998). Impact of organochlorine contamination on levels of Dex hormones and external morphology of common snapping turtle in Ontario, Canada. *Environ. Health. Perspect.* 106: 253 – 260.

Dua, V.K., Pant, C.S. & Sharma, V.P. (1996). Determination of levels of HCH and DDT in soil, water and whole blood from bioenvironmental and insecticide-sprayed areas of malaria control. *Indian. J. Malariol.* 33: 7 – 15.

Dunstan, R.H., Roberts, T.K., Donohoe, M., McGregor, N.R., Hope, D., Taylor, W.G., Watkin, J.A., Murdoch, R.N. & Butt, H.L. (1996). Bioaccumulated chlorinated lydrocarbons and red / white blood cell parameters. *Biochem. Mol. Med.* 58: 77 – 84.

El-Saeed, W.Y. & Hassan, M.H. (2000). Chronic lymphocytic leukemia in Egyptian farm workers exposed to pesticides. *East Mediterr. Health. J.* 5: 960 – 966.

Ferrer, A., Bona, A.M. Castellano, M., ToFigueras, J. & Brunet, M. (1992). Organochlorine residues in human adipose tissue of the population of Zaragoza (Spain). *Bull. Environ. Centam.* 48: 561- 566.

Georghiou, G.P. & Pasteur, N. (1978). Electrophoretic esterase pattern in insecticide resistant and susceptible mosquitoes. J. Econ. Ent. 71: 201 – 205.

Georghiou, G.P. & Mellon, R.B. (1983). Pest resistance to pesticides. Edited by G.P. Georghiou and P. Siato. pp. 1 – 66 and 769 – 792.

Guardino, X., Serra, C., Obiols, J., Rosell, M.G., Berenguer, M., Lopes, F. & Porossa, J. (1996). Determinotion of DDT and related compounds in blood samples from agricultural workers. *J. Chromatogr. A.* 719: 141 -147.

Heath, D.F. (1961). Organophosphorus poisons. *Pargamon Press.* Oxford.

Hemingway, J. (1982). The biochemical nature of malathiion resistance in *An. Stephensi.* Pestic. Biochem. Physiol. 17: 149 – 155.

Heudorf, U., Angerer, J. & Drexler, H. (2003). Current internal exposure to pesticide in children and adolescents in Germany: blood plasma levels of pentachlorophenol (PCP), lindane (gamma – HCH) and dichlorodiphenylethylene (DDE), a biostable metabolite of dichlorodiphenyl trichloroethane (DDT). *Int. J. Hyg. Environ Health.* 206: 1816 -1820.

Howitt, R.E. & Moore, C.V. (1975). Pesticide injury reporting: One study; Econ. Social Issue, Univ. Claif. Cooperative Extension, Davis, CA. 05616.

Husin, L.S., Ultaman, A., Hisham, H.J., Hussain, I.H & Jamil, M.R. (1999). The effect of pesticide on the activity of serum choloinesterase and current perception threshold on the paddy farmers in the Muda agricultural development area, Mada, Kedah, Malaysia. Med. J. Malaysia. 54: 320 – 324.

Jahan, M. (1995). Pesticide residues in random blood samples of human population in Karachi. *JCPSF.* 6: 151 – 153.

Jarrer, J., Goomen, A., Foster, W., Brant, R., Cham, S. & Sevcik, M. (1998). Evaluation of reproductive outcomes in women inadvertently exposed to hexachlorobenzene in southern Turkey in 1980's. *Reprod. Toxicol.* 12: 469 -476.

Kahkashan, A. & Naqvi, S.N.H. (1997). Effect of DDT and malathion on macromolecules (proteins) of susceptible and resistant strains of *Musca domestica* L. Pak. J. Entomol. 12: 37 – 42.

Kahkashan, A. & Naqvi, S.N.H., Ahmad, I. & Azmi, M.A. (2001). Effect of resistance on the activity of acid and alkaline phosphatases after treatment with DDT and malathion in three different strains of *Musea domestica* L. *Bulletin. Pure. Applied Sci.* 20: 41 -50.

Kanja, L.W., Skaare, J.U., Ojwang, S.B. & Maitai, C.K. (1992). A comparison of organochlorine pesticide residues in maternal adipose tissues, maternal blood, cord

blood and human milk from mother / infant pairs. *Arch. Environ. Contam.* Toxicol. 22: 21 -24.

Khan, M.F., Rehana, A. & Baig, M.M.H. (1987). Determination of resistance in local *Drosophila* against DDT in comparison with four different strains of *Drosophila melanogaster.* Pak. J. entomol. *Kar.* 2: 109 -120.

Khan, M.F., Rehana, A. & Naqvi, S.N.H. (1993). Detection of DDT resistance level in *Drosophila melanogaster* of Karachi East region. *Zoologica. Pak.* 3: 57 – 60.

Khan, M.F., Naqvi, S.N.H., Azmi, M.A., Khan, M.I. & Qadri, S.S. (1996). DDT reswidues on apple after spray for codling moth control. Pak. J. Entomol. *Kar.* 11: 21 -22.

Khan, M.F., Khan, M.I., Aslam, M. & Naqvi, S.N.H. (2000). Study of cholinesterase level in blood of cotton field workers exposed to pesticides. *J. Baqai. Med. Univ.* 3: 12 -16.

Khan, M.Z., Naqvi, S.N.H., Khan, M.F., Tabassum, R., Ahmad, I., Farina, F. & Tariq, R.M. (2003). Determination of induced effect of biosal (Neem-based formulation) on cholinesterase and protein in kidney and liver of *Calotes versicolor* Damdin. J. Exp. zool. *India.* 6: 175 -179.

Kocan, A., Petrik, J., Drobna, B. & Chovancova, J. (1994). Levels of PCB's and some organochlorine pesticides in human population of selected areas of Slovak Republic. I. Blood. *Chemosphere.* 29: 2315 -2325.

Krawinkel, M.B., Plehn, G., Kruse, H. & Kasi, A.M. (1989). Organochlorine residues in Baluchistan / Pakistan: blood and fat concentration in human. *Bull. Environ. Contam.* Toxicol. 43: 821 – 826.

Krieger, R.I. & Dinoff, T.M.N. (2000). Malathion deposition, metabolite clearance and cholinesterase status of date dusters and harvesters in California. *Arch. Environ. Contam.* Toxicol. 38: 546 – 553.

Krauthacker, B., Tanja, A.K., Kralj, M, Thalcevic, B. & Reiner, E. (1980). Organochlorine pesticides in blood serum of the general population of Yoguslavia and in occupationally exposed workers. Nt. Arch. Occup. Environ. Health. 45: 217 – 220.

Kusevitsky, I.A., Kirlich, A.Y & Khovayera, L.A. (1970). Inhibitory effect of organochlorines on thyroid gland. *Veterinanariya.* 4: 73 - 77.

Luo, X.W., Foo, S.C. & Ong, H.Y. (1997). Serum DDT and DDE levels in Singapore general population. Sci. Total. Environ. 208: 97 – 104.

Malcolm, C.A. (1983). The genertic basis of pyrethrioid and DDT resistance inter-relationship in *Aedes aegypti. Genetica.* 60: 213 -219.

Matsumura, F. & Hogendijk, C.J. (1984). The enzymatic degradation of malathion in organophosphate resistant and susceptible strain of *Musca domestica. Ento. Exp. Appl.* 7: 179 -193.

Matuo, Y.K., Lopes, J.N.C., Carsanova, I.C., Matuo, T. & Lopes, J.L.C. (1992). Organochlorine pesticide residues in human milk in the Ribeuao Preto region. State of Sao Pauto, Brazil. *Arch. Environ. Contam. Toxicol.* 22: 167 – 175.

Mercedes, B. & Thiel, R. (1986). DDT and polychlorinated biphenyl residues in human milk. *Rev. Costrarric Cienc. Med.* 7: 1333 -136.

Mughal, H.M. & Rahman, M.A. (1973). Organochlorine pesticide content of human adipose tissue in Karachi. *Arch. Environ. Health.* 27: 396 – 398.

Musshoff, F., Junker, H. Madea, B. (2002). Simple determination of 22 organophosphorus pesticides in human blood using head space solid – phase micro extraction and gas chromatography with mars spectrometric detection. *J.Chromatogr. Sci.* 40: 29 -34.

Naqvi, S.N.H., Muzaffar, S.A. & Ashrafi, S.H. (1970). Detoxification of DDT and its relation with inhibition of phosphomonesterase in desert laust, *Schistocerca gregaria* (Forskal). *Pglanzenk. Pflanzenschutz.* 77: 578 -581.

Naqvi, S.N.H. & Tabassum, R. (1992). Probable development of resistance against neem extract (RB-A) and cyfluthrin (solfac 10% E.C.) in *Musca domestica* L. (PCSIR Strain). Pak. J. Entomol. *Kar.*7: 9 -16.

Naqvi, S.N.H., Kahkashan, A. Azmi, M.A. (1993). Determination of toxicity and resistance in different strains of *Musca domestica* L. against DDT and malathion. *Pak. J. Entomol. Kar.* 8: 27 - 34.

Naqvi, S.N.H., Jahan, M., Tabassum, R., Qamar. S. J. & Ahmad, I. (1995). Toxicity and teratogeny caused by coopex 25 EC and a neem extract (n-7) against 3rd instar larvae of *Musca domestica* L. *Pak. J. Entomol. Kar.* 27: 27 -31.

Naqvi, S.N.H. & Jahan, M. (1995). Pesticide residues in random blood samples of human population in Karachi. *JCPSF*. 6: 151 – 153.

Naqvi, S.N.H. & Jahan, M. (1996). Detection of some pesticides in blood of some people of Karachi by HPLC. *JCPSP*. 6: 151 – 153.

Naqvi, S.N.H. & Jahan, M. (1999). Pesticide residues in serum and blood samples of the people of Karachi. J. Environ. Biol. 20: 241 – 244.

Ntow, W.J. (2001). Organochlorine pesticides in water, sediment, crops and human fluids in a farming community in China. *Arch. Environ. Contam. Toxicol.* 40: 557 – 563.

O' Brien, R.D. (1967). Insecticides, Action and Metabolism,. Academic press. Inc., Newyork.

O' Brien, R.D. (1976). Acctylcholinesterase and its inhibition. In wikinson, C.F. (ed): Insecticides, Biochemistry and Physiology. Plenum Press., Newyork. pp. 271 – 296.

Padungtod, C., Lasley, B.G.L., Christiani, D.C., Raayan, L.M. & Xu, X. (1998). Reproductive hormone profile among pesticide factory workers. J. Occup. Environ. Med. 40: 1038 – 1047.

Rubin, C.H., Lanier, A., Socha, M., Brock, J.W., Kieszak, S. & Zahm, S. (2001). Exposure to presistant organochlorines among Alaska Native women. *Int. J. Circumpolar. Health.* 60: 157 - 169.

Rahila, T., Naqvi, S.N.H., Masarrat, J. & Shaista, R. (1993). Determination of DDT and malathion resistance in 4th instar larvae of *Culex fahigans* collected from NIPA and Urdu College Strations. *Pak, J. Entomol. Kar.* 8: 71 -78.

Rahila, T., Naqvi, S.N.H., Shaista, R., Masarrat, J. & Khan, M.F. (1994). Susceptibility of different populations of *Musca domestica* against malathion and DDT in Karachi region. *Zoologica. Pak.* 4: 43 – 50.

Rahman, M.F., Siddiqui, M.K. & Jamil, K. (2002). Acid and alkaline phosphatase activities in a novel phosphorothionate (RPR – 11) treated male and female rats. Evidence of dose and time – dependent response. *Drug. Chem. Toxicol.* 23: 497 -509.

Saad, J.J., Robert, L.P. & Alphonse, P. (1992). Measurement of DDT and DDE from serum fat and stool specimens. *J. Environ. Health.* 27: 967 – 981.

Sabbah, S., Jemaa, Z. & Bouguerra, M.L. (1987). Gas chromatography study of organochlorine pesticide residues in human milk and umbilical cord and adult blood. *Analysis.* 15: 399 – 403.

Saddy, J.J., Robert, L.F. & Alphouse, P. (1992). Measurement of DDT and DDE from Serum, fat and stool specimens. *J. Environ. Sci. Health Part A, Environ. Sci. Eng.* 27: 967 – 981.

Saqib, T.A., Naqvi, S.N.H., Siddiqui, P.A. & Azmi, M.A. (2005). Detection of pesticide residues in muscles, fat and liver of three species of *Labeo* found in Karli and Haleji lakes. *J. Environ. Biol.* 26: 4333 – 438.

Sandra., G.A., Norma, N.A., Austin, O., Fernando, G., Sergio, L. & Rafael, V.P. (1992). Sister-chromatid exchange analysis in a rural population of Mexico exposed to pesticides. *Mutat. Res.* 281: 173 – 179.

Saltatos, L.G. & Gagliardi, C.L. (1990). Desulfuration of the insecticide parathion by human placenta *in vitro. Biochem. Pharmacol.* 39: 799 – 801.

Saxena, M.C., Seth, T.D. & Mahajan, P.L. (1980). Organochlorine pesticides in human placemta and accompanying fluids. *Int. J. Environ. Anal. Chem. 7:* 245 – 251.

Shahida, P. (2008). Determination of pesticide residues in blood samples from patients in rural Sindh and their effects on biochemical and hematological levels. *Ph.D. Thesis.*

Swaen, G.M.H., Varvliet, C., Slangen; J. J. M. & Sturmans, F. (1992). Cancer mortality among licensed pesticide applicators. *Scand. J. Work* Environ. 18: 201 – 204.

Van Oastdam, J.C. Dewaitly, E., Gilman, J.C., Hamsen, J.O., Odland, V., Chaschchin, J. *et al.* (2004). Circumpolar maternal blood contaminant survey, 1994 – 1997 organochlorine compounds. Sci. Total. Environ. 330: 55 – 70.

Vilar, O. & Tullner, W.W. (1959). Effect of DDT on sex hormones. Endocrinology. 65: 80 – 84.

Wachendoorf – Neumann, U. (1984). Diagnosis of insecticide resistance in aphids. *Proc. XVII. Int. Cong. Entomol. Hamburg.* 17: 1 -3.

Wakeling, A.E., Schmidt, T.J. & visek, W.J. (1972). Inhibition of 5-dihydrotestosterone binding with specific protein receptors by dieldrin treatment. *Fed. Proc.* 31: 725.

Waliszewski, S.M., Aquirre, A.A., Infanzon, R.N., Silva, C.S. & Silico, J. (2001). Organochlorine pesticide levels in maternal adipose tissue, maternal blood serum, unbilical blood serum and milk form inhabitants of Veravwz, Mexico. *Arch. Environ. Contam. Toxicol.* 40: 432 – 438.

Wang T.C., Feng. H.T. & You, B.Y. (1984). Malathion resistance of peach aphid in Taiwan. *Proc. XVII. Int. Cong. Entomol. Hamburg.* 17: 1 – 2.

WHO, (1962). Toxic hazards of pesticides to man. Twelfth report of the Expert Committee on insecticides. *Wld. Hlth. Org. Tech. Rept. Ser.* No. 227.

Wood, R.J. (1983). Genetics. New Frontiers. Proc. XV. Int. Cong. Genetics. 125 -135.

Wood, R.J., Pasteur, N. & Singere G. (1984). Carbamate and organophosphate resistance in *Culex pipiens* L. (*Diptera Culicidae)* in France and significance of Est-3-A. *Bull. Ent. Res.* 74: 677 – 687.

Yastomi, K. (1970). Studies on organophosphate resistance and esterase activity in mosquitoes of *C. Pipiens* group. Jap. J. Sanit, Zool. 21: 41 – 45.

You, I., Casonova, m., Archibeque-Engle, S., Sar, M., Fam, I.Q. & Heck, H.A. (1998). Impaired male sex development in prenatal spargue Dawley and long Evans hooded rats, exposed to utero and loctationally functional samples to P′ P′ DDE. *Toxicol. Sci.* 45: 162 – 173.

Zafar, S.M.N. (2009). Determination of possible resistance in different populations of *Sitophilus oryzae* against biosol, cypermethrin and phosphine on the basis of enzyme activity. *Ph.D. Thesis Part-A.*

2

Understanding the Full Costs of Pesticides: Experience from the Field, with a Focus on Africa

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1. Introduction

Pesticide use in Africa accounts for less than 5% of global pesticide use and per hectare averages are low, estimated at around 1 kg/ha active ingredient applied (compared with 3-7kg/ha in Latin America and Asia (Repetto and Baliga, 1996; Agrow, 2006; CropLife International, 2009). However, low use volumes do not necessarily equate to low risk, particularly as some of the most toxic pesticides continue to be applied in Africa, often under extremely dangerous conditions. African studies on pesticide impacts on health frequently highlight poor pesticide practice (e.g. Sibanda el al., 2000 in vegetables; Ngowi et al., 2001, in coffee; Matthews et al., 2003 in tree crops), in the case of both farm workers on large farms and smallholders on their own farms. These studies make general recommendations for better education in handling practices and sometimes stricter controls on pesticide distribution but tend to focus on health effects on those directly spraying pesticides – usually men.

This chapter takes a broader look at the impacts of pesticide poisoning, from case studies mainly of West African smallholders. The findings are discussed in terms of exposure for farm families and the social and economic costs of ill health and environmental harm, to affected households and to society at large. Detailed information is provided on endosulfan and cotton systems, before exploring the effectiveness of regulatory controls and governmental pesticide policies to reduce harm. A final section examines efforts in food supply chains to reduce hazard, risks and use of pesticides and the chapter concludes with examples of action research with farming communities to address pesticide harm and promote safer alternatives.

2. Health impacts

In the early 1990s, the World Health Organisation (WHO) estimated that there were 3 million acute pesticide poisonings a year worldwide, almost all in developing countries: 700,000 occupational; 300,000 accidental; and 2 million by intent (WHO, 1990). Jeyaratnam (1990) estimated 25 million occupational pesticide poisonings each year among agricultural workers in developing countries alone. The International Labour Organisation estimated 2-5 million occupational poisonings per year, with 40,000 fatalities (ILO, 1994). The discrepancies between these estimates reveal how little is known about the actual incidence

and scale of poisonings. WHO 1990 figures are considered a severe underestimate mainly because many cases are not formally documented in health surveillance statistics: estimates for Thailand put likely poisonings at thirteen times higher than official records (Jungbluth, 1996). Murray et al. (2002) provide an overview of under-reporting with a focus on Central America, estimating 98% under-reporting. Kishi's review (2005) confirms the difficulties in obtaining an accurate picture of pesticide-related health impacts, the significant underestimations of occupational ill health, and includes specific country studies which indicate much higher incidence than previously thought. Clinical records often seriously over-represent suicides, thus tending to downplay occupational and accidental exposure (Murray et al. 2002; London et al. 2005). Increasing health surveillance reveals a more realistic estimate of actual poisoning levels: in South Africa, intensive monitoring found a ten-fold increase in poisoning rates, many from occupational exposure (London and Baillie, 2001).
Kishi (2005) suggests there is little sign of poisonings decreasing. After nearly ten years of efforts to implement the FAO/WHO International Code of Conduct on the Distribution & Use of Pesticides, an FAO survey found very limited improvement in health problems and 'substantially worse' environmental problems (Dinham, 2005). A further assessment for developing countries suggests up to 2.9 million cases of acute poisoning per year, with acute poisoning a major public health problem for those countries where much of the workforce is employed in farming (Kangas and Tuomainen, 1999). Poorer farmers and women workers may be particularly affected (Mancini et al., 2005).

2.1 Collecting pesticide impact data from the field in Africa

Pesticide Action Network (PAN) UK, PAN Africa Regional Centre (based in Senegal) and several of its national affiliates (most notably the Beninese Organisation for Promotion of Organic Agriculture, OBEPAB), have been collecting information on human and animal pesticide poisoning incidents for over a decade in Africa. The rationale has been to raise awareness among decision makers in Ministries of Agriculture, Health and Environment, Environmental Protection Agencies, the media, crop protection researchers, international donors and development partners, and farmers themselves, of the unacceptably high levels of pesticide-related ill health and environmental damage in African farming communities (Thiam and Touni, 2009). By providing concrete figures, along with qualitative data that illustrate the main pesticide exposure routes and risk scenarios, this work has helped to fill a critical information gap, particularly as only 2% of human poisoning incidents documented reported seeking medical attention and thereby entering official health records.
This informal data collection started in 1999, triggered by a sudden increase in serious and fatal poisonings in cotton growing areas in Benin, following the introduction of the insecticide endosulfan (see case study in section 3). Working in local languages, PAN Africa and OBEPAB staff visit villages and through a process of meeting village leaders, word of mouth and direct questioning identify farm families who have possibly been adversely affected. These families are then interviewed in their home with a standard questionnaire to identify the pesticides implicated, record symptoms and to assess likely exposure routes, as well as to warn farm families of the danger of handling hazardous pesticides without safety precautions. During 1999-2001, 703 human poisoning incidents were documented in Benin and Senegal by interviewing farm families mainly, but not exclusively, in cotton growing areas, and the data entered in a database run by PAN Africa. Where minimum factual information was unavailable for a supposed case, it was not included. Data was analysed by gender, age, compounds responsible (where known) and exposure 'scenario'. Twelve

different scenarios by which family members were poisoned were identified, of which application in the field accounted for 33% in Senegal and 24% in Benin. Contamination of food and re-use of empty containers for food and drink accounted for 57% of all cases in Benin and 86% of all fatal poisonings, showing how important this route is in putting families in danger. Other routes included unsafe storage and inhalation in rooms, children playing with pesticides, confusing pesticides for other products, inappropriate use for treating headlice or ticks, stomach ache, as well as 67 suicide attempts and 2 cases of murder. On average, 16% of the 619 incidents in Benin were fatal and 23% of the 84 cases in Senegal (Williamson, 2005; PAN UK, 2007; Williamson et al., 2008).

Data collection continued during 2002-2009, with 128 villages in Senegal and Mali visited, documenting 305 poisoning cases, In this round of data collection the questionnaire protocols were updated to bring them closer in line with the Health Incidents Reporting Form developed by the Rotterdam Prior Informed Consent (PIC) Convention for identifying 'severely hazardous pesticide formulations' in the field *(http://www.pic.int/home.php?type=t&id=38&sid=34*). The PIC monitoring methodology is not designed to use statistically representative samples but relies on self-selection, combined with experience and judgement to document individual incidents of ill health which are likely to be related to the rough pesticide exposure data recorded for each case. Qualitative data gathered in interviews on what happened and over what time periods, any information from local press and radio reports and discussion with independent experts enables PAN Africa to build up a picture of the main poisoning scenarios. The PIC methodology does not include medical verification of symptoms, indeed many of the incidents recorded took place weeks, if not months, earlier. However, symptoms reported by farmers are checked to verify if they demonstrate typical results of acute exposure to organophosphate, carbamate, pyrethoid and organochlorine insecticides- the main pesticide families implicated in the research. Table 1. summarises the data for the 1,008 cases recorded from the two survey rounds, disaggregated by age, gender and incident severity. Table 2. Summarises data on the most commonly implicated active ingredients.

Further survey work conducted in Senegal, Mali and Tanzania in 2007-09 as part of PAN's global community-based health monitoring, generated useful figures on most frequent symptoms of acute, temporary ill health experienced by smallholders, based on interviews with 420 farmers (PAN International, 2010). In Senegal, rice and cotton farmers suffered most from headaches (57% and 61%, respectively) and blurred vision (49% and 59%), while Malian cotton farmers' two most frequent symptoms were headaches (21%) and dizziness (including blackouts, 8%). Vegetable farmers in Tanzania, in contrast, reported skin rashes (66%) and excessive salivation (58%).

2.2 Issues arising from poisonings research

People often assume that poisoning risk is highest for those handling pesticides directly yet the data from Benin and Senegal shows that women and children feature significantly even though they generally are not the ones doing pesticide application. In Benin, children under 10 years old made up 20% and 30% of poisoning cases recorded in 2000 and 2001. High poisoning rates among women and children were also documented in Ethiopia, from statistics provided by the Amhara Regional Health Bureau for 2001 from hospital records. Women made up 51% of these 185 cases even though pesticides are almost exclusively sprayed by men in Ethiopia, while children 5-14 years old accounted for 20% of cases.

Country/Period	Male	Female	Adult	Children	Fatality rate
Benin / '99-'00 n=148 cases	86%	14%	72%	28%	7%
Benin / '00-'01 n=265	75%	25%	65%	35%	9%
Benin / '01-'02 n=206	61%	39%	44%	56%	32%
Senegal / '99-'01 n=84	67%	33%	68%	32%	23%
Senegal / '02-'09 n=258	86%	4% (10% gender not specified)	90%	5% (5% age not specified)	10%
Mali / '02-'09 n=47	100%	0%	100%	0%	0%

Sources: PAN UK, 2003; PAN UK, 2004; Williamson, 2005; Thiam and Touni, 2009

Table 1. Summary of poisoning cases collected in West Africa, 1999-2009

Active ingredient and/or formulated product	**Benin / '99-'00**	**Benin / '00-'01**	**Benin / '01-'02**	**Senegal / '99-'01**	**Senegal + Mali / '02-'09**
endosulfan	60%	83%	53%	12%	24%
methamidophos					21%
dimethoate					6%
dimethoate+cypermethrin	13%		4.5%	1%	
cypermethrin +profenofos					6%
Nurelle (cypermethrin + chlorpyrifos)	6%				
chlorpyrifos		2%	10%		
lambda-cyhalothrin +profenos or cypermethrin (Cotalm)	4%	10%	16.5%		
Granox (carbofuran + thiram + benomyl)				6%	
diamine +propanil					6%
cypermethrin +acetamiprid +triazophos					6%
methamidophos + methomyl					4%
deltamethrin					3%
Other named products	17%	1.5%	4.5%	8%	
Undetermined pesticides		3.5%	11.5%	73%	24%
NB: % figures relate to total number of poisoning cases documented, NOT to % cases where a compound was implicated.					

Sources: PAN UK, 2003; Williamson, 2005; Thiam and Touni, 2009

Table 2. Active ingredients or products implicated in poisoning cases

Similar frequency of poisonings among women and children has been documented in recent studies in Ecuador (Sherwood et al., 2005) and in India (Mancini et al., 2005), emphasising

how pesticide-related ill health can seriously affect farm families and rural communities, yet government risk assessment generally only considers scenarios for male spray operators.

Widespread use of hazardous insecticides in the home, unsafe storage in kitchens and bedrooms, dangerous treatment of grains and beans and use of empty insecticide containers all contribute to these tragic figures. Washing pesticide-contaminated work clothing poses another risk. Using insecticides for home 'remedies' is especially dangerous- in Ethiopia, farmers used highly toxic insecticides to treat headlice, fleas and bedbugs, and even to try and cure open wounds, using malathion or DDT, sometimes with fatal results (PAN UK, 2003). Farmers explained that it was the poorest people who resorted to this potentially lethal 'cure'. Easy availability of such hazardous chemicals in rural areas contributes to increased suicide rates, particularly of women and teenage girls, mentioned as a growing worry by farmers in Ethiopia, and cotton farmers in Senegal and Benin.

While the Benin and Senegal poisoning data are rather small case studies, purposively sampled and therefore not statistically representative, similar findings were reflected by qualitative and quantitative data from Ghana (Williamson, 2005). Crude estimates of incidence were made from those villages studied where we had data on population levels. For incidents recorded in 77 Beninese villages, average annual frequency estimates of 21.3 serious poisonings per 100,000 population in 2000-01 (the season with highest documented cases) and 11.9 per 100,000 in 1999-00 (the lowest) were made. Fatality incidence per year ranged from 0.8 to 1.9 deaths per 100,000 people. Calculations from the official figures from Amhara Regional Bureau of Health give 1.1 poisoning cases per 100,000 population, for those attended at clinics and hospitals.

Regular ill health from pesticide exposure may not be as dramatic or as visible as serious poisonings but can be far more widespread. Cotton and cowpea farmers in Ghana estimated that 33-60% of economically active people in their villages were adversely affected each season after spraying pesticides. Although farmers were worried about the immediate effects in terms of losing days off work, they viewed the symptoms as temporary 'mild' poisoning. However, scientific studies provide growing evidence that regular exposure to neurotoxic and other pesticides can lead to chronic impairment of the nervous, immune, reproductive and hormone systems in humans. Children are particularly vulnerable as their organs are still developing (Ecobichon, 2001; Szmedra, 2001; Meredith, 2003; Colborn, 2006).

More recent studies by other researchers confirm PAN assessment that poisonings are commonplace. In Benin, 105 cases, including 9 fatalities, were documented during May 2007-July 2008, due to endosulfan (Badarou and Coppieters, 2009). In market gardening in Côte d'Ivoire , only 27% of pesticides used by growers were authorized for such use and a range of poisoning symptoms reported, with 55% suffering headaches and stomach pains (Doumbia and Kwadjo, 2009). Researchers hypothesised that 65% of illnesses suffered by these market gardeners could be linked to pesticide use.

Lack of adequate, or in most cases, any personal protective equipment (PPE) stands out as another key factor in the high levels of pesticide poisoning documented. Most farmers are aware that they should be protecting themselves but the vast majority do not, mainly for reasons of lack of availability or affordability of suitable kit (PAN International, 2010). This problem extends to those selling and distributing pesticides too, as evidenced by a survey of 35 pesticide stores in Mali. Only 63% of these held a relevant licence to sell pesticides and less than 50% had received training. Those who had been trained reported topics covered mainly precautions for mixing and storing pesticides at retail level. Less than a quarter of

stores stocked some form of PPE, demonstrating the woeful lack of consideration given to farmer protection by either regulatory agencies or pesticide distributors.

3. External costs of pesticide use

3.1 Studies on pesticide externalities

Ill health impacts are not just sad incidents for farm families - they also impose serious economic costs on farming communities, in terms of time off work and treatment costs. The work of Cole, Sherwood and colleagues in smallholder potato production in Ecuador is possibly the best and most detailed multidisciplinary study to analyse the costs of acute and chronic health impacts (Cole et al., 2000; Sherwood et al., 2005). Using a combination of questionnaire surveys, focus groups, bioassay, physical tests and household exposure sampling, their findings highlighted the 'invisible' face of chronic exposure to hazardous insecticides, from low-level but cumulative effects on the nervous system, motor coordination and behavioural function. Levels and patterns of exposure to some of the insecticides were found to adversely affect farmer decision-making capacity to a level that would justify worker disability payments in developed countries. That study revealed alarming levels of fatalities at 21 deaths per 100,000, among the highest reported in the world. In economic terms, while increased use of carbofuran insecticide improved crop production, it also lowered neurobehavioural function and thus productivity. Treatment costs imposed a significant financial burden on the public health system, with each non-fatal poisoning costing six worker days.

Factoring externalities into the equation shows that full costs of pesticide use can be enormous. Recent research shows that a very conservative estimate of these costs in Germany, UK, US and China (rice only) amounts to between US$8-47 per hectare of arable land, or an average US$4.28 per kg of pesticide active ingredient applied (Pretty & Waibel, 2005).In the Chinese case, these external costs exceeded the market value of the pesticides-for every US$1.0 worth of pesticide applied, costs to society in the form of health and environmental damage averaged US$1.86. This may be a good reflection of the situation in other developing countries, where the majority of global pesticide poisonings occur.

3.2 Data from Africa

Pesticides can and do cause serious human and environmental damage throughout Africa. Numerous studies over the last 15 years have shown that a considerable proportion of farm workers suffer regular ill health. Others have documented frequent incidence of health problems among smallholder farmers using pesticides, particularly those growing vegetables, coffee or cotton. Table 3 summarises what is known about the economic burden of these hidden health and other costs from the few studies published.

A recent study for the UN Food & Agriculture Organisation (FAO) analysed externalities caused by spraying high concentrations of organophosphate insecticides (mainly malathion and fenitrothion) for locust control operations in Senegal during the last outbreak in 2003-2005 (Leach et al., 2008). It estimated external costs of over 8 million euros: 2.75 million for environmental costs; 2.5 million on human health; 2.1 million in agricultural production losses; and 0.7 million in damage prevention costs. The researchers concluded that failure to recognize and factor in such externalities can result in inappropriate balances of net costs and benefits of pesticide use decisions, favouring 'cheap' solutions that incur higher net costs for society than safer alternatives which are perceived as 'more expensive'.

Country of study	Estimated external costs	Date of study	Reference
Zimbabwe	Cotton smallholders lost US$3-6 per year in acute health effects, equivalent to 45-83% of annual pesticide expenditure. Time spent recuperating from illnesses attributed to pesticides averaged 2- 4 days.	1998-1999	Maumbe et al., 2003
Cote d'Ivoire	Average US$2-5 pesticide-related health expenses incurred by cotton and rice growing households. Cotton farmers suffer at least one adverse health effect 20% of the time.	1996-1997	Ajayi, 2000
Niger	Health costs, livestock losses and costs of obsolete stocks disposal = US$2 per hectare treated	1996	Houndekon et al., 2006
Mali	Annual national poisoning health costs= US$0.25-1.5 million Costs to farming from ineffective pest management due to pesticide resistance and destruction of natural pest control organisms = US$8.5 million	2000	Ajayi et al., 2002

Table 3. External cost studies from Africa

Interviews conducted by PAN UK with cotton and cowpea farmers in Northern Region, Ghana, in 2003 revealed that insecticide-related ill health was widespread and considered by most to be a "fact of farming life". Farmers reported that exposure during spraying made them so weak and sick that they had to stay in bed for 2-7 days afterwards to recover. Table 4 details the number of days off sick after spraying insecticides per season, routine preventative costs (mainly purchase of milk drunk before or after spraying to mitigate poisoning symptoms) and costs of more severe poisoning treatment at the local clinic or hospital (usually administration of saline drips). Active ingredients in products most often associated by farmers with these health effects included endosulfan, chlorpyrifos and lambda-cyhalothrin.

Average no. days off sick after spraying cotton (n=26)	**Cost in terms of average daily farm labour rate**	**Average no. days off sick after spraying cowpea** (n=19)	**Cost in terms of average daily farm labour rate**	**Preventative treatment costs** (n=13)	**Medical treatment costs** (n=30)
21.7	33	15.1	17	0.9	51

Source: adapted from Williamson, 2005

Table 4. Estimated costs of days off work and treatment following insecticide spraying by Ghanaian farmers *(in euros, adapted from Ghanaian cedis, 2003 rate: 1 euro equivalent to approx. 8,895 cedis)*

These health costs are underestimates because they do not include chronic pesticide health effects, or suffering and other non-monetary costs. Taking preventative measures to avoid getting ill after spraying also costs money, for purchase of protective clothing (in the minority of smallholders who use it), or for purchasing milk to drink before application to try and mitigate harmful effects. Ivorian (Ajayi, 2000) and Ghanaian farm families accept temporary episodes of illness as almost an inevitable part of using pesticides and seriously underestimate the real costs to their household, as they only consider cash outlay on medicines, and ignore the costs of days off sick. PAN's 2007 Tanzanian study identified smallholder vegetable production as a high risk situation, with 73% farmers applying pesticides weekly. Over 65% reported suffering some form of poisoning in the previous season, with 22% experiencing symptoms more than three times and 58% had been admitted to hospital for poisoning (PAN International, 2010).

A further issue relates to managing pesticide poisoning in rural communities, especially in the cotton producing areas of northern Benin. In this area, there is complete lack of capacity and expertise by medical personnel in rural clinics, hospitals or medical centres to accurately recognize even the basic and simplest symptoms of pesticide poisoning. Therefore wrong diagnoses of pesticide poisoning cases are common, resulting in giving the wrong treatment to people who experience pesticide poisoning and, who continue to suffer (A Youdeowei, pers.comm, 2010). State-run poison information centres exist in only 13 countries in Sub-Saharan Africa.

4. Case study: endosulfan and cotton systems

4.1 Health and environmental harm from endosulfan use

The persistent organochlorine insecticide endosulfan was introduced in cotton production in francophone West Africa over the 1999/00 season, as part of a regional programme to combat pyrethroid insecticide resistance in the bollworm *Helicoverpa armigera*. Endosulfan already had a reputation as a highly toxic and dangerous pesticide, particularly under poor spraying conditions without any use of protective clothing, and was banned in a number of countries. In the first season of its introduction, cases of acute poisonings, including fatalities, were picked up: official sources in Benin stated that at least 37 people died over the 1999/2000 season in the northern Borgou province due to endosulfan poisoning, while another 36 people experienced serious ill health. In view of the relative share of the Borgou province in national cotton crop area, PAN UK's partner NGO in Benin, OBEPAB, estimated that at least 70 people may in fact have died in Benin over that single season from endosulfan poisoning. From that year OBEPAB started careful documentation of poisoning cases in different parts of the country. Their work has proven invaluable in alerting West African decision makers to the real problems of endosulfan and other hazardous pesticides in widespread use in smallholder production under conditions which can never be 'safe' (Thiam and Touni, 2009).

Over the last ten years, endosulfan is increasingly viewed globally as a priority for phase-out (Watts, 2008).The EU withdrew its approval in 2006 and notified it to the UN Prior Informed Consent (PIC) Rotterdam Convention as banned for agricultural use in Europe for health and environmental reasons (PAN UK, 2008a). Partly as a result of poisoning data collection by PAN partners in Benin, Senegal, Mali and Burkina Faso, in 2007 the CILSS regional Sahel Pesticides Committee decided to stop endosulfan distribution and ban its use a year later (Thiam, 2009). Apart from human health incidents, regional monitoring studies

on water and aquatic fauna indicated endosulfan is a common water pollutant, contaminating surface, groundwater and wells for drinking water. Benin, which is not part of CILSS, also decided to ban endosulfan use in 2008. Other West African studies also implicate endosulfan as a major culprit of serious, sometimes fatal poisonings, in Benin (Badarou and Coppieters, 2009) and the Toxicology Division of the Public Hospital of Lomé-Tokoin in Togo has registered over 500 annual poisoning cases linked to endosulfan (Kodjo, 2007).

The endosulfan-generated cases of deaths and poisoning in West Africa are an unforeseen consequence of the dominant narrative discourse on pesticide 'indispensability' of those responsible for regional decision-making on cotton pest management. Solutions to technical problems with crop protection were decided upon without adequate consideration of the wider contexts in which cotton pesticides are being managed and used (Ton et al., 2000). Successful use of endosulfan in Australian cotton to combat bollworm resistance to pyrethroids was taken as a blueprint for the situation in West Africa, without apparent recognition that the socioeconomic, literacy, education, cropping systems and pesticide regulatory and distribution systems are worlds apart in poor, developing countries like Benin. The case illustrates well what can happen when broad stakeholder consultation is not factored into decision making on pesticide regulation and pesticide use recommendations from research and extension. Yet some in the cotton sector continued to applaud the use of endosulfan (Martin et al., 2005) even when evidence against its appropriateness was well documented.

4.2 Health and environmental issues in conventional cotton in West Africa

West Africa also provides an illuminating case of the health and environmental impacts of current levels of reliance on pesticides commonplace in conventional cotton production. Distributing large volumes of hazardous insecticides through both public and private cotton supply chains without adequate farmer and field agent training, nor understanding of the real risks, has ended up with serious negative consequences (Ton, 2001; Silvie et al 2001). FAO's recent Regional Pollution Reduction & Sustainable Production Program is the first effort to monitor pesticides in the environment and communities of the Senegal and Niger River basins, studying 30 locations in six countries where cotton and vegetable production are the main pesticide users and polluters. Researchers found 19 pesticides regularly contaminating watercourses, including the banned organochlorine dieldrin, along with problematic active ingredients methyl parathion, monocrotophos, endosulfan and lindane (Poisot, 2007). European drinking water standards were exceeded in 90% of samples and the same percentage exceeded Maximum Tolerable Risk levels for ecological effects. The study predicted that such levels of water contamination would have acute effects on fish and aquatic invertebrates - an assessment which is supported by numerous reports of large fish kills, especially following run-off incidents from fields recently sprayed for cotton (e.g. Youdeowei, 2001; Okoumassoun et al., 2002; PAN Africa, 2009).

Insufficient consideration of hazards and risks has undermined productivity and farm family welfare by encouraging the development of pest resistance to commonly used insecticides; killing livestock, effective natural enemies and pollinators; contaminating soil, water and food; and exacerbating gender and income inequalities within rural areas (Youdeowei, 2001; Ajayi et al., 2002; Williamson et al., 2005). Cotton insecticide diversion onto food crops for domestic and local use abounds, as documented in our field work in

Ghana, Senegal and Benin (Williamson, 2003), with negative consequences for food safety and the productivity of cotton in smallholder systems. This is particularly true for the poorer farmers who often sell some of the insecticides and fertilizers they receive on credit from the cotton companies, in order to buy food during the 'lean' season.

4.3 Safer and more sustainable alternatives for cotton production

PAN UK, PAN Germany and PAN Africa have been working for 15 years to promote organic cotton in Africa, as a practical move to deliver social, environmental and economic benefits through safer alternatives in pest management (Box 1). PAN Africa also promotes Integrated Pest Management (IPM), running cotton IPM Farmer Field Schools in Senegal. PAN UK is a member of the Better Cotton Initiative, a new multi-stakeholder initiative in mainstream cotton, which recognizes serious pesticide impact challenges and aims to reduce use and hazards through IPM strategies (*www.bettercotton.org*).

Box 1. Promoting organic cotton systems in Africa

Details of PAN work in African organic cotton can be found elsewhere (PAN Germany, 2004; Ferrigno et al, 2005; Williamson et al., 2005; Sanfilippo, 2007) via *http://www.pan-uk.org/organic-cotton/wearorganic-homepage*. It should be noted that net income is usually higher for cotton farmers engaged in organic supply chains, as a result of cost savings on inputs and organic premiums of 10-20% on average. Yields obtained by some of the most experienced organic cotton farmers can approach those of good conventional ones. Our research in West Africa also shows that actual yields in conventional cotton are often much lower than research station averages, due to bad husbandry and poorer farmers 'selling on' their cotton agrochemical inputs (Williamson, 2003). Further benefits expressed by organic farm families are that they no longer suffer poisonings, they enjoy safer food and grow a wider range of food crops as part of the organic rotation (Truscott, 2009).

"It has been 5 years now since I decided to convert to organic cotton. I made this decision in 2001 because I had just suffered a miscarriage due to the use of pesticides. Organic cotton has given me more independence as a woman, because I receive a better income, and I am paid immediately after the harvest. I am now able to buy luxuries, clothing, crockery, something which is a real pleasure because I couldn't do it before. And more importantly, my children's health is no longer at risk."

Evelyn Ate Kokale, organic cotton farmer, Glazoué District, Benin.

Problem-solving research and development for sustainable organic cotton systems is woefully neglected by governments in cotton-producing countries and international donors. Conventional cotton systems tend to reward quantity (tons of cotton fibre at national level) rather than sustainability or social or environmental goods or services - putting the interests of ginners, exporters and foreign currency generation before those of cotton farming communities (Ferrigno et al., 2005). PAN has identified a list of R&D needs to improve organic cotton yields and systems from a farmer perspective, including: organic seed treatments; varietal improvement for resistance to pests and diseases; best practices for organic fertilization, weed management and tillage regimes in rainfed systems; and manipulating predator populations for more effective control of key pests. PAN's participatory research with farmers in Benin is adapting use of food sprays to attract key predators of cotton bollworm, first developed for large-scale cotton farms in Australia, to the resources and capacity of smallholders (Vodouhê et al., 2009).

Gaining better national or export markets for some of the numerous food crops grown by farmers as part of their organic cotton rotation is another important route for building sustainable cropping systems, livelihoods and enhancing local food security. Current work focuses on cashew and sheanut in Benin, with sesame, hibiscus and the millet-like *fonio* grain in Senegal (PAN UK, 2010).

5. Regulation into practice?

5.1 Stakeholder perceptions and policy coherence

While quantitative data is important for policymakers to make good informed decisions, qualitative and participatory methods are also essential for exploring important perceptions and experiences of farmers on pesticide use issues, which need to be considered by all those working to reduce pesticide externalities and promote IPM. One quote from a 30-year old cotton and cowpea farmer's wife from Voggu village in Ghana, obtained in 2001 fieldwork, conveys the family costs, personal tragedy and sense of disempowerment expressed by many about levels of pesticide dependency:

"*I have to look after my husband and provide him with food and water on the days when he has sprayed. I don't do any spraying myself but I still get affected, I still breathe in the pesticide. Once I came back from the farm and was vomiting and I had a miscarriage from inhaling the spray and had to go to hospital. The pesticide does its job but it's the side effects we don't like. There is no option- we have to do this.*"

Just as important is to understand the perceptions, viewpoints and attitudes of key stakeholders, which may pose obstacles to change at policy and programme levels. Table 5 summarises ten key issues of poor policy coherence or implementation, identified from open-ended interviewing of 80 stakeholders from government, private sector, research, donor, grower associations and NGO sectors in four African countries.

As one example of poor coherence at programme and broader policy levels, research in cowpea in Ghana and on cereal/legume systems in Ethiopia revealed increasing pest control problems on higher yielding varieties. These had been introduced by government and donor programmes in an attempt to improve local food security yet these varieties were far more susceptible to attack in the field and in storage by weevil and other pests, in comparison with local landraces. However, the energetic promotion of higher yielding varieties had not been accompanied by information on their pest control needs or the associated costs, nor by training in appropriate, affordable and safe pest control methods. Farmers interviewed attempted, not always successfully, to reduce yield losses by resorting to applications of unauthorized and often dangerous insecticides. Government crop protection staff in Ethiopia complained that pest control needs had been ignored in the policy focus on potential yield increases and their department was not allocated sufficient resources to address the urgent need for better pest management (Williamson et al., 2008).

5.2 Effective implementation?

The last decade has witnessed substantial and welcome legislation enacted on pesticide controls even in the poorest countries, yet there is very little implementation or monitoring of its effectiveness (Ramirez & Mumford, 1995; Williamson, 2007; Amera & Abate, 2008; SP-IPM, 2008). For example, several studies have reported significant 'leakage' of DDT from East African malaria control programmes -its only permitted use since 2004 under the Stockholm Persistent Organic Pollutants (POPs) Convention (Gebre-Medhin, 2003; Katima & Mng'anya,

Area	Issue
Pesticide policy	1. Wide recognition of pesticide misuse yet subsidy & direct provision of pesticides by governments & donors continue.
	2. Hazardous pesticides are not controlled effectively at regulatory level.
	3. The informal pesticide dealing network is flourishing yet controls concentrate on formal distribution systems.
	4. Emphasis on improving regulation on paper, rather than putting it into practice.
	5. Banned & restricted pesticides remain easily accessible in informal markets.
Agricultural policy	6. Food security programmes promote higher yielding crop varieties which are more susceptible to pests and therefore less affordable to poorer farmers, notably women.
	7. Agricultural intensification assumes higher use of external inputs without adequately addressing health, environmental or economic costs.
	8. Lack of mainstreaming of IPM principles & projects which promote safer alternatives.
	9. Disjuncture between recognizing need to address pesticide hazards & incorporating this into institutional practice.
Health policy	10. Health policy fails to address pesticide-related ill health adequately or recognize it as a major public health burden.

Source: Williamson, 2006

Table 5. Poor policy coherence and implementation identified in PAN research 2000-2005 in Benin, Ethiopia, Ghana and Senegal

2009; Amera, 2009). Why are pesticide controls so poorly implemented or enforced? Our research findings suggest a combination of lack of resources, possibly of political will too, and incoherence between environmental, health, rural development, agriculture and trade policy making (Williamson, 2005). The relative 'invisibility' of pesticide external costs in policy making may contribute too- Sherwood and colleagues concluded that poisoning impacts in Ecuador may well be equivalent to the public health burden posed by some important infectious diseases in that country (Sherwood et al., 2005).

Efforts to put tougher controls in place on the ground may face fierce opposition from vested interests in commercial and public organizations. In 2000, the Health Ministries of six Central American countries identified a regional 'Dirty Dozen' active ingredients responsible for the most frequent occupational and accidental poisonings, based on evidence gathered via the Latin American World Health Organisation (WHO) expanded health surveillance programme. Their list included nine WHO Class I and three Class II compounds along with a proposal for withdrawing approvals of these top problem pesticides at regional level. Unfortunately, implementation of the phase-out was blocked by agrochemical companies, the US and national Finance/Trade Ministries under the pretext of permitting 'free trade' in the Central American Free Trade treaty (Rosenthal, 2005). More recently, inclusion of endosulfan in the Stockholm POPs Convention has been thwarted by India and its state-funded pesticides manufacturer, despite almost universal consensus from technical experts and governments worldwide that this insecticide should no longer play a role in 21st century crop protection (PAN UK, 2009a).

Nevertheless, several UN agencies recognize the continued problem of pesticide externalities and have set up initiatives and awareness raising activities to tackle them. In 2004 FAO, WHO and the UN Environment Programme (UNEP) jointly published the report '*Childhood Pesticide Poisoning: Information for Advocacy and Action*' estimating up to 5 million cases of child pesticide poisoning occur each year, resulting in thousands of fatalities. Agency experts highlighted how children face higher risks from pesticides than adults because they are exposed more to such chemicals over the course of their lifetime and because they are more susceptible in physiological terms (FAO/WHO/UNEP, 2004). Stakeholder reflection on the failure of existing pesticide controls to reduce the incidence of damage to human health and environment led FAO and WHO to launch a new initiative for a progressive ban on Highly Hazardous Pesticides (HHP) in 2006 *http://www.fao.org/agriculture/crops/core-themes/theme/pests/pm/code/hhp/en/*. The HHP initiative recognizes that WHO Class II pesticide active ingredients ('moderately hazardous' in terms of acute mammalian toxicity as determined in laboratory testing), such as endosulfan, paraquat and chlorpyrifos, can be as problematic in reality as the 'extremely' and 'highly' hazardous pesticides which make up WHO Class I. This conclusion is also drawn from PAN's poisoning cases data in West Africa (PAN UK, 2008) and locust cost externality assessment in Senegal (Leach et al., 2008).

In 2009 PAN International published its 'List of Highly Hazardous Pesticides' as a contribution to these UN discussions (PAN Germany, 2009). It provides a catalogue of the most harmful pesticides that is more comprehensive, and takes into account more potential pesticide hazards, than current listings by official bodies (for example, endocrine disrupting properties, ecotoxicity and operator inhalation toxicity are not included in the latter). PAN believes it is essential to include chronic health hazard in the definition of HHPs. WHO very conservatively estimated at least 735,000 people annually suffer specific chronic defects and a possible 37,000 cases of cancer in developing countries (WHO, 1990). The PAN International HHP list also includes five environmental hazard criteria.

In its latest global report, PAN International assesses the very limited achievements of regulations, at global, regional and national levels to prevent pesticide poisonings and reduce harmful health and environmental impacts (PAN International, 2010). To redress this poor performance, report authors recommend a series of measures for governments to put in place (Box 2.), supporting the call by international agencies, including FAO and WHO, for more assertive action on pesticide hazards.

6. Pesticide use and impact reduction in supply chains

The regulatory approach, especially at international level, can be slow and tortuous. Meanwhile, some parts of the private sector have been taking action, via voluntary standards which prohibit and restrict the use of specific hazardous pesticides. Table 6 summarises WHO pesticide classes and hazard listings prohibited in six private schemes related to coffee production. Several UK supermarkets have similar prohibitions and restrictions (PAN UK, 2009b).

A concrete example of how supply chains can take positive action to phase out specific hazardous pesticides, using the PAN Highly Hazardous Pesticides list, is given in Table 7. In 2009, PAN UK was requested by British retailer Marks and Spencer to advise on prioritising top pesticides of concern from a list of 38 substances that remained in fairly common use in the retailer's non-EU supply base. Running these 38 through the HHP list

Box 2. PAN International recommendations for government action

1. Adopt and practice good governance regarding development and implementation of plant protection policies and regulations.
2. Invest in research and participatory, community-based training in agroecological systems, especially in Africa.
3. Insist on an agroecological approach in relevant policy measures and support, including incentives for rapid adoption of agroecological production (e.g. reducing taxes for land managed with agroecological approaches, ensuring access to credit and markets for agroecological producers).
4. Promote ecological, safer and non-chemical alternatives for pest management, as recommended by UNEP's Strategic Approach to International Chemicals Management (SAICM).
5. Strengthen consumer movements on food security and food safety, especially in Africa.
6. Adopt PAN International list of HHPs as the basis for a progressive ban on highly hazardous pesticides, and identify additional risky active ingredients to target for elimination, such as 'Pesticides whose handling and application require the use of personal protective equipment that is uncomfortable, expensive or not readily available' (Article 3.5, FAO/WHO Code of Conduct).
7. Base policy decisions on hazard assessment rather than risk assessment.
8. Adopt a pro-public health approach to eliminating pesticide poisonings, that takes action based on the intrinsic hazardous properties of pesticides, rather than considering pesticides on a case-by-case or incident-based approaches.
9. Adopt a precautionary approach to pesticide regulation.
10. Place liability onto pesticide manufacturers and distributors for human health and ecosystems harm. People and governments should not be left bearing the costs.
11. Legally require those who employ pesticide sprayers to provide full personal protective equipment (PPE), along with training and retraining on a regular basis.
12. Support establishment through WHO of poisoning information centres in developing countries.
13. Promote the use of community–based monitoring of pesticides worldwide. Adopt innovative strategies for measuring pesticide exposure and identifying priority areas for action.
14. Insist upon the implementation of international conventions related to chemicals.
15. Enact regulations on "right to information" and "right to know" to ensure that communities and agricultural workers are provided with full information on the pesticides that they exposed to or spray.
16. Implement legislation and regulations on pesticide management on national and regional levels, especially in Africa.

'screen' identified 28 with one or more HHP hazards. To produce a 'top 10' priority, PAN UK selected the nine pesticides which scored under three or more HHP criteria, plus endosulfan, based on PAN documentation of its major role in pesticide poisonings in many crops across the developing world. Marks and Spencer has now committed in its environmental responsibility Plan A to develop plans to phase these out in food production based on assessments of operator safety and environmental impact by 2012 (Marks and

Pesticide hazard category or list	Rainforest Alliance (SAN)	Utz Certified	FairTrade (FLO)	GlobalGAP	Common Code for Coffee (4CA)	C.A.F.É. Practices (Starbucks)
POPs list	**Yes**	**Yes**	No	No	**Yes**	No
PIC list	**Yes**	**Yes**	**Yes**	No (except 15 PIC pesticides also on EU 79/117 directive)	**Yes**	No (except those also WHO 1a/1b)
WHO Class 1a & 1b	No immediate prohibition but growers must phase out after 3 years of certification	No	Yes (with some specific exemption possible on certain crops but not coffee)	No	No immediate prohibition but growers must phase out within 3-5 years	Yes (with some possible specific exemption requests for nematicides)
WHO Class II	No	No	No	No	No	No
PAN "Dirty Dozen" list	**Yes**	No	Yes (with one exception possible for paraquat)	No	No	No
EU or US prohibited lists	**Yes** (some)	**Yes** (some)	No	**Yes** (some)	No	No
Methyl bromide	**Yes**	No	No	**Yes**	No	No

Yes *indicates when a specific scheme DOES prohibit pesticides in the particular hazard class*

Source: PAN UK, 2008c

Table 6. Pesticide prohibitions in six private coffee assurance standards

Spencer, 2010). Since most of these phase-out priorities are already banned in the EU, this type of unilateral phase-out action by a retail company makes a significant contribution to reducing hazardous exposure of farmers and farm workers in developing countries growing crops for export. Over half of these priority top 10 are either on the Rotterdam PIC list, or have been notified to PIC by the EU as qualifying as regional bans for health or environmental reasons (see PAN UK, 2008d for explanation of PIC notification in terms of which pesticides are banned in the EU). Taking action on PIC list and notified substances is a practical way in which food companies can support the aims of the Rotterdam Convention and their obligations under the FAO/WHO Pesticide Code of Conduct to address pesticide problems in developing countries (PAN Germany, 2005).

Active ingredient	No. hazard criteria under PAN HHP list	HHP hazard criteria
1. Aldicarb	3	WHO Ia; EU operator inhalation risk (R26 risk phrase); EU endocrine disrupting chemical (EDC)
2. Benomyl	4	Poss. cancer (US EPA); EU mutagen; EU reprotoxic; PIC list
3. Cadusafos	3	WHO Ib; v. persistent-sediment; highly toxic to bees
4. Lambda-cyhalothrin	3	R26; EDC; bees
5. Fentin hydroxide	3	R26; prob. cancer (EPA); poss. cancer (EU)
6. Metolachlor	3	Poss. cancer (EPA) v. persistent- water; v. persistent-sediment
7. Parathion-methyl	4	WHO Ia; R26; EDC; PIC list
8. Procymidone	4	Prob. cancer (EPA); poss.cancer (EU); EU reprotoxic; EDC
9. Trifluralin	4	Poss. cancer (US +EU); EDC; v. bioaccumulative
10. Endosulfan	2	R26; EDC

Table 7. Ten priority pesticides recommended by PAN UK for priority phase-out by Marks and Spencer retailer

Taking out specific pesticides based on intrinsic hazard is criticized by some as overcautious, economically risky or even unscientific (FERA, 2008; Farmers Weekly, 2008). Such voices advocate instead an approach based on risk management and mitigation, while some private sector initiatives blend hazard and risk-based approaches (e.g. Unilever, 2010). Reliance on probabilistic risk assessment based mainly on current known facts, often extrapolated from laboratory studies and based on overoptimistic assumptions about compliance with good agricultural practices, simply cannot tackle the scientific uncertainties around the extent of health and environmental exposure and the complex and largely unknown interactions inside non-target organisms, including humans, between pesticides and other chemicals at ecologically relevant concentrations in the field. Critics point out that pesticide regulation policy is a value-laden process and the narrative space around it dynamic and highly contested (Bro-Rasmussen, 1999; Watterson, 2001; and Irwin & Rothstein, 2003). The stance of the agrochemical industry and some governments on pesticide exposure and risk minimization stands in contrast to the industrial hygiene approach used in many other occupational health and safety fields, where the most effective option is to 'remove the hazard', recognizing that human error can never be eliminated (Sherwood et al., 2002; Gee, 2004). The authors of PAN International's latest global overview of poisonings stress how the current regulatory approach of delaying action until evidence of health or environmental impacts becomes apparent places an enormous and unfair burden on pesticide users, farm workers and rural communities, particularly in developing countries. It also causes environmental damage and incurs hidden economic costs (PAN International, 2010).

Beyond debate over the merits of reducing risk versus hazard, PAN UK's assessment is that prohibiting or phasing out a set of pesticides can trigger useful change in pest management practice- 'what do you do instead of using pesticides x, y and z'? Such actions can be powerful drivers for IPM, for example, British retailer Marks & Spencer are trialling alternatives to hazardous compounds used in large-scale viticulture, as well as safer pest management in rice cultivation by Indian smallholders (Franklin, 2009). Unilever's sustainable agriculture programme has focused on reducing pesticide reliance in general and in India has supported its smallholder gherkin growers to reduce fungicide use by 78% mainly by better agronomic practices and changing attitudes among farmers and advisers (Ramesh, 2008). Helping farmers to put IPM into practice does require the food and fibre sectors to invest in technical R&D and advice. PAN UK would like to see much more private investment, with public research institutes and farmer associations, into a more ecologically-informed Integrated Production approach, addressing not just pesticide use but also fertilizers, energy, carbon footprints, climate change, soil and water management, as for example, Unilever is doing in its supply chains (Smith, 2008).

7. Conclusion

Evidence from field documentation of poisonings and results from the few programmes of increased health surveillance show clearly that in the 21st century, hazardous pesticides are still routinely used in unsafe situations. African farmers are possibly the least equipped among the developing world to protect themselves and their community against the hazards of pesticide use, in terms of literacy, education, access to information and poverty. While pesticide use in Africa appears lower than in other parts of the world, rural populations and the environment are likely to suffer significant exposure. To date, while most African countries have ratified the major pesticide-relevant global conventions, they lack the resources to implement these properly.

Researchers, policy makers and donors need to pay more attention to external costs and implement a variety of policy and programme measures to cut back on use of hazardous pesticides and implement safer alternatives. This is also a major conclusion from the global report of the International Assessment of Agricultural Science, Knowledge and Technology for Development (IAASTD, the UN expert assessment 'equivalent' to the Millenium Ecosystem Assessment), to which 58 countries have signed up (IAASTD, 2009). The African IAASTD regional chapter highlights "*the economic, environmental and health costs associated with greater use of agrochemicals suggests that agricultural knowledge, science and technology options involve reorienting research away from high-input blanket doses towards technologies that enable technically efficient applications specific to local soil conditions and towards integrated nutrient management approaches*" (IAASTD 2009b). The need to address external costs as one of the priorities also features in a key review on food security challenges published in *Science* this year (Godfray et al., 2010) and in recent assessments conducted for better decision making on appropriate pest control choices in Mediterranean citrus production (Leach and Mumford, 2008).

Innovative ecotoxicology monitoring with government agencies and staff of PAN Africa affiliate NGOs in Ethiopia and Tanzania, under the auspices of the FAO and World Bank-funded African Stockpiles Program, illustrates the research and policy value of community-based monitoring methods, backed up with expert technical support. In Ethiopia secondary

school students were trained as data collectors for an assessment of pesticide use and IPM impact in cotton and subsequently engaged enthusiastically in hazard awareness-raising in their villages and as local 'champions' for IPM (Amera, 2009). Ecotoxicology experts provided a Rapid Risk Assessment (RRA) for the two most widely used pesticides as reported in the survey: the herbicide 2,4-D and illegal use of DDT insecticide. The RRA highlighted serious risks to certain wildlife in the Rift Valley's unique alkaline salt marshes, an important passage zone for aquatic and insectivorous Palaearctic migrant birds (Amera & Abate, 2008).

In Tanzania, PAN has pioneered new ways to reduce the distance between policymakers, implementing agencies and communities affected by pesticides (Touni, 2009). Conducting training in community-based monitoring with local NGOs, villagers and government officers led to the joint development of an Environmental Incident Reporting procedure – the first attempt in the world to establish an upward reporting chain from community level to the Secretariat of the Rotterdam PIC Convention. Village environment committees are the key link in the chain, with an 'open door' to report directly to Tanzania's Designated National Authority for the Rotterdam Convention, hosted in the Ministry of Agriculture, Food Security & Cooperatives. A pilot project is adapting a suitable health monitoring form, combining elements of the PIC health incident reporting form with community monitoring tools developed by PAN Asia Pacific, which are more suitable for rural communities with low literacy levels. Such work enables international Conventions to reflect concerns and problems identified at field level.

Research has a crucial role in making farming a safer, as well as a more sustainable and rewarding, livelihood for the millions of small-scale farmers and farm workers in developing countries (Murray et al., 2002; Pretty and Waibel, 2005; Kishi, 2005; Leach et al., 2008). This requires researchers to work closely with farmer groups and food and fibre supply chain actors, plus civil society stakeholders, in liaison with relevant government and donor programmes for poverty reduction, health and environmental improvement. Undertaking small surveys combining quantitative and qualitative methods to estimate human health, livestock and wildlife impacts from acute toxicity can serve as an invaluable first step to opening the eyes of farmers themselves and decision makers about the reality of external costs. Such research is most effective when using multidisciplinary and participatory approaches, not relying on questionnaire surveys alone, but adding social science methods that can identify important perceptions behind people's opinions and concerns. PAN's experience is that a fruitful marriage of natural and social science methods, with community participation of this kind, is true 'action research' and one which can lead to real change at policy and practical levels in reducing the burden of pesticide-related harm. Ultimately, the huge gap between aspirational standards in international pesticide policy recommendations and conventions and the reality of those living and working near pesticide use can only be bridged by promoting safe and sustainable strategies for agricultural development.

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9. References

Agrow. (2006). World agchem market steady. *AGROW* 497, 9 June 2006 p.17.

Ajayi, O.O.C. (2000). Pesticide use practices, productivity and farmers' health: the case of cotton-rice systems in Côte d'Ivoire, West Africa. Pesticide Policy Project Special Issue Series no. 3, GTZ/University of Hannover, Germany.

Ajayi, O., Camara, M.., Fleischer, G.., Haidara, F., Sow, M.., Traoré, A. and van der Valk, H. (2002). Socio-economic assessment of pesticide use in Mali. Pesticide Policy Project Special Issue no. 6, GTZ/ University of Hannover, Germany.

Amera, T. (2008). A comprehensive study of impacts of cotton IPM in the Rift Valley of southern Ethiopia. Institute for Sustainable Development, Ethiopia, and PAN UK, Addis Ababa.

Amera, T. and Abate, A. (2008). An assessment of pesticide use, practice and hazards in the Ethiopian Rift Valley. Report for the African Stockpiles Program. Institute for Sustainable Development, Addis Ababa, and PAN UK. London.

Badarou, S. and Coppieters, Y. (2009). Intoxications alimentaires dues à l'endosulfan : mise en place d'un système de notification et de prise en charge au Bénin. *Environnement risques et santé* 8 (2) 133-136.

Bro-Rasmussen, F. (1999). Precautionary Principle and/or risk assessment. A penitence in contemporary political culture. *Environmental Science & Pollution Research* 6 (4) 188-192.

Colborn, T. (2006). A case for revisiting the safety of pesticides: a closer look at neurodevelopment. *Environmental Health Perspectives* 114 (1) 10-17. doi 10.1289/ehp.7940

Cole, D.C., Carpio, F. and León, N. (2000). Economic burden of illness from pesticide poisonings in highland Ecuador. *Pan American Review of Public Health* 8 (3) 196-201.

CropLife International. (2009). Facts and figures: the status of global agriculture. *www.croplife.org*

Dinham, B. (2005). Corporations and pesticides. In: *The Pesticide Detox. Towards a more sustainable agriculture*. Pretty, J. (Ed.) Earthscan, London, pp 55-69.

Doumbia, M. and Kwadjo, K.E. (2009) Practices of use and management of pesticides by market gardeners in Côte d'Ivoire: the case of the city of Abidjan and two of its suburbs. *Journal of Applied Biosciences* 18 992-1002. ISSN 1997–5902

Ecobichon, D. (2001). Pesticide use in developing countries. *Toxicology* 160 27-33.

FAO/WHO/UNEP (2004). UN warns of children poisoned by pesticides. FAO press release, October 2004, Rome.

Farmers Weekly (2009). Pesticides ban: How it will affect your crops. *Farmers Weekly* 29/01/2009

FERA (2008). Evaluation of the impact of the proposed revisions to EU Directive 91/414/EEC on wheat production in the UK. A report commissioned by the Department for Environment, Food and Rural Affairs . Central Science Laboratory, York .

Ferrigno, S., Ratter, SG., Ton. P., Vodouhê, SD., Williamson, S. and Wilson, J. (2005). Organic cotton: a new development path for African smallholders? Gatekeeper series no. 120, International Institute for Environment & Development, London.

Franklin, S. (2009). UK retailer steps up action on pesticides. *Pesticides News* 85 6-7

Gebre Medhin, H. (2003). Facts on DDT use in Ethiopia. *Pesticides News* 62 12-13.

Gee, D. (2004). Late Lessons From Early Warnings. *Epidemiology* 15 (4) S113

Godfray, HCJ, Beddington, JR, Crute, IR, Haddad, L, Lawrence, D, Muir, JF, Pretty, J, Robinson, S, Thomas, SM and Toulmin, C. (2010). Food security: The challenge of feeding 9 billion people. *Science* 327 812-818 doi 10.1126/science.1185383

Houndekon, V.A., De Groote, H. and Lomer, C. (2006). Health costs and externalities of pesticide use in the Sahel. *Outlook on Agriculture* 35 (1) 25-31.

IAASTD (2009a). Agriculture at a crossroads. Global summary for decision makers. International Assessment of Agricultural Knowledge, Science & Technology for Development. Island Press, Washington. *www.agassessment.org*

IAASTD (2009b). Summary for decision makers of the Sub-Saharan Africa (SSA) report. International Assessment of Agricultural Knowledge, Science & Technology for Development. Island Press, Washington.

ILO (1994). Chemicals in the working environment. In: World Labour Report 1994, International Labour Organisation, Geneva.

Irwin, A. and Rothstein, H. (2003). Regulatory science in an international regime. In: *Pesticides: Problems, Improvements, Alternatives.* Den Hond, F., Groenewegen, P. and van Straalen, N.M. (Eds). Blackwell Science, Oxford, pp 77-86.

Jeyaratnam, J. (1990). Acute pesticide poisoning: a major global health problem. *World Health Statistics Quarterly* 43 139-144.

Jungbluth, F. (1996). Crop protection policy in Thailand-economic and political factors influencing pesticide use. Pesticide Policy Project Publication Series no. 5, Institut fur Gartenbauokonomie, University of Hannover, Germany.

Kangas, J. and Tuomainen, A. (1999). Occupational exposure and biological monitoring of pesticides. African Newsletter on Occupational Health & Safety, 1/99. *http://www.occuphealth.fi/e/info/anl/199/biolog05.htm*

Katima, JHY & Mng'anya, S. (2009). African NGOs outline commitment to malaria control without DDT. *Pesticides News* 84 p.5

Kishi, M. (2005). The health impacts of pesticides: what do we now know? In: *The Pesticide Detox. Towards a more sustainable agriculture.* Pretty, J. (Ed). Earthscan, London, pp 23-38.

Kodjo, E.A. (2007). ANCE fights for the prohibition of the use of endosulfan in Togo. International POPs Elimination Network (IPEN) Newsletter 57, November 2007. *http://www.ipen.org/ipenweb/news/*

Leach, AW and Mumford, JD. (2008). Pesticide Environmental Accounting: a method for assessing the external costs of individual pesticide applications. *Environmental Pollution* 151 139-147 doi 10.1016/j.envpol.2007.02.019

Leach, A., Mullié, WC, Mumford, JD and Waibel, H. (2008). Spatial and historical analysis of pesticide externalities in locust control in Senegal- first steps. Imperial College London, University of Hanover and FAO.

London, L. and Baillie, R. (2001). Challenges for improving surveillance for pesticide poisoning: policy implications for developing countries. *International Journal of Epidemiology* 30 564-570

London, L., Flisher, A.J., Wesseling, C., Mergler, D. and Kromhout, H. (2005). Suicide and exposure to organophosphate insecticides: cause or effect? *American Journal of Industrial Medicine* 47 302-321. doi 10.1002/ajim.20147

Mancini, F., Van Bruggen, A.H.C., Jiggins, J.L.S., Ambatipudi, A.C. and Murphy, H. (2005). Acute pesticide poisoning among female and male cotton growers in India. *International Journal of Occupational and Environmental Health* 11 221-232.

Marks and Spencer. (2010). How we are doing. Annual report on corporate social responsibility. Via *http://plana.marksandspencer.com/media/pdf/we_are_doing/sustainable-raw-materials/sustainable_raw_materials_2010.pdf*

Martin, T., Ochou, G.O., Djihinto, A., Traore, D., Togola, M., Vassal, J-M., Vaissayre, M. and Fournier, D. (2005). Controlling an insecticide-resistant bollworm in West Africa. *Agriculture, Ecosystems and Environment* 107 (4) 409-411. doi10.1016/ j.agee.2004.11.006

Matthews, G., Wiles, T. and Baleguel, P. (2003). A survey of pesticide application in Cameroon. *Crop Protection* 22 707-714. Doi 10.1016?S0261-2194(03)00008-5

Maumbe, B. M. and Swinton, S. M. (2003). Hidden health costs of pesticide use in Zimbabwe's smallholder cotton growers. *Social Science and Medicine* 57 (9) 1559-1571. doi 10.1016/SO277-9536(03)00016-9

Meredith, K.F.S. (2003). Poisonings in developing countries- challenges and opportunities. *Toxicology* 191 (3) 2-3.

Murray, D., Wesseling, C., Keifer, M., Corriols, M. and Henao, S. (2002) Surveillance of pesticide-related illness in the developing world. Putting the data to work. *Int. J Occupational & Environmental Health* 8 243-248.

Ngowi, A.V.F., Maeda, D.N., Wesseling, C., Partanen, T.J., Sanga, M.P. and Mbise, G. (2001). Pesticide-handling practices in agriculture in Tanzania: observational data from 27 coffee and cotton farms. *International Journal of Occupational and Environmental Health* 7 (4) 326-332.

Okoumassoun, L., Brochu, C., Deblois, C., Akponan, S., Marion, M., Averill-Bates, D. and Denizeua, F. (2002). Vitellogenin in Tilapia male fishes exposed to OC pesticides in Oueme river in the Republic of Benin. *Science of the Total Environment* 299 (1-3) 163-171. doi 10.1016/S0048-9697(01)01053-1

PAN Africa. (2009). Atelier International *Eau et Pollution des Eaux par les Pesticides en Afrique*. Hôtel Laguna Beach - Mbodiène (Sénégal) 2-6 juin 2008. Rapport narratif. PAN Afrique, Dakar, Senegal.

PAN Germany. (2004). Proceedings 04 Cotton: a European conference on developing the organic cotton market, held 10-13 February 2004, Hamburg, Germany.

PAN Germany. (2005). The FAO Code: Grab it for Action! Via *http://fao-code-action.info/action_centre.html*

PAN Germany. (2009). *PAN International List of Highly Hazardous Pesticides* Via *http://www.pan-germany.org/*

PAN International. (2010). *Communities in Peril: Global report on health impacts of pesticide use in agriculture*. PAN Asia Pacific, Penang. ISBN 978-983-9381-52-8

PAN UK. (2004). Living with Poison: endosulfan problems in West African cotton systems. PAN UK, London. *http://www.pan-uk.org/publications/living-with-poison*

PAN UK. (2007). Hazardous pesticides and health impacts in Africa. Food & Fairness briefing no. 6. London. *https://secure.virtuality.net/panukcom/PDFs/Hazardous_pesticides_and_health_impacts_in_Africa.pdf*

PAN UK. (2008a). Which pesticides are banned in Europe? Food & Fairness briefing no. 1. London. *http://www.pan-uk.org/Publications/*

PAN UK. (2008b). Hazardous pesticides and health impacts in Africa. Food & Fairness briefing no. 6, London. Via *http://www.pan-uk.org/Publications/*

PAN UK. (2008c). Reducing hazardous pesticide practice in coffee supply chains, Food & Fairness case study, PAN UK, London. *http://www.pan-uk.org/PDFs/f&f-coffee.pdf*

PAN UK. (2008d). Which pesticides are banned in Europe? Food & Fairness briefing no. 1, updated April 2008, London.

PAN UK. (2009a). Stockholm Review Committee action on India. *Pesticides News* 85 p.9

PAN UK. (2009b). Comparison of UK supermarket performance on pesticide problems, March 2009 website update. Via *http://www.pan-uk.org/Supermarkets*

PAN UK. (2010). Organic cotton systems reduce poverty and food insecurity for African farm families. Fibre, Food & Beauty project briefing. PAN UK, London. Via *http://www.pan-uk.org/foodAfrica/PDFs/*

Poisot, A-S. (2007). Implementing safer pest and pesticide management in supply chain interventions: some considerations from African experiences. *Presentation at PAN UK workshop 'How can supply chains foster safer pest management in African local and export markets?' held in Utrecht, NL, 31 Oct-1 Nov 2007.*

Pretty, J. and Waibel, H. (2005). Paying the price: the full cost of pesticides. In: *The Pesticide Detox. Towards a more sustainable agriculture*. Pretty, J. (Ed). Earthscan, London, pp 39-54.

Ramesh, A. (2008). Unilever work with Indian gherkin growers to reduce pesticide use. *Pesticides News* 80 10-11.

Ramirez, O.A. and Mumford, J.D. (1995). The role of public policy in implementing IPM. *Crop Protection* 14 (7) 565-572. doi 10.1016/0261-2194(95)00051-8

Repetto, R. and Baliga, S.S. (1996). *Pesticides and the immune system: the public health risks*. World Resources Institute, Washington.

Rosenthal, E. (2005). Who's afraid of national laws? Pesticide corporations use trade negotiations to avoid bans and undercut public health protections in Central America. *International Journal of Occupational and Environmental Health* 11 437-443.

Sanfilippo, D. (2007). *When organic means fair. The case of cotton*. PAN UK leaflet, London.

Sherwood, S., Crissman, C. and Cole, D. (2002). Pesticide exposure and poisonings in Ecuador: a call for action. *Pesticides News* 55 3-6.

Sherwood, S., Cole, D., Crissman, C. and Paredes, M. (2005). From pesticides to people: Improving ecosystem health in the Northern Andes. In: *The Pesticide Detox. Towards a more sustainable agriculture*. Pretty, J. (Ed.) Earthscan, London, pp 147-164.

Sibanda, T., Dobson, H.M., Cooper, J.F., Manyangarirwa, W. and Chiimba, W. (2000). Pest management challenges for smallholder vegetable farmers in Zimbabwe. *Crop Protection* 19 807-815. doi 10.1016/S0261-2194(00)00108-3

Silvie, P., Deguine, J.P., Nibouche, S., Michel, B. and Vaissayre, M. (2001). Potential of threshold-based interventions for cotton pest control by small farmers in West Africa. *Crop Protection* 20 297-301. doi 10.1016/SO261-2194(00)00146-0

SP-IPM. (2008). Incorporating Integrated Pest Management into national policies. IPM Research Brief no. 6, Systemwide Program on Integrated Pest Management, IITA, Ibadan, Nigeria.

Smith, G. (2008). Developing sustainable food supply chains. *Phil. Trans. R. Soc. B* 363 849–861 doi 10.1098/rstb.2007.2194

Szmedra, P. (2001). What price agricultural productivity? Pesticides and the health of sugar farmers in Fiji. *Development in Practice* 11 (1) 91-96.

Thiam, A. (2009). Endosulfan banned in West Africa. *Pesticides News* 83 14-15.

Thiam, M. and Touni, E. (2009). Pesticide poisoning in West Africa. *Pesticides News* 85 3-4. Ton, P. et al., (2000). Endosulfan deaths and poisonings in Benin. *Pesticides News* 47 12-14

Ton, P. (2001). Cotton production and rural livelihoods in West Africa. Situation and trends in the context of trade liberalization and structural adjustment. Oxfam GB and P. Ton, Dakar, Senegal.

Touni, E. (2008). Linking rural African communities with global policy-makers. *Pesticides News* 80 16-17

Truscott, L. (2009). Improving livelihoods for organic cotton farmers. *Pesticides News* 83 10-13

Unilever. (2010). Unilever Sustainable Agriculture Code 2010.
Via *http://www.unilever.com/sustainability/environment/agriculture/approach/*

Vodouhê, DS, Mensah, R., Sanfilippo, D and Assogba, G. (2009). A new tool for improving organic cotton yields in Africa. *Pesticides News* 84 6-9

Watterson, A. (2001). Pesticide health and safety and the work and impact of international agencies: partial successes and major failures. *International Journal of Occupational & Environmental Health* 7 (4) 339-347.

Watts, M. (2008). Time to act on endosulfan. *Pesticides News* 81 3-7

WHO. (1990). The public health impact of pesticides use in agriculture. World Health Organisation, Geneva.

Williamson, S. (2003). Pesticide provision in liberalised Africa: Out of control? *Agricultural Research & Extension Network Paper no. 126*, Overseas Development Institute, London.

Williamson, S. (2005). Breaking the barriers to IPM in Africa: Evidence from Benin, Ethiopia, Ghana and Senegal. In: *The Pesticide Detox. Towards a more sustainable agriculture*. Pretty, J. (Ed.) Earthscan, London, pp 165-180.

Williamson, S. (2006) .Agricultural policy and pesticides in Africa: a study of the impacts on health and livelihoods. Thesis submitted for the degree of Doctor of Philosophy, Dept. Biological Sciences, University of Essex.

Williamson, S. (2007). The Hidden Costs of Pesticide Use in Africa. *Pesticides Management in West Africa, FAO/ ECOWAS newsletter no.6*, December 2007, FAO, Accra, Ghana.

Williamson, S., Ferrigno, S. and Vodouhe, S.D. (2005). Needs-based decision-making for cotton problems in Africa: a response to Hillocks. *International Journal of Pest Management* 51 (4) 219-224.

Williamson, S., Ball, A. and Pretty, J. (2008). Trends in pesticide use and drivers for safer pest management in four African countries. *Crop Protection* 27 1327-1334 doi 10.1016/j.cropro.2008.04.006

Youdeowei, A. (2001). Pest management plan for cotton production. Consultant's report for the Benin Cotton Sector Reform Program. World Bank/FAO, Accra, Ghana.

3

Dietary Intake of Environmentally Persistent Pesticides in the European Population

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1. Introduction

The pesticide industry has contributed to increase the benefits of the agricultural sector by controlling plagues and vegetable diseases. Moreover, several human diseases as malaria, encephalitis or phylariasis, have been effectively controlled with this type of products (Abhilash & Singh, 2009). The need to increase the food production, due to the fast growth of the global population, involves the use of pesticides to prevent the loss of 45% of global food production due to pests. This fact is more important in tropical countries where humidity and temperature are higher (Abhilash & Singh, 2009). Because the use and abuse of such substances, global pollution has become one of the most important problems of the modern societies and pesticides play a major role among the chemical contaminants that are released to the environment every year. According to the Food and Agriculture Organization (FAO) data, more than 500.000 T of obsolete and non-used pesticides represent a real threat and a risk for environment and global public health. In late 1990, more than 100.000 chemical substances were registered in the European catalogue of commercialized chemical substances (European Environmental Agency, 2007). It must be highlighted that less is known about late toxicity effects of the majority of these compounds. Thus, only the 14% of these 100.000 substances have enough toxicological data to ensure its safety. Several studies have reported that human beings could have 300 synthetic chemical substances in blood and different tissues, some of them clearly established as adverse for health. Some of these adverse effects are:

- In males: testicular cancer, cryptorchidism, lymphoma non-Hodgkin, multiple myeloma, decrease in spermatozoa quality, occupational disease (asthma, some kind of cancer types…), etc.
- In females: breast cancer, lymphoma non-Hodgkin, spontaneous abortion, birth defects (mainly in the genito-urinary tract), occupational disease, etc.
- In children: increment of childhood cancer, immunology impairment, early puberty, learning problems, etc.

These substances act over environment and over living at low concentrations and, more important, in combination. Each mix of substances is different according to many factors, giving to the situation a higher dimension and hindering their understanding and resolution. Obviously, pesticide residues affect not only humans but also wildlife, pets, fish, livestock, and the environment in general. It is the real globalization.

Of all the pesticides, organochlorine compounds, used as insecticides, are particularly relevant from the point of view of waste. Although banned in developed countries, substances as dichlorodiphenyltrichloroethane (DDT), hexachlorocyclohexane (HCH), aldrin and dieldrin are used in developing countries because its low costs, efficacy and versatility against pests and disease-carrying insects (Lallas, 2001). Due to their high lipophilicity, stability and resistance to degradation, their levels remain high in the environment as persistent organic pollutants (POPs). Their presence in the environment leads to their introduction into the food chain, especially affecting food of animal origin with higher fat content. These substances tend to be bioaccumulated into the fat tissues of living beings along their entire lives, and to be biomagnificated across trophic levels in the food chain.

The situation has reached a high degree of relevance. In 2006, the European Union started a legislative project entitled "Registration, Evaluation, Authorisation and Restriction of Chemical substances" (REACH), with the aim of to improve the protection of human health and the environment through the better and earlier identification of the intrinsic properties of chemical substances (Williams et al., 2009). The new law entered into force on 1 June 2007. Nonetheless, the REACH project does not solve the background of the problem. That is, what can we do with all the persistent substances discharged into the environment for decades?

2. Routes of exposure

The main problem of exposure to pollutants, such pesticides, lies in the chronic nature of it. Humans are exposed to low amounts of several substances for long periods of life. Thus, acute intoxications as well as chronic exposure in certain labours remain less important.

There have been identified different sources of exposure. Without doubt, the way of food should be considered the most important one. Moreover, due to the high lipophility of these substances, foods with higher amounts of fat become the most dangerous. Thus, some products that must be considered essential for life, such as milk, fish or meat, become important risk factors for health (Yaktine et al., 2006). The contribution of specific food groups to daily intake of chemical contaminants may vary by country or geographic region (Sasamoto et al., 2006). Eating habits, the chemical composition of food, and the period in which the studies are done can explain the differences in the contribution of food to the global chemical exposure among different human populations. In this way, the results obtained from different studies are not easily comparable from one region to another. Moreover, there are several social and demographic factors such as sex, age, ethnicity, urban or rural residence and economic status that are strongly associated with changes in eating habits (i.e., fat intake may vary from 30 to 130 g/day (FAO/WHO, 1994)). Furthermore, the levels of residues into food vary in relation to the geographical area. The local pattern of use, the environmental practises and the food security strategies modify this parameter (Chikuni et al., 1997; Koopman-Esseboom et al., 1994). In that way, the levels of DDT, DDE and HCH

found in butter from 23 different countries were higher in those countries that used these substances for longer periods of time (Kalantzi et al., 2001). Finally, although the time to do the study may influence the results, when we talk about persistent pollutants, factors such as seasonality become less important (Ryan et al., 1990).

3. Environmentally persistent pesticides

The most important pesticides, from the standpoint of environmental and health, are the persistent ones: DDT (and its metabolismsm, DDE and DDD), HCH, aldrin, dieldrin, ... Those substances, due to their liposolubility, tend to be bioaccumulated into the fat tissues and to be biomagnificated in the food chain (Gray, 2002). The exposure starts even at embryonic stages (Luzardo et al., 2009). It has been reported that Eskimos population, who eat very fatty foods like salmon or seal, ingest levels of these compounds that are well above the acceptable daily intake (ADI) established by the World Health Organization (WHO) (Bonefeld-Jorgensen et al., 2006). The same results have been observed in groups of population who present a high intake of milk (i.e. baby infants) (Trapp et al., 2008). Most POPs are banned, severely restricted or carefully managed in industrialized countries; however, some of them are still manufactured and used in developing countries. The physical and chemical characteristics of these substances make possible that a POP released anywhere, can eventually grow to anywhere in the world. These pesticides are propagated by evaporation into the atmosphere from warmer regions of the world -tropical countries, which is precisely where they are most commonly used- to the cooler regions, where they are deposited by condensation (Scheringer, 2009). Evaporation rates in these cold regions of the planet are very low, therefore, such substances are "trapped" there, and deposited in living organisms (Scheringer, 2009). Anyhow, in contrast to expectations, the levels of POPs in tropical countries are similar to those found in the coolest regions (Weber et al., 2008). This observation could be explained because these types of pesticides are indeed effective, cheap and safe from the point of view of acute poisoning. The cultural level of the user is very low, and the controls to restrict the illegal trade of these substances are ineffective (Ssebugere et al., 2010). The problem grows because some of these substances (i.e. DDT) are extremely effective against certain insect-borne diseases. Thus, it requires the management of pesticides and banned products already manufactured. Some countries, in collaboration with FAO, have made an inventory of obsolete pesticides, which have been subsequently destroyed. Anyway, due to loss of labeling, leakage and poor storage conditions, is impossible to know the real amount of these uncontrolled substances, which thus become a major source of pollution (Felsot et al., 2003; Haylamicheal & Dalvie, 2009). As stated above, it follows that no country can combat the problem independently. A comprehensive strategy is needed to tackle a global problem.

Different governments have developed many projects and agreements in order to control the exposure and emission of POPs. Some of the most important are the following:

- Candidate Substances List for Bans and Phase-Outs (Ontario, Canada; 1992)
- Accelerated Reduction/Elimination of Toxics (ARET) Program (Canada; 1994)
- Toxic Substances Management Program (TSMP) (Canada; 1995)
- Sound Management of Chemicals (SMOC) (North American Commission for Environmental Cooperation; 1995)
- Waste Minimization Program (US Environmental Protection Agency; 1998)

Most of these programs had ambitious goals that were not achieved. On May 23, 2001, more than 90 countries signed the Stockholm Convention on Persistent Organic Pollutants.

Substance	Comments	Commercial use
Aldrin	Annex A *	Pesticide
Chlordane	Annex A *	Pesticide
Chlordecone	Annex A **	Pesticide
DDT	Annex B *	Pesticide
Dieldrin	Annex A *	Pesticide
Endosulfan	***	Pesticide
Endrin	Annex A *	Pesticide
Heptachloro	Annex A *	Pesticide
Hexabromobiphenyl	Annex A **	Industrial chemical
Hexabromocyclododecane	***	Industrial chemical
Hexabromodiphenyl ether and heptabromodiphenyl ether	Annex A **	Industrial chemical
Hexachlorobenzene (HCB)	Annex A *	Pesticide/Industrial chemical
Alpha hexachlorocyclohexane	Annex A **	Pesticide/By-product
Beta hexachlorocyclohexane	Annex A **	Pesticide/By-product
Lindane (γ HCH)	Annex A **	Pesticide
Mirex	Annex A *	Pesticide
Toxaphene	Annex A *	Pesticide
Pentachlorobenzene (PeCB)	Annex A **	Pesticide/Industrial chemical
Perfluorooctane sulfonic acid (PFOS), its salts and perfluorooctane sulfonyl fluoride (PFOS-F)	Annex B **	Industrial chemical
Polychlorinated biphenyls (PCB)	Annex A *	Industrial chemical
Polychlorinated dibenzo-p-dioxins (PCDD)	Annex C *	By products
Polychlorinated dibenzofurans (PCDF)	Annex C *	By products
Short-chained chlorinated paraffins	***	Industrial chemical
Tetrabromodiphenyl ether and pentabromodiphenyl ether	Annex A **	Industrial chemical

* Initial POPs from the dirty dozen.
** New substances added on May 2009. *** Chemicals proposed for listing.

Table 1. Complete list of substances included in the Stockholm Convention.

The Convention entered into force on May 17, 2004 with a main goal: "Protecting human health and the environment of persistent organic pollutants, reducing or eliminating their emissions into the environment". The list of POPs that is currently considered is the following:

- Annex A (substances that must be eliminated): pesticides (aldrin, chlordane, dieldrin, endrin, heptachlor, CHC, mirex, hexachlorobenzene or toxaphene) and industrial chemicals (i.e. polychlorinated biphenyls (PCBs)).
- Annex B (restricted substances): pesticides (DDT) and other industrial chemicals included.
- Annex C (reduction of emissions of unintentionally produced substances): industrial sub-products included.

Initially, signatory countries aimed at reducing and / or eliminate the production and use of the most dangerous POPs, known as "Dirty Dozen". It was established a mechanism by which they could add other POPs to the Convention in the future. Thus, in May 2009, nine more dangerous substances were listed. Actually, three new compounds are under revision.

The following is a brief description of the pesticides, as published in the official website of the Stockholm Convention (www.pops.int):

- Aldrin: A pesticide applied to soils to kill termites, grasshoppers, corn rootworm, and other insect pests, aldrin can also kill birds, fish, and humans. In humans, the fatal dose for an adult male is estimated to be about five grams. Humans are mostly exposed to aldrin through dairy products and animal meats. Studies in India indicate that the average daily intake of aldrin and its byproduct dieldrin is about 19 micrograms per person.
- Chlordane: Used extensively to control termites and as a broad-spectrum insecticide on a range of agricultural crops, chlordane remains in the soil for a long time and has a reported half-life of one year. The lethal effects of chlordane on fish and birds vary according to the species. Chlordane may affect the human immune system and is classified as a possible human carcinogen. It is believed that human exposure occurs mainly through the air, and chlordane has been detected in the indoor air of residences in the US and Japan.
- Chlordecone: It was first produced in 1951 and introduced commercially in 1958. Chlordecone is highly persistent in the environment, has a high potential for bioaccumulation and biomagnifications and based on physico-chemical properties and modelling data, chlordecone can be transported for long distances. It is classified as a possible human carcinogen and is very toxic to aquatic organisms.
- DDT: It was widely used during World War II to protect soldiers and civilians from malaria, typhus, and other diseases spread by insects. DDT continued to be used to control disease, and it was sprayed on a variety of agricultural crops, especially cotton. DDT continues to be applied against mosquitoes in several countries to control malaria. Its stability, its persistence (as much as 50% can remain in the soil 10-15 years after application), and its widespread use have meant that DDT residues can be found everywhere; residual DDT has even been detected in the Arctic. The short-term acute effects of DDT on humans are limited, but long-term exposures have been associated with chronic health effects. DDT has been detected in breast milk, raising serious concerns about infant health.
- Dieldrin: Used principally to control termites and textile pests, dieldrin has also been used to control insect-borne diseases and insects living in agricultural soils. The

pesticide aldrin rapidly converts to dieldrin, so concentrations of dieldrin in the environment are higher than dieldrin use alone would indicate. Dieldrin is highly toxic to fish and other aquatic animals, particularly frogs, whose embryos can develop spinal deformities after exposure to low levels. Dieldrin residues have been found in air, water, soil, fish, birds, and mammals, including humans. Food represents the primary source of exposure to the general population, being the second most common pesticide detected in a US survey of pasteurized milk.

- Endosulfan: It is a synthetic organochlorine compound commonly used as an agricultural insecticide. It has been sold from the mid 1950s but it is now banned in at least 60 countries with former uses replaced and its production is decreasing. It has the potential for long-range transport of endosulfan residues. Endosulfan is in the Arctic at increasing levels in water, air and biota.It is highly acutely toxic via oral, dermal and inhalation routes of exposure and it is associated to human poisoning. Contradictory opinions on the potential for endocrine disruption have been presented. a benchmark approach has been performed with lindane having similar toxicity than endosulfan.
- Heptachloro: Primarily used to kill soil insects and termites, heptachlor has also been used more widely to kill cotton insects, grasshoppers, other crop pests, and malaria-carrying mosquitoes. Laboratory tests have shown high doses of heptachlor to be fatal to mink, rats, and rabbits, with lower doses causing adverse behavioral changes and reduced reproductive success.Heptachlor is classified as a possible human carcinogen. Food is the major source of exposure for humans, and residues have been detected in the blood of cattle from the US and from Australia.
- Hexachlorobenzene: First introduced in 1945 to treat seeds, HCB kills fungi that affect food crops. It was widely used to control wheat bunt. It is also a byproduct of the manufacture of certain industrial chemicals and exists as an impurity in several pesticide formulations. Mothers passed HCB to their infants through the placenta and through breast milk. In high doses, HCB is lethal to some animals and, at lower levels, adversely affects their reproductive success. HCB has been found in food of all types. A study of Spanish meat found HCB present in all samples. In India, the estimated average daily intake of HCB is 0.13 micrograms per kilogram of body weight.
- Alpha and beta hexachlorocyclohexane: Although the intentional use of alpha-HCH as an insecticide was phased out years ago, this chemical is still produced as unintentional by-product of lindane. It is highly persistent in water in colder regions and may bioaccumulate and biomagnify in biota and arctic food webs. This chemical is subject to long-range transport, is classified as potentially carcinogenic to humans and adversely affects wildlife and human health in contaminated regions.
- Lindane: It has been used as a broad-spectrum insecticide for seed and soil treatment, foliar applications, tree and wood treatment and against ectoparasites in both veterinary and human applications. The production of lindane has decreased rapidly in the last few years. Lindane is persistent, bioaccumulates easily in the food chain and bioconcentrates rapidly. There is evidence for long-range transport and toxic effects in laboratory animals and aquatic organisms.
- Mirex: This insecticide is used mainly to combat ants and termites. Direct exposure to mirex does not appear to cause injury to humans, but studies on laboratory animals have caused it to be classified as a possible human carcinogen. It is considered to be one of the most stable and persistent pesticides. The main route of human exposure to mirex is through food, particularly meat, fish, and wild game.

- Toxaphene: This insecticide is used on cotton, cereal grains, fruits, nuts, and vegetables. Toxaphene was the most widely used pesticide in the US in 1975. For humans, the most likely source of toxaphene exposure is food. While the toxicity to humans of direct exposure is not high, toxaphene has been listed as a possible human carcinogen due to its effects on laboratory animals.
- Pentachlorobenzene: PeCB was used in PCB products, in dyestuff carriers, as a fungicide, a flame retardant and as a chemical intermédiate. It also present as impurities in products such as solvents or pesticides. PeCB is persistent in the environment, highly bioaccumulative and has a potential for long-range environmental transport. It is moderately toxic to humans and very toxic to aquatic organisms.

Restrictions on the use of these organochlorine compounds have resulted in a marked decrease in the average concentration of these pesticides in human tissues. Thus, in 1960s the mean concentration of DDT in fat tissue from USA population was 5 ppm, while in 1990s this value decreased one hundred times. However, 35 years after the banned of DDT, is possible to measure residues of this product and DDE in serum from different populations (Jakszyn et al., 2009; Zumbado et al., 2005). Organochlorine pesticides can be classified in order to the chemical structure, the manufacture system, and other factors (O'Neil, 2006). Anyway, all of these substances share a number of common characteristics as its lipid solubility and resistance to degradation. If we consider that the technical pesticides that were applied in past decades was a mixture of isomers among which the most abundant was 4,4 '-DDT, and that the pesticide undergoes slow degradation in the environment to its more persistent metabolite (4.4 '-DDE) (Safe, 1994), it is normal to observe that those countries which had a more controlled use of DDT, and where they were banned for longer, the 4.4 '-DDE is the metabolite found most frequently in foods and in serum of the population.

Pesticide	Approximate half-life
Aldrin	5 years in temperate soil
Campheclor (Toxaphene)	3 months - 12 years
Chlordane	2 - 4 years
DDT	10 - 15 years
Dieldrin	5 years in temperate soil
Endrin	Until 12 years
HCB	3 - 6 years
Heptachloro	Until 2 years
Mirex	Until 10 years
HCH	Days - 3 years

Table 2. Half-life in soil of the POPs listed in Stockholm Convention.

4. Potential adverse consequences of POPs. The phenomenon of endocrine disruption

The US Environmental Protection Agency (EPA) defines an endocrine disruptor (ED) as "an exogenous agent which interferes with the synthesis, secretion, transport, metabolism,

binding or elimination of hormones that are naturally present in the body and that are responsible for homeostasis, reproduction and development" (Diamanti-Kandarakis et al., 2009). This phenomenon was first described in 1968, when the 4,4´-DDE (an active metabolite of DDT) was considered responsible for the reproductive abnormalities of birds feeding on fish contaminated with this product (Hickey & Anderson, 1968). Another important fact observed was that occurred with the population of alligators of Lake Apopka in Florida. This animal population was exposed to high levels of dicofol in 1980s due to an accidental release. Ten years later, alligator population had dropped significantly, had increased mortality in eggs and half of newborn languished and died. Moreover, significant sexual abnormalities were observed in both male and females: decreased penis size, increased levels of estrogen, and ovarian malformations (Guillette et al., 1995a; Guillette et al., 1995b). Similar findings have been reported in other species reaching the same conclusion: disorders of sexual phenotype expression (Quintela et al., 2002; Sumpter & Jobling, 1993).

The group of molecules identified as ED is big, and includes several pesticides as DDT, chlordane or methoxychlor. Although some "endocrine disrupters "can be of natural origin, it must be highlighted that the majority of them are anthropogenic substances. Actually, the knowledge about the action mechanisms of ED is deep and wide. First studies reported that ED joined to nuclear receptors related with hormone signalling: estrogen, androgen and progesterone receptors, mainly. Today, it is known that these interactions are more complex. ED join to several membrane receptors for steroid hormones, as well as to orphan receptors (i.e. aryl hydrocarbon receptor), and other types of proteins (i.e. dopamine receptor, serotonine receptor). Thus, from a physiological perspective, an endocrine disruptor is a compound, either natural or synthetic, which through environmental exposure alters hormonal and homeostatic systems that allow the organization to communicate and respond to their environment (Diamanti-Kandarakis, Bourguignon et al., 2009). While most of the effects of ED could be categorized as "estrogenic", there are many substances that have androgenic effects (or more often anti-estrogenics); as well as agonists or antagonists of thyroid hormones. The sources of pollution and exposure to ED are highly variable, but may be considered a global phenomenon that affects all living beings on the planet, that are constantly exposed to mixtures, highly variable from individual to individual and from region to region, of these substances. The situation is also changing, because many of these compounds have been banned decades ago, while others are still permitted for certain uses or have been banned recently. Food and water intake must be considered the main routes of exposure. Different factors must be considered at this point:

- Age of exposure: the substances in the environment in which an organism develops interact with individual genes to determine their propensity to develop a disease or dysfunction in the future (Barker, 2003)
- Latency period from exposure: the consequences of exposure to ED may not appear immediately or early in life (Barker, 2003)
- Importance of mixtures: interactions, potentiation, synergism or addition (Kortenkamp, 2007)
- Non-conventional dose-response effects: in some cases, low doses may produce more powerful effects that higher doses (Boada et al., 2007; vom Saal et al., 2007)
- Trans-generational effects: ED not only can affect the exposed person but also to subsequent generations, possibly through non-genomic pathways (epigenetics)

The exposure to ED has been related with different health problems: testicular cancer, cryptorchidism, lymphoma non-Hodgkin, multiple myeloma, decrease in spermatozoa quality, breast cancer and other cancer types, spontaneous abortion, birth defects, immunology impairment, early puberty, or learning problems. Literature also shows that ED play a role in the aetiology of complex diseases such as obesity, diabetes mellitus or cardiovascular disease, although the scale of its implications are still poorly known. Nonetheless, to find associations between exposure to ED and the development of different pathologies is a very hard and complicate work, taking into account that the extent of chemical compounds in isolation may not give the required information on the biological effect to be surveyed. The evidences obtained from in vitro and animal models are quite concise, not the case of humans. Meanwhile, the precautionary principle should be applied in any case.

5. Legal regulations on waste food for persistent organochlorine pesticides

With the aim to protect the animal and human health, all food intended for human or animal consumption in the European Union is subject to legislation that determines the maximum residue level (MRL) that may be present in its composition. The MRL is defined as the maximum concentration of pesticide residue which is legally permitted or acceptable in food under the laws of the EU, based on good agricultural practices and the lowest consumer exposure necessary to protect all vulnerable consumers. This parameter is based in the acceptable daily intake (ADI) of the regulated substances. Thus, ADI is a measure of the amount of a specific substance in food or drinking water that can be ingested over a lifetime without an appreciable health risk, according to all the facts known at the time of evaluation, taking into account vulnerable groups of population.

Persistent organochlorine pesticides	ADI (mg/kg)	MRL (mg/kg)
Aldrin and dieldrin	0.0001	0.006
Chlordane (sum of *cis*- and *trans*- chlordane)	0.0005	0.002
DDT (sum of 4,4'-DDT, 2,4'-DDT, 4,4'-DDE and 4,4'-DDD)	0.01	0.04
Endosulfan (sum of α- and β- endosulfán and endosulfan sulphate)	0.006	0.05
Endrin	0.0002	0.0008
Heptacloro	0.0001	0.004
HCB	Not available	0.01
HCH (isomere α)	Not available	0.004
HCH (isomere β)	Not available	0.003
HCH (isomere γ) – lindane	0.005	0.001
Metoxicloro	0.1	0.01
Canfeclor (Toxaphene)	Not available	0.01

Table 3. MRL and ADI of the persistent organochlorine pesticides in not concentrated milk and cream (not containing added sugar or other sweetening matter), butter and other fats from milk, cheese and curd.

Laws related to MRL of pesticides were different between countries until 2005. Before this year, the UE had set different limits of pesticide for each type of product: fruit and vegetables (Directive 76/895/EEC), cereals (Directive 86/362/EEC), products of animal origin (Directive 86/363/EEC) and products of plant, including fruit and vegetables (Directive 90/642/EEC). The EC Regulation 396/2005 of the European Parliament and the Council, repeals all previous directives and gathers in one text the limits for various products for human or animal, and sets a default ceiling in the case that no MRL has been fixed. In general, the maximum level of pesticide residues in food is 0.01 mg/kg. However, all the organochlorine compounds have its MRL. Due to its persistent nature, is very important to ensure the protection of all consumers, especially those belonging to the "vulnerable population groups". It follows that MRLs in food must not only ensure the direct protection of the consumer but also to ensure that does not be undesirable accumulation or concentration in the food chain.

6. Current levels of dietary exposure of EU citizens to persistent organochlorine pesticides

Dietary modelling is a scientific method for estimating the levels of pesticide residues, contaminants or other substances that a person or a population may be consuming. Dietary modeling techniques have been used by international food regulators for years to determine whether dietary exposure to pesticide residues, contaminants and other substances represents an unacceptable risk to public health (Kroes et al., 2002; Lopez et al., 2003). In a recent publication, it has been reported an estimation of theoretical maximum daily intake (TMDI) of 421 pesticides, including POPs, among different populations from Europe with several dietary habits (Van Audenhaege et al., 2009). TMDI, expressed as the percentage of the ADI, was estimated taking into account the MRL for each pesticide as well as the dietary habits of each population. It should be noted that this is a theoretical estimate, which means assuming that the controls work perfectly and that it is not marketed in the EU any food

Persistent organochlorine pesticides	ADI (mg/kg)	TMDI (% ADI)
Aldrin	0.0001	348.8
Dieldrin	0.0001	348.8
Chlordane (sum of *cis*- and *trans*- chlordane)	0.0005	38.5
DDT (sum of 4,4'-DDT, 2,4'-DDT, 4,4'-DDE and 4,4'-DDD)	0.01	17.5
Endosulfan (sum of α- and β- endosulfán and endosulfan sulphate)	0.006	27.1
Endrin	0.0002	77.7
Heptacloro	0.0001	331.0
HCH (isomere γ) – lindane	0.005	4.2
Metoxicloro	0.1	0.1

Table 4. Theoretical estimation of the exposure (TMDI) to POPs through the diet in terms of the eating habits in European population.

which present POPs pollution levels exceeding the MRL established. Also, implies assuming that all food consumed have a residue level equal to the MRL for each pollutant. To achieve the real intake of POPs would be necessary to know the data from total diet or to work with a random sampling of foods from all groups.

A total diet study considers the total number of foods that make up the diet of an individual in the population studied, and analyzes the levels of each chemical contaminant. Thus, it is possible to know the total intake of residues from toxic substances that are commonly present in the food that take part of the diet. Therefore, this kind of studies determines the overall exposure of an individual to contaminants, analyzing whether this exposure has an unacceptable risk to human health. Because the preparation of food affects the concentration of pollutants and other substances, total diet studies use the analyses made over food ready to eat. Actually, there is a lack of total diet studies and global reports in EU regarding to contamination of food by POPs. For this, we can only provide rough estimates based on recently published works (Van Audenhaege et al., 2009). According to this, the levels of ADI for aldrin, dieldrin and heptachlor could be three times over the recommendations made from the WHO. Ten different groups of food were included: fruits, vegetables, cereals, potatoes, vegetable products, milk and dairy products, eggs, meat and other animal products, water and wine. Milk consumption represents about 13% of the total diet in EU countries. Daily contribution intake of milk and dairy products would be around 45% of the ADI for the case of aldrin and dieldrin, and 42% of the ADI in the case of heptachlor.

Dietary modelling is an important part of studies estimating the exposure of a population to a particular group of pollutants. It makes that the analytical results achieved in food dietary exposure can be compared to health benchmarks established. This comparison is crucial in determining whether the estimated dietary exposure to contaminants in food presents an unacceptable risk to the health of any population group (Kroes et al., 2002; Lambe, 2002). In this type of studies, the amount of chemical in each food is multiplied by the amount of food consumed. The sum of the amount raised for all food products determines the exposure to this substance from the total diet. Once the exposure to chemicals is estimated, it is compared with the reference health standards for assessing the potential risk that such exposure poses to human health (Lambe, 2002). The health standards of reference are the ADI for residues of pesticides, and tolerable intake limits for pollutants and other substances. These are the amounts of substance which, according to scientific evidence, can be consumed daily or weekly without posing a significant risk to human health. The levels of chemicals used are representative levels obtained from the analytical testing of food samples in the study. However, one of the most important steps is to match the food samples with more than one-thousand foods that are identified in a food survey. In total/partial diet studies is impossible to analyze all foods consumed, or all which are part of a particular food group. Therefore, each food tested should be considered as representative of a food group, with appropriate adjustments for the concentration (Kroes et al., 2002). The European Food Safety Authority (EFSA) established the Concise European Food Consumption Database in 2008 with the aim of to support estimations of exposure to toxic substances through the diet in the countries of the Union. In order to obtain comparable results, data were aggregated into fifteen major categories, based on the precautionary principle, to provide a conservative estimate of exposure levels. The study also includes subcategories of food in some of the major groups, providing data from consumption of a total of twenty-eight kinds of food. The database was created to provide a

tool for exposure assessment, and it allows the evaluation of total exposure of the population groups. Although it has the limitations of categorization in large food groups and differences in methodologies employed for data collection, it is sufficient when calculating exposure based on conservative estimates of concentrations are below the level of concern (ESFA, 2008). Data referred to these fifteen categories are summarized in Table 5. Sixteen countries took part in the study: Belgium (BE), Bulgaria (BG), Czech Republic (CZ), Denmark (DK), Finland (FI), France (FR), Germany (DE), Hungary (HU), Iceland (IS), Ireland (IE), Italy (IT), The Netherlands (NL), Norway (NO), Slovakia (SK), Sweden (SE),

Group	BE	BG	CZ	DK	FI	FR	DE	HU	IS	IE	IT	NL	NO	SK	SE	GB	M
Cereals	245	257	274	217	153	317	287	252	276	227	271	220	192	345	291	249	**257**
Sugar products and chocolate	31	40	39	43	42	31	45	39	31	41	19	43	47	69	28	27	**43**
Fats	46	39	48	36	40	28	29	54	33	36	36	48	41	29	24	20	**38**
Vegetables	230	210	131	166	135	210	252	191	125	244	249	193	140	164	118	163	**194**
Starchy roots and potatoes	95	83	103	112	95	67	125	110	79	229	48	128	133	96	138	112	**129**
Fruits	113	70	122	150	121	132	190	180	71	106	203	107	119	116	119	95	**92**
Fruit juices and soft drinks	945	207	618	340	213	389	947	280	426	179	384	296	491	382	329	325	**439**
Coffee, te and cocoa	432	120	559	836	580	282	691	176	429	714	124	887	604	461	575	724	**601**
Alcoholic beverages	214	102	413	292	139	163	231	76	104	335	126	206	123	64	191	313	**413**
Meat and meat products	121	108	175	128	120	134	127	173	93	122	134	141	103	179	150	49	**234**
Offal	2	7	9	7		8	11	13	6	1	3	5	6	12		2	**12**
Fish and seafood	25	20	19	18	27	37	19	9	37	24	43	13	63	9	34	31	**62**
Eggs	10	21	20	16	17	18	23	27	11	20	18	15	21	13	14	19	**25**
Milk and diary products	203	169	186	386	437	265	313	265	442	306	212	388	522	91	386	251	287
Dietary uses	2	13	15	5	24	2	36	17	23	13	5	6	12	6	16	17	**14**
Tap Water	100		288	840	886	283	71	1	670	284	206	209	312	224	480	205	**349**

Table 5. Average food consumption (g/day) in the total adult population of sixteen EU countries (M, median).

and United Kingdom (UK). Other countries, as Poland (PO), Estonia (ES) and Austria (AU) have sent data, but they are not included in the database, yet. Finally, countries as Spain (SP), Portugal (PT) or Greece (GR) have not sent any data.
The need for such data at European level was raised at the colloquium on "European Food Consumption Database - Current and medium to long-term strategies" organised by EFSA in Brussels in April 2005. Exposure assessment is a crucial part of the risk assessment and the quality of available data both on food consumption and on occurrence levels may have a major impact on the outcome of the risk assessment. Other European important projects are underway. It is the case of the "Benefit-Risk Assessment for Food" (Beneris). The main objective of the project is to forge major advancements in food benefit-risk analysis on human health. Beneris brings together a team of epidemiologists, toxicologists, nutrition scientists, exposure assessors, risk analysts, and authorities from five European countries (Finland, The Netherlands, Ireland, Denmark and Spain) with crucial access to contacts and data.
The Beneris project forms a cluster with another project in the same call, namely Qalibra. The two projects are tackling the same problems but using complementary methods and approaches. Qalibra is more focused on developing web-based technical tools for risk assessment, while Beneris concentrates on developing useful approaches and strategies (including extensive case studies).

6.1 The potentially hazardous foods: milk and dairy products

Although the POPs can reach humans due to direct environmental exposures (such as occupational exposure), the main source of exposure to these compounds is the consumption of contaminated food. Milk is a food of animal origin where POPs are detected at higher concentrations, making this product a food "at risk" for the consumer (Focant et al., 2002). However, other food groups are involved as sources of exposure, and relevant data have been published recently. Thus, vegetable consumption has been related to lindane, and fruit intake with endosulfan (Mariscal-Arcas et al., 2010)
The concentration of POPs found in the milk brands tested was low and always lower than the MRLs established by the laws. It must be highlighted that, actually, the levels of POPs residues in milk throughout the world have been significantly reduced, as a result of prohibition or restriction of use of this substances (Nag & Raikwar, 2008). In that way, the level of contamination by 4,4'-DDT in the European environment has declined drastically in recent years following the ban on its use forty years ago. In the case of 4,4'-DDE, residue levels of this substance in samples of milk, are similar among different Western countries (U.S. Total Diet Study, 2001). But this does not apply in the developing countries. Both 4,4'-DDE and 4,4'-DDT are routinely detected in 100% of milk and other dairy products sold in China, Ghana and India (Darko & Acquaah, 2008; Nag & Raikwar, 2008; Zhang et al., 2006). In these countries the amounts of these residues are high, and reach worrying levels. In milk samples from India were measured average levels of 4 μg/kg and 5 μg/kg for 4,4'-DDT and 4,4'-DDE, respectively; results that suggest a recent and continued use of the pesticide DDT. The same profile has been observed for lindane (HCH), whose levels in samples of milk from India reached values of 10 μg/kg (Nag & Raikwar, 2008). On the contrary, the higher values of lindane described in the Total Diet Study of the United States in 2001 were 0.00002 μg/kg, which is indicative of the high levels of exposure in the population from developing countries. Thus, current intakes are at least 5- to 100 fold greater than those observed in more developed nations, suggesting a greater risk from organochlorine exposure. Moreover,

the estimated intake of DDT by infants from these Asian regions is at least 100 fold greater than the ADI of the FAO/WHO, being the milk a major source of exposure.
Consumption of dairy products in occidental countries has experienced a sharp increase in recent decades. Thus, consumption of cheese, yogurt or butter is vastly superiors who had thirty years ago. Since milk appears to be an important "food of risk" in terms of pesticide residues, it is necessary to study dairy products as sources of exposure to organochlorine pesticides. Just as with milk, the levels of POPs contamination detected in cheese are low, and are always below the maximum residue limit established by law in Western countries (DOUE, 1993 and 1994). However, lindane seems to appear frequently as residue in cheese. Although it levels have decreased in recent years, they vary according to the world region, being highest in developing countries as Jordan or Ghana (Darko & Acquaah, 2008; Mallatou et al., 1997; Salem et al., 2009). Results from Western countries show that HCH pollution is due to the persistence of lindane degradation products (α and β isomers), indicating a past use of the compound. In addition, relationships have been established between high levels of lindane found on products like cheese, and high levels of lindane in the serum of the population consuming these products (Luzardo et al., 2006). It is clearly established in this case a direct connection between the presence of contaminants in food consumption and high pollution levels in the population. Although its use is banned several decades ago, studies have found residues of DDT and its metabolites also in cheese. Again, the relationship between food waste and waste in the population has been established, this time for the case of DDT (Zumbado et al., 2005).

6.2 The potentially hazardous foods: fish and seafood products. Role of sea and river contamination

Although fish and seafood present detectable levels of organochlorine pesticides, which vary depending on geographic location and other parameters as fat amount, these levels, in Western countries are always below the permitted maximum residue limits for each substance. The National Contaminant Biomonitoring Program periodically determined concentrations of organochlorine chemical residues from the U.S. Fish and Wildlife Service. The mean concentration of total DDT and its homologues (p,p'-constituents) in 1999, declined from 1984 to 1986, thus continuing a trend that began in 1970. Averaged 74% of total DDT residues, up from 70% in 1974-1979, are essentially unchanged from 1984, suggesting a low rate of influx and continued weathering of DDT in the environment (Schmitt et al., 1999). As cited previously, in most Southeast Asian countries DDT was a common contaminant in animal origin foodstuffs. The higher percentage of p,p'-DDT in meat and fish from Southeast Asian countries, except Japan and Korea, indicated the recent use of DDT in vector control operations. Aquatic food products from more industrialized countries, such as Japan, South Korea, Hong Kong, and Taiwan, contained significant levels of other POPs from industrial origin, such as PCBs. In South Pacific countries, particularly in Australia and New Zealand, chlordanes and PCBs were the most prevalent organochlorines in foodstuffs (Kannan et al., 1997), being meat and fish the major sources of organochlorine exposure by Australians. Human dietary intake of organochlorines has been declining more slowly in Asian developing countries (Kannan, et al., 1997).
Sea and river pollution have been extensively studied due to the intrinsic relation with fish and seafood contamination. Several researches have been published reporting levels of contaminations by POPs in different seas and rivers all over the world. In a recent study, it has been estimated the residue levels of organochlorine compounds (and other contaminants as PCBs) in Northwestern Mediterranean Sea from the Ebro River (Gomez-

Gutierrez et al., 2006). Based on the contaminant concentrations and on hydrological data, contaminant discharges into the sea were estimated amounting in total to 167 kg per year of organochlorine compounds. Concentrations ranged from 0.4 to 19.5 ng/l for the organochlorine compounds (Gomez-Gutierrez et al., 2006). Similar data have been reported from other European regions, reporting a modest contaminated status of rivers and seas, that must be under observation (Neamtu et al., 2009).

It has been observed that among several substances, the main contaminants in the muscles of both pikeperch and perch species from the Sulejowski Reservoir in central Poland was p,p'- DDE, reaching an average of 1,072 and 694 ng/g lipid, respectively. Nonetheless, based on the contaminant levels in the sediment and fish, this region compares well with other, freshwater environments relatively uncontaminated with persistent organic pollutants.

In relation to other POPs, temporal trends from ice/snow cores as well as mountain lake sediments reveal a marked increase in endosulfan accumulation from the 1980s onwards. Levels of alpha-endosulfan do not show a decline in atmospheric monitoring data, reflecting ongoing use of this pesticide in the northern hemisphere. Endosulfan is present at low concentrations (relative to the pesticide, lindane) in surface Arctic Ocean waters. Residues of endosulfan have been detected in marine biota for different geographical regions of the Arctic, with higher bioaccumulation factors ($>10^3$-10^7) for zooplankton and various species of fish, compared to studies in warmer/temperate systems. Endosulfan is present in marine mammals, and biomagnification factors for alpha-endosulfan are >1, notably from fish to seal (Weber et al., 2009).

7. Conclusion

Pollution is inherent to human progress. For decades, persistent pesticides have been used, and actually they are accumulated in the global environment. Food is the main source of exposure to these substances for human beings. Although the exposure levels vary by geographic area, in Europe, levels of organochlorine pesticides detected in foods are below the maximum residue limits established by laws. In general, levels of such waste have been decreasing over the years, mainly due to the creation of laws for the regulation or prohibition of such substances. However, nowadays World population is still environmentally exposed to low levels of these contaminants and the future consequences of such exposure are unknown and a matter of concern.

8. References

Abhilash, P.C. & Singh, N. (2009). Pesticide use and application: an Indian scenario. *J Hazard Mater,* 165, (1-3), (1-12)

Barker, D.J. (2003). The developmental origins of adult disease. *Eur J Epidemiol,* 18, (8), (733-736)

Boada, L.D., Lara, P.C., Alvarez-Leon, E.E., Losada, A., Zumbado, M.L., Liminana-Canal, J.M., Apolinario, R., Serra-Majem, L. & Luzardo, O.P. (2007). Serum levels of insulin-like growth factor-I in relation to organochlorine pesticides exposure. *Growth Horm IGF Res,* 17, (6), (506-511)

Bonefeld-Jorgensen, E.C., Hjelmborg, P.S., Reinert, T.S., Andersen, B.S., Lesovoy, V., Lindh, C.H., Hagmar, L., Giwercman, A., Erlandsen, M., Manicardi, G.C., Spano, M., Toft, G. & Bonde, J.P. (2006). Xenoestrogenic activity in blood of European and Inuit populations. *Environ Health,* 5, (12)

Chikuni, O., Nhachi, C.F., Nyazema, N.Z., Polder, A., Nafstad, I. & Skaare, J.U. (1997). Assessment of environmental pollution by PCBs, DDT and its metabolites using human milk of mothers in Zimbabwe. *Sci Total Environ,* 199, (1-2), (183-190)

Darko, G. & Acquaah, S.O. (2008). Levels of organochlorine pesticides residues in dairy products in Kumasi, Ghana. *Chemosphere,* 71, (2), (294-298)

Diamanti-Kandarakis, E., Bourguignon, J.P., Giudice, L.C., Hauser, R., Prins, G.S., Soto, A.M., Zoeller, R.T. & Gore, A.C. (2009). Endocrine-disrupting chemicals: an Endocrine Society scientific statement. *Endocr Rev,* 30, (4), (293-342)

Felsot, A.S., Racke, K.D. & Hamilton, D.J. (2003). Disposal and degradation of pesticide waste. *Rev Environ Contam Toxicol,* 177, (123-200)

Focant, J.F., Eppe, G., Pirard, C., Massart, A.C., Andre, J.E. & De Pauw, E. (2002). Levels and congener distributions of PCDDs, PCDFs and non-ortho PCBs in Belgian foodstuffs--assessment of dietary intake. *Chemosphere,* 48, (2), (167-179)

Gomez-Gutierrez, A.I., Jover, E., Bodineau, L., Albaiges, J. & Bayona, J.M. (2006). Organic contaminant loads into the Western Mediterranean Sea: estimate of Ebro River inputs. *Chemosphere,* 65, (2), (224-236)

Gray, J.S. (2002). Biomagnification in marine systems: the perspective of an ecologist. *Mar Pollut Bull,* 45, (1-12), (46-52)

Guillette, L.J., Jr., Crain, D.A., Rooney, A.A. & Pickford, D.B. (1995a). Organization versus activation: the role of endocrine-disrupting contaminants (EDCs) during embryonic development in wildlife. *Environ Health Perspect,* 103 Suppl 7, (157-164)

Guillette, L.J., Jr., Gross, T.S., Gross, D.A., Rooney, A.A. & Percival, H.F. (1995b). Gonadal steroidogenesis in vitro from juvenile alligators obtained from contaminated or control lakes. *Environ Health Perspect,* 103 Suppl 4, (31-36)

Haylamicheal, I.D. & Dalvie, M.A. (2009). Disposal of obsolete pesticides, the case of Ethiopia. *Environ Int,* 35, (3), (667-673)

Hickey, J.J. & Anderson, D.W. (1968). Chlorinated hydrocarbons and eggshell changes in raptorial and fish-eating birds. *Science,* 162, (850), (271-273)

Jakszyn, P., Goni, F., Etxeandia, A., Vives, A., Millan, E., Lopez, R., Amiano, P., Ardanaz, E., Barricarte, A., Chirlaque, M.D., Dorronsoro, M., Larranaga, N., Martinez, C., Navarro, C., Rodriguez, L., Sanchez, M.J., Tormo, M.J., Gonzalez, C.A. & Agudo, A. (2009). Serum levels of organochlorine pesticides in healthy adults from five regions of Spain. *Chemosphere,* 76, (11), (1518-1524)

Kalantzi, O.I., Alcock, R.E., Johnston, P.A., Santillo, D., Stringer, R.L., Thomas, G.O. & Jones, K.C. (2001). The global distribution of PCBs and organochlorine pesticides in butter. *Environ Sci Technol,* 35, (6), (1013-1018)

Kannan, K., Tanabe, S., Giesy, J.P. & Tatsukawa, R. (1997). Organochlorine pesticides and polychlorinated biphenyls in foodstuffs from Asian and oceanic countries. *Rev Environ Contam Toxicol,* 152, (1-55)

Koopman-Esseboom, C., Huisman, M., Weisglas-Kuperus, N., Boersma, E.R., de Ridder, M.A., Van der Paauw, C.G., Tuinstra, L.G. & Sauer, P.J. (1994). Dioxin and PCB levels in blood and human milk in relation to living areas in The Netherlands. *Chemosphere,* 29, (9-11), (2327-2338)

Kortenkamp, A. (2007). Ten years of mixing cocktails: a review of combination effects of endocrine-disrupting chemicals. *Environ Health Perspect,* 115 Suppl 1, (98-105)

Kroes, R., Muller, D., Lambe, J., Lowik, M.R., van Klaveren, J., Kleiner, J., Massey, R., Mayer, S., Urieta, I., Verger, P. & Visconti, A. (2002). Assessment of intake from the diet. *Food Chem Toxicol,* 40, (2-3), (327-385)

Lambe, J. (2002). The use of food consumption data in assessments of exposure to food chemicals including the application of probabilistic modelling. *Proc Nutr Soc,* 61, (1), (11-18)

Lopez, A., Rueda, C., Armentia, A., Rodriguez, M., Cuervo, L. & Ocio, J.A. (2003). Validation and sensitivity analysis of a probabilistic model for dietary exposure assessment to pesticide residues with a Basque Country duplicate diet study. *Food Addit Contam,* 20 Suppl 1, (S87-101)

Luzardo, O.P., Goethals, M., Zumbado, M., Alvarez-Leon, E.E., Cabrera, F., Serra-Majem, L. & Boada, L.D. (2006). Increasing serum levels of non-DDT-derivative organochlorine pesticides in the younger population of the Canary Islands (Spain). *Sci Total Environ,* 367, (1), (129-138)

Luzardo, O.P., Mahtani, V., Troyano, J.M., Alvarez de la Rosa, M., Padilla-Perez, A.I., Zumbado, M., Almeida, M., Burillo-Putze, G., Boada, C. & Boada, L.D. (2009). Determinants of organochlorine levels detectable in the amniotic fluid of women from Tenerife Island (Canary Islands, Spain). *Environ Res,* 109, (5), (607-613)

Mallatou, H., Pappas, C.P., Kondyli, E. & Albanis, T.A. (1997). Pesticide residues in milk and cheeses from Greece. *Sci Total Environ,* 196, (2), (111-117)

Mariscal-Arcas, M., Lopez-Martinez, C., Granada, A., Olea, N., Lorenzo-Tovar, M.L. & Olea-Serrano, F. (2010). Organochlorine pesticides in umbilical cord blood serum of women from Southern Spain and adherence to the Mediterranean diet. *Food Chem Toxicol,* 48, (5), (1311-1315)

Nag, S.K. & Raikwar, M.K. (2008). Organochlorine pesticide residues in bovine milk. *Bull Environ Contam Toxicol,* 80, (1), (5-9)

Neamtu, M., Ciumasu, I.M., Costica, N., Costica, M., Bobu, M., Nicoara, M.N., Catrinescu, C., van Slooten, K.B. & De Alencastro, L.F. (2009). Chemical, biological, and ecotoxicological assessment of pesticides and persistent organic pollutants in the Bahlui River, Romania. *Environ Sci Pollut Res Int,* 16 Suppl 1, (S76-85)

O'Neil, M.J. (2006). *The Merck index : an encyclopedia of chemicals, drugs, and biologicals*. Merck. 9780911910001 (hbk.),Whitehouse Station, NJ

Quintela, M., Barreiro, R. & Ruiz, J.M. (2002). Dumpton syndrome reduces the tributyltin (TBT) sterilising effect on Nucella lapillus (L.) by limiting the development of the imposed vas deferens. *Mar Environ Res,* 54, (3-5), (657-660)

Ryan, J.J., Gasiewicz, T.A. & Brown, J.F., Jr. (1990). Human body burden of polychlorinated dibenzofurans associated with toxicity based on the yusho and yucheng incidents. *Fundam Appl Toxicol,* 15, (4), (722-731)

Safe, S.H. (1994). Polychlorinated biphenyls (PCBs): environmental impact, biochemical and toxic responses, and implications for risk assessment. *Crit Rev Toxicol,* 24, (2), (87-149)

Salem, N.M., Ahmad, R. & Estaitieh, H. (2009). Organochlorine pesticide residues in dairy products in Jordan. *Chemosphere,* 77, (5), (673-678)

Sasamoto, T., Ushio, F., Kikutani, N., Saitoh, Y., Yamaki, Y., Hashimoto, T., Horii, S., Nakagawa, J. & Ibe, A. (2006). Estimation of 1999-2004 dietary daily intake of PCDDs, PCDFs and dioxin-like PCBs by a total diet study in metropolitan Tokyo, Japan. *Chemosphere,* 64, (4), (634-641)

Scheringer, M. (2009). Long-range transport of organic chemicals in the environment. *Environ Toxicol Chem,* 28, (4), (677-690)

Schmitt, C.J., Zajicek, J.L., May, T.W. & Cowman, D.F. (1999). Organochlorine residues and elemental contaminants in U.S. freshwater fish, 1976-1986: National Contaminant Biomonitoring Program. *Rev Environ Contam Toxicol,* 162, (43-104)

Ssebugere, P., Wasswa, J., Mbabazi, J., Nyanzi, S.A., Kiremire, B.T. & Marco, J.A. (2010) Organochlorine pesticides in soils from south-western Uganda. *Chemosphere,* 78, (10), (1250-1255)

Sumpter, J.P. & Jobling, S. (1993). Male sexual development in "a sea of oestrogen". *Lancet,* 342, (8863), (124-125)

Trapp, S., Bomholtz, L.M. & Legind, C.N. (2008). Coupled mother-child model for bioaccumulation of POPs in nursing infants. *Environ Pollut,* 156, (1), (90-98)

Van Audenhaege, M., Heraud, F., Menard, C., Bouyrie, J., Morois, S., Calamassi-Tran, G., Lesterle, S., Volatier, J.L. & Leblanc, J.C. (2009). Impact of food consumption habits on the pesticide dietary intake: Comparison between a French vegetarian and the general population. *Food Addit Contam Part A Chem Anal Control Expo Risk Assess,* 26, (10), (1372-1388)

vom Saal, F.S., Akingbemi, B.T., Belcher, S.M., Birnbaum, L.S., Crain, D.A., Eriksen, M., Farabollini, F., Guillette, L.J., Jr., Hauser, R., Heindel, J.J., Ho, S.M., Hunt, P.A., Iguchi, T., Jobling, S., Kanno, J., Keri, R.A., Knudsen, K.E., Laufer, H., LeBlanc, G.A., Marcus, M., McLachlan, J.A., Myers, J.P., Nadal, A., Newbold, R.R., Olea, N., Prins, G.S., Richter, C.A., Rubin, B.S., Sonnenschein, C., Soto, A.M., Talsness, C.E., Vandenbergh, J.G., Vandenberg, L.N., Walser-Kuntz, D.R., Watson, C.S., Welshons, W.V., Wetherill, Y. & Zoeller, R.T. (2007). Chapel Hill bisphenol A expert panel consensus statement: integration of mechanisms, effects in animals and potential to impact human health at current levels of exposure. *Reprod Toxicol,* 24, (2), (131-138)

Weber, J., Halsall, C.J., Muir, D., Teixeira, C., Small, J., Solomon, K., Hermanson, M., Hung, H. & Bidleman, T. (2009). Endosulfan, a global pesticide: A review of its fate in the environment and occurrence in the Arctic. *Sci Total Environ,* [Epub Ahead of Print]

Weber, R., Gaus, C., Tysklind, M., Johnston, P., Forter, M., Hollert, H., Heinisch, E., Holoubek, I., Lloyd-Smith, M., Masunaga, S., Moccarelli, P., Santillo, D., Seike, N., Symons, R., Torres, J.P., Verta, M., Varbelow, G., Vijgen, J., Watson, A., Costner, P., Woelz, J., Wycisk, P. & Zennegg, M. (2008). Dioxin- and POP-contaminated sites--contemporary and future relevance and challenges: overview on background, aims and scope of the series. *Environ Sci Pollut Res Int,* 15, (5), (363-393)

Williams, E.S., Panko, J. & Paustenbach, D.J. (2009). The European Union's REACH regulation: a review of its history and requirements. *Crit Rev Toxicol,* 39, (7), (553-575)

Yaktine, A.L., Harrison, G.G. & Lawrence, R.S. (2006). Reducing exposure to dioxins and related compounds through foods in the next generation. *Nutr Rev,* 64, (9), (403-409)

Zhang, H., Chai, Z.F., Sun, H.B. & Zhang, J.L. (2006). A survey of extractable persistent organochlorine pollutants in Chinese commercial yogurt. *J Dairy Sci,* 89, (5), (1413-1419)

Zumbado, M., Goethals, M., Alvarez-Leon, E.E., Luzardo, O.P., Cabrera, F., Serra-Majem, L. & Dominguez-Boada, L. (2005). Inadvertent exposure to organochlorine pesticides DDT and derivatives in people from the Canary Islands (Spain). *Sci Total Environ,* 339, (1-3), (49-62)

4

Combined Exposure to Mixture of Chemicals. An Impossible Challenge?

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1. Introduction

The harmful effects of the human activities on health and the environment are known for a very long time but the public awareness is recent and dates from second half of the 20th century. Living organisms are almost constantly exposed to many stressors. Among them, chemical pollutants play a major role. A wide range of chemical substances act as pollutants, ranging from simple inorganic ions to complex organic molecules. Some metals such as cadmium, mercury, lead provoke adverse effects of human health when they are present at high level of exposure. Radioactive isotopes may be harmful to organisms, depending on the dose and type of radiation. Numerous organic compounds are also known to be noxious: hydrocarbons (i.e. benzene, polycyclic aromatic hydrocarbons, PAHs), polychlorinated phenols (PCPs) polychlorinated biphenyls (PCBs), polychlorinated dibenzodioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), polybrominated biphenyls (PBBs), organochlorine pesticides (OCs), organophosphorus pesticides (OPs), carbamates, pyrethroid insecticides, phenoxy herbicides, rodenticides, organometallic compounds and so on (Walker et al., 2001). Some of these chemicals are of concern because of their human toxicity. Other chemicals cause damages to non-human biota but are not believed to be harmful to humans. Finally, some other pollutants are not directly toxic to humans or other biota at current environmental concentrations, but are capable to modify environmental features causing major environmental damage (i.e. chlorinated fluorinated carbons, CFCs, known to drastically disturb the chemistry of the stratosphere). In this chapter, only the pollutants harmful to living organisms are considered, keeping in mind that non chemical stressors may act at the same time on biota.

Any substance can have adverse effects on cell biology and/or on whole organism, but this depend on dose and chemical speciation. Toxicity and ecotoxicity are defined as the capacity to cause injury to a living organism (human or not) defined with reference to the quantity of substance absorbed, the way in which the substance is taken up and distributed in time (single or repeated doses), the type and severity of injury, the time needed to produce the injury, the nature of the organism(s) affected, and other relevant conditions (Duffus et al., 2009).

Any chemical of concern has to be taken up by an organism before it can produce an effect. Once absorbed, the potentially toxic substance will be distributed throughout the organism and the absorption of the toxicant will result in a toxic effect and a response defined as the percentage of the exposed population showing the defined toxic effect. To quantitatively

describe toxicological effects of a given substance, one has to define a reference value that characterizes safe exposure. Very often, median dose lethal to 50% (LD_{50}) of a test population was used as a reference, whereas an increasing number of toxicologists and ecotoxicologists favour now the benchmark concentration (BMC) or dose (BMD). The benchmark concentration (or dose) is the statistical lower confidence limit on the concentration (or dose) that produces a defined response (called the benchmark response or BMR, usually 5 or 10 %) for an adverse effect compared to the background, defined as 0%.
After pollutant uptake, subsequent elimination and clearance of the substance from the organism will occur due to various biological and biochemical processes. The biological half-life is the parameter used to describe the progressive reduction in the pollutant internal concentration. Similarly, in environmental compartments such as air, water, soil, sediment, the pollutant concentration may decrease or not depending on various ecological processes and chemical properties of the pollutant. Persistence is the key concept which describes the ability of a substance to stay in a given environmental compartment. The way in which the substance is distributed in time (single or repeated doses) is also a key factor modulating toxicity of chemicals. Consequently, one has to distinguish between acute, subchronic or chronic toxicity, which may be very different for a given chemical. Therefore, LD_{50}, BMD, biological half-life, persistence, and ways of exposure are very important issues in risk assessment for toxicant effects on humans or non-human biota.
Toxicological and ecotoxicological studies have produced a considerable corpus of knowledge which has been used to draw rules and regulations for managing chemicals of concern. However, most toxicological and ecotoxicological studies focus on exposure and effects of single compounds, whereas in real world, organisms are submitted to many pollutants often acting at low doses and at the same time. The chemical substances do not act independently. The living organisms are permanently exposed to multiple substances acting in a concomitant way. It is therefore crucial for scientists and policy-makers involved in the field of (eco)toxicology to develop, use and refine efficient methods for risk assessment of combined exposures to various toxicants and chemical mixtures. Up to now, most of the methods for the management of chemical compounds are based on single-substance risk evaluations. When risk assessment of multiple chemicals are required, single-substance toxicity data are used to derive mixture toxicity using a limited number of methods and models. The objective of the present chapter is to give a brief overview of the methods currently available to assess combined exposure toxicity. We will first give some basic concepts and terminology, and we will review the state-of-the-art of the current available tools and methodologies. Then, we will use the case-study of wood preservative toxicity to illustrate some of the difficulties and gaps of the current methodologies.

2. The general framework of chemical risk assessment. Basic concepts and terminology.

2.1 The four stages of the environmental risk assessment of chemicals.

Numerous biological, physical, and chemical *stressors* are harmful to humans, biota, and ecosystems. These agents are perceived like threats and cause concerns within the human society. They may exert *adverse effects* at different biological levels: they disturb molecular and cell biology, but also the physiology of whole organism. *Responses* occur at population, community, and/or ecosystem levels. Various means have been implemented to face these environmental and health problems.

Chemical risk assessment may pursue various objectives. One may try to reduce human exposure to chemicals of concern. An other frequent goal is the reduction of health effects. Risk assessment may also be devoted to the mitigation of ecological impacts, or to the protection of vulnerable populations. Evaluation can be done before (*a priori* assessment) or after (*a posteriori* assessment) exposure to toxicants.

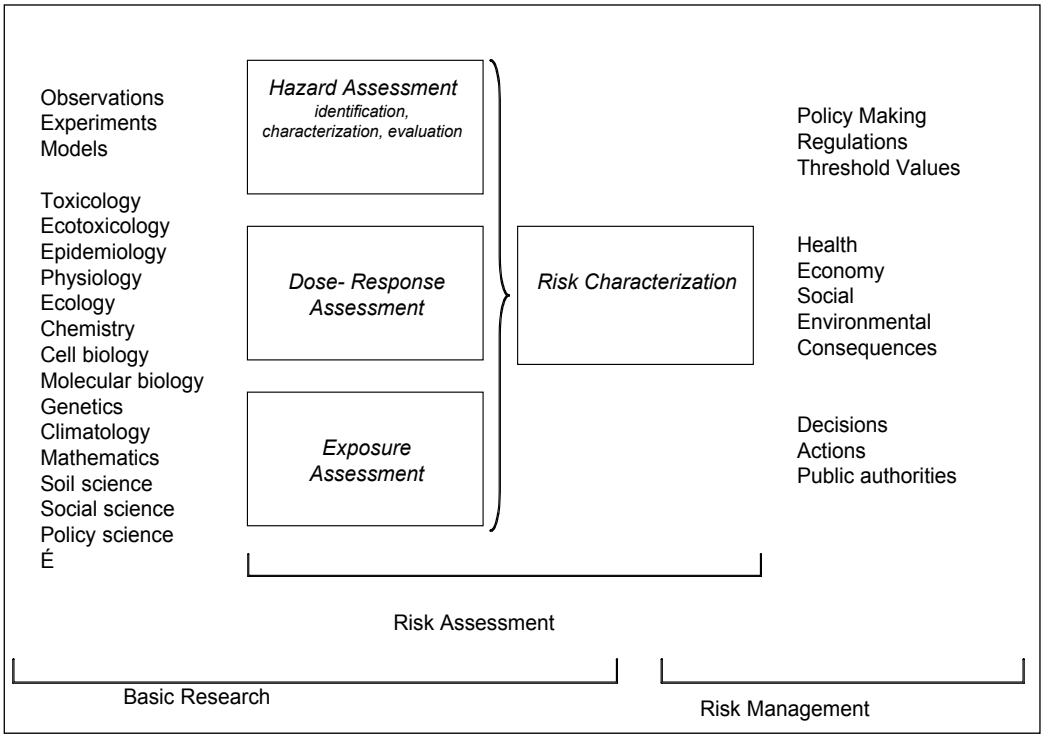

Fig. 1. The four stages of the environmental risk assessment of chemicals.

Whatever the objective, the Environmental Risk Assessment (ERA, Fig. 1) is usually carried out in four stages. Hazard is a set of inherent properties of a substance, a mixture of substances, or a process that make it capable of causing adverse effects to living organisms or the environment. Hazard is a source of danger: during *hazard assessment*, the dangerosity of a chemical is evaluated independently of the probability of occurrence of the damage. During this stage, the potential causes of damage are exhaustively reviewed and clearly identified. The substances of concern and the adverse effects that they may produce are identified and a list is made. All the relevant informations relating to toxicity are gathered in the form of material safety data sheets (MSDS). Then, the hazard characterization consists of the qualitative and quantitative description of the hazard associated with the agent of concern. During the second stage, the relationship between exposure to a hazard (dose) and the resultant adverse effects (responses) are comprehensively described and *dose-response assessment* is produced. Dose-response assessment always involves extrapolation of results from an experimental or observation group to an entire population. This stage necessarily includes a part of uncertainty, which has to be clearly stated before managing decisions. The third stage is devoted to *exposure assessment*: a complete description of exposure is

performed. Exposed populations, levels and pathways of exposure are studied. Then, all these data are integrated during the *risk assessment* stage which aims to produce a quantitative description of the probabilities of the damage. Risk assessment provides quantitative estimation, including uncertainties, of the severity and probability of occurrence of known and potential adverse effects of a substance in a given population.
The environmental risk assessment is based on multidisciplinary approaches involving observations, experiments, and models from various fields of science. Once an ERA is available, *policy makers* have to define regulations, which often result in threshold values. *Public authorities* are in charge of the *risk management* involving relevant decisions and actions. Such procedure is used worldwide, but one has to keep in mind that it has been implemented for single-chemical toxicity. It fails to be fully efficient to predict risks linked to combined exposure to multiple chemicals. Although some potential environmental hazards involve significant exposure to only a single chemical, most instances of environmental contaminations involve concurrent or sequential exposures to several compounds that may induce similar or dissimilar effects over exposure periods ranging from short-term to lifetime (U.S. EPA, 2000).

2.2 Basic concepts and terminology.

A quick survey of the scientific literature may convince anybody that there is a very rich terminology in the field of mixture toxicity, but this terminology remains sometimes unclear and sometimes contradictory. In the following paragraphs, we try to summarize the main concepts and definitions.
A very high number of terms are applied to toxic substances in the scientific literature. We will consider that a *contaminant* is any substance detected in a place where it is not normally found. *Pollutant* is any chemical found in the environment causing adverse effects or harms to living organisms, or disturbances to the ecosystem structure and function. Toxicologists often refer to *toxicant* for any substance that is capable to provoke injuries to living organisms as a result of physicochemical interactions under circumstances which are thought likeky to happen. *Poison* is nearly a synonym of toxicant, but is usually applied to substances deliberately used to impair the health of the organism or to kill it. *Drug* is any substance that, when absorbed into a living organism, may alter its functions. *Biocides* are substances intended to kill living organisms. *Pesticides* are specific biocides intended to kill pests.
Following the U.S. Environmental Protection Agency (1986, 1987), a *mixture* will be defined as any combination of two or more chemical substances, regardless of source or of spatial or temporal proximity, that can influence the risk of chemical toxicity in the target population. Mixtures may be highly complex originating for a single source or process as by-products (diesel exhaust, municipal incinerator, etc.). In other instances, chemical mixtures are man-made commercial products (e.g. pesticide formulations, PCBs, gazoline). In some other cases, environmental releases, waste disposals, or storages of various chemical compounds cause combined exposures. Multichemical exposures are ubiquitous, including air, water, soil and food contaminations from various sources.
Scientific literature contains many definitions, about chemical mixtures and mixture toxicity. Therefore, key concepts must be clearly listed and defined before describing toxicity of chemical mixtures. Table 1 gives a summary of the most commonly used definitions. A *chemical mixture* corresponds to any set of multiple chemical substances regardless of their sources that may jointly cause toxicity in the target population. The *components* of the

mixture may or may not be identifiable. *Similar components* are components with the same or similar biological activities. Literature often refers to this chemical mixture as whole mixture or mixture of concern. A mixture can be simple or complex. A *simple mixture* is considered as any mixture that toxicity can be adequately characterized with the help of the combination of the single toxicities and interactions of its components. Usually, such simple mixture contains a small number of identifiable single chemicals. Unfortunately, real world case studies most often involve complex mixtures. One has to consider as a *complex mixture* any mixture containing so many components that it is not possible to properly characterize its toxicity from data based on components' toxicities and interactions. Risk assessment of complex mixture are based on toxicity and exposure data on the whole mixture. Mixtures that displayed similar characteristics for transport, fate, physiological processes, and toxicity are known as *similar mixtures*. Very often, they only differs by a small number of features. Moreover, similar mixtures frequently contain groups of components that are similar in chemical structure and biological activity and also originate together from the same kind of sources (e.g. diesel exhaust, municipal incinerator). Such similar components belong to the same *chemical class*.

component	single chemical that may enter in the composition of a chemical mixture
similar component	single chemicals with the same or similar biological activities.
chemical mixture	any set of multiple chemical substances regardless of their sources that may jointly cause toxicity in the target population.
simple mixture	any mixture containing two or more identifiable single chemicals, but few enough that the mixture toxicity can be adequately characterized.
complex mixture	any mixture containing so many components that any estimation of its toxicity contains too much uncertainties and error to be useful.
similar mixtures	mixtures that are expected to have comparable characteristics for toxicity.
chemical class	any group of components displaying similar chemical structure and biological activity.

Table 1. Definitions and key concepts widely used in assessment of mixture toxicity. Components and mixtures (U.S. EPA, 1987)

The concept of toxicological similarity is based on data dealing with the biological activities of chemicals. In this matter, the literature frequently refers to the *mode of action* as a series of events and processes starting with interaction of an agent with a cell, causing disturbances and damages (Table 2). The sequence of events has to be supported by experimental evidences and a clear link must be identified between the adverse effect and the chemical. The reference to the *mechanism of action* implies a more detailed understanding and a deeper description of the cascade of events. One has to clearly distinguish between aggregate and cumulative exposures. *Aggregate exposure* refers to single chemical toxicity. It is the whole exposure to a single chemical whatever the *exposure pathways* (food, water, air, residential uses, occupational) and the *exposure routes* (oral, dermal, inhalatory, external). The associated risk is the *aggregate risk*. *Cumulative exposure* and corresponding *cumulative risk* refer to multiple chemicals, whatever the pathways and routes. One has to emphasize that temporality of exposure/effects plays a key role in aggregate and cumulative exposure assessments.

mode of action	series of events or processes resulting in an adverse effect.
mechanism of action	detailed description and understanding of the molecular events explaining biological activity
aggregate exposure	demographic, spatial and temporal characteristics of exposure to a single chemical through all relevant pathways and routes.
aggregate risk	risk associated with aggregate exposure.
cumulative exposure	aggregate exposure to multiple chemicals.
cumulative risk	risk associated with cumulative exposure
exposure pathway	any physical way that contributes to a physical interaction between chemicals and living organisms
exposure route	any process that permits the entry of a chemical into an organism or the interaction between the toxicant and the organism

Table 2. Definitions and key concepts widely used in assessment of mixture toxicity. Biological action, exposures, risks. (U.S. EPA, 1987 & WHO ICPS, 2009)

Several other concepts may be remembered, because of their importance in predicting toxicity and assessing risks. It is now well established that *speciation*, the occurrence of an element in different forms, is crucial to understanding its toxicity (Duffus et al., 2009). The *chemical species* include isotopic composition, electronic or oxidation state, and/or complex or molecular structure. *Bioaccessibility* is the potential for a substance to come in contact with a living organism. For instance a substance trapped inside a particule is not bioaccessible, whereas a part of the substance adsorbed on the surface of a particule are accessible. *Bioavaibility* describes the potential for a substance to be taken up by a living organism. Bioavaibility depends on both physicochemical properties and biological capabilities.

3. Non interactive chemicals. Additivity.

Very early, Plackett & Hewlett (1952) have identified the four possible types of joint action for mixtures (Table 3).

Types	Similar joint action	Dissimilar joint action
Non interactive	Simple similar action (concentration addition)	Independent joint action (response addition)
Interactive	Complex similar action	Dependent joint action.

Table 3. The four possible types of joint action for mixtures (Plackett & Hewlett, 1952).

The four types essentially refer to binary mixtures. In real world, chemical mixtures often contain numerous substances. Moreover, interactions are thought at the molecular level in terms of mode of action. Other interactions between chemicals may occur at other biological levels. Nevertheless, these authors have clearly distinguished two key points of joint action: (i) the similarity or dissimilarity of the modes of action and (ii) the dependence or independence of chemical actions. Indeed, mixture components exert their toxicity independently or not. They may also have toxicological interactions or not. These properties have been used to define different ways of assessing mixture toxicity.

Revisiting the concepts from Plackett & Hewlett, Ashford (1981) has distinguished six possible combination mechanisms for the joint action of mixtures or drugs (Table 4). The

author considers that the different subsystems (i.e. nervous, cardiovascular, endocrine subsystems…) have to be studied independently. Thus, it becomes possible to estimate the respective contributions of the different subsystems to the response of the whole organism. He has also proposed to take into account possible interactions between chemical substances occurring into the different subsystems. The model also identifies the possibilities of partial interactions between chemicals. Such partial interactions better correspond to the real physiology of the organisms.

Correspondence between compounds in the mixture	None	Some	All
Common sites of action (similarity)	Dissimilar (and noninteractive	Partially similar	Fully similar
Common subsystems (dependence)	Independent (and noninteractive)	Partially dependent	Fully dependent

Table 4. The six possible combination mechanisms for the joint action of toxicants (Ashford, 1981)

A key concept in understanding mixture risk assessment is *toxicologic similarity*. In this case, one assumes a similar mode of action across mixture components. Sometimes, the mode of action is not the same and components act only on the same target organ.
In contrast, *independence of action* is defined as mixture components that cause different kinds of toxicity, or effects in different target organs. The term *additivity* is used when the toxicity of the combination of chemicals can be estimated directly for the sum of the exposure levels (*dose additivity*) ot of the responses (*response additivity*).

3.1 Dose additivity or concentration addition.

When the components of a chemical mixture have the same mode of action, the mixture toxicity is assessed by the sum of the dose of the components (Loewe & Muischnek, 1926). The dose additivity or concentration addition (CA) concept is devoted to similarly acting toxicants. Sprague (1970) proposed a derived concept: the toxic unit approach (TU). In this hypothesis any component can be replaced by another if they display the same action mechanism as long as the corresponding relative toxic potency allows to obtain a similar toxic unit.
This method has been refined and is currently used to assess the toxicity of several chemical classes (US EPA, 2000). One considers that each component of the mixture behaves as a concentration or dilution of every other chemical in the mixture. The response of the combination is the response expected from the equivalent dose of an index chemical. This index chemical is selected as the basis for standardization of toxicity of components in a mixture. The index chemical must have a clearly defined dose-response relationship. The equivalent dose is the sum of component doses scaled by their toxic potency relative to the index chemical.

$$C_m = \sum_{k=1}^{n} C_k \times RPF_k \quad (1)$$

where
C_m is the mixture concentration expressed as an equivalent of the index chemical,
C_1 is the concentration of the index chemical,
C_k is the concentration of the k component,
RPF_k is the relative potency factor relative to the index chemical ($RPF_1 = 1$).
PCDDs and PCDFs commonly called dioxins, are by-products of combustion processes. PCBs were manufactured in the past for a variety of industrial uses, as electric insulators, dielectric fluids and hydraulic fluids. Most countries banned the manufacture and use of PCBs in the 1970s. Improper handling of PCBs is responsible of a continuing source of environmental contamination. Dioxins, furannes, and co-planar polychlorobiphenyls (PCBs) are Persistent Organic Pollutants (POPs), which are known to have the same mechanism of action since they are Aryl hydrocarbon Receptor (AhR) ligands. AhR is a cytosolic transcription factor that is normally inactive, bound to several chaperones. Toxicity results of the activation of AhR signaling pathways.
2,3,7,8-tetrachlorodibenzodioxin (TCDD) is the most potent congener of this group and is considered one of the most potent toxicants and carcinogens known to date. Since PCDDs, PCDFs, and PCBs occur as complex mixtures in food, this chemical class of compounds poses some risks for humans. Consequently, methods have been developed to assess cumulative risk related to dioxins and dioxin-like compounds thanks to the World Health Organization.

$$TEQ = \sum_{i=1}^{n} Ci \times TEFi \tag{2}$$

where
TEQ, toxic equivalency quantity is expressed in toxic equivalents of the 2,3,7,8-TCDD, i.e. the index chemical,
C_1 is the concentration of the 2,3,7,8-TCDD,
C_i is the concentration of the i component,
TEF_i is the toxic equivalency factor, that is the relative potency factor relative to the index chemical ($TEF_1 = 1$).
TEFs values are estimates derived from experimental data (see for instance Van den Berg, 1998). TEFs have been recently reevaluated (Van den Berg, 2006) and uncertainties were assumed to be within 1 order of magnitude. The underlying principle of effect additivity has been confirmed by recent data.
When the chemical components have the same mode of action, but the mechanism of action is not accurately known, it is not possible to use the RPF or TEQ methods with a high level of confidence. In such cases, an alternative method has been proposed. The hazard quotient is the ratio of the potential exposure to the substance to the level at which no adverse effects are expected. The hazard quotient is based on the estimation of exposure and its comparison with a reference level supposed to be acceptable.

$$HQ_i = \frac{E_i}{RfD_i} \tag{3}$$

where
HQ_i is hazard quotient for the substance i,

E_i is the exposure to the substance i,
RfD_i is the reference dose (acceptable level) for the substance i.
This hazard index method is a simple addition method: the hazard index is the sum of hazard quotients for substances that affect the same target organ.

$$HI = \sum_{i=1}^{n} HQ_i \tag{4}$$

where HI is the hazard index for the chemical mixture.
A more simple additive method has also been used. The *point of departure index* (PDI) consists in the addition of the *no observed adverse effect level*s (NOAEL) or benchmark doses (BMD). All these methods require additivity of the doses or concentrations.
The *margin of exposure* (MOE) method is rather close to the HI and PDI methods. It is based on the estimation of the ratio of the no-observed adverse effect level to the estimated exposure dose. Similarly, margins of exposure of components of a mixture may be summed.
Basic concepts supporting dose additivity or response additivity are briefly summarized in Table 5. Unfortunately, none of these methods takes into account possible interactions between the components of the mixture.

index chemical	The chemical selected as the basis for standardization of toxicity of components in a mixture. The index chemical must have a clearly defined dose-response relationship.
dose additivity concentration addition	When each component of the mixture behaves as a concentration or dilution of every other chemical in the mixture, the response of the combination is the response expected from the equivalent dose of an index chemical. The equivalent dose is the sum of component doses scaled by their toxic potency relative to the index chemical.
response additivity independence of action	The toxic response from the combination of chemicals is equal to the conditional sum of components responses as defined by the formula for the sum of independent event probabilities.
RPF	Relative Potency Factor (see Eq. 1)
TEF	Toxic Equivalency Factor (see Eq. 2)
TEQ	Toxic Equivalency Quantity (see Eq. 2)
HQ	Hazard Quotient (see Eq. 3)
HI	Hazard Index (see Eq. 4)
PDI	The Point of departure index is the simple addition of the no observed adverse effect levels (NOAEL) or benchmark doses (BMD).
MOE	The margin of exposure is the ratio of the no-observed adverse effect level to the estimated exposure dose.

Table 5. Definitions and key concepts used for mixture toxicity assessment when components of the mixture do not interact. Additivity. Independence of action. (U.S. EPA, 2000 & WHO ICPS, 2009)

3.2 Response additivity or independence of action.

One of the first paper dealing with mixture toxicity is the one from Bliss (1939), who proposes the method known as *response additivity*. This approach is used when the mixture

components act independently on different targets. The response of the mixture is given by the sum of the responses of its components. For the noninteractive or independent types of joint action, one has to keep in mind that it is assumed that the components of the mixture do not affect the toxicity of one another.
In such a case, the toxic response from the combination of chemicals is equal to the conditional sum of components responses as defined by the formula for the sum of independent event probabilities (ATSDR, 2004). For instance, for a binary mixture, the cumulative risk may be given by Eq. 5:

$$p_m = 1 - (1-p_1) \times (1-p_2) \quad (5)$$

where
p_m is the risk related to the exposure to the mixture,
p_1 is the risk related to the exposure to component 1,
p_2 is the risk related to the exposure to component 2.

3.3 Critical overview of the CA and IA models

Two basic concepts have been generally used for predicting multiple mixture toxicity: concentration addition (CA, Loewe and Muischnek, 1926) and independent action (IA, Bliss, 1939).
It has been proved that the CA model provides highly accurate predictions of mixture toxicity when all of the components have a strictly similar mode of action, regardless of their levels and ratios in the mixture (Faust et al., 2001; Zwart and Posthuma, 2005; Junghans et al., 2006). However the CA model is not adapted to mixtures with components having dissimilar modes of action because it leads to an overestimation of the toxicity of such mixtures (Faust et al., 2003).
The independent action (IA) model is based on dissimilar actions of mixture components. In this approach, the toxicity of each component is independent and cannot be replaced by another. The basic idea of this approach is that different compounds act on different physiological systems within the exposed organisms and lead to a common toxicological endpoint. This model provides accurate predictions of the mixture toxicity when all of the components have dissimilar modes of action, regardless of their levels and ratios in the mixture (Faust et al., 2003). However the IA model is not adapted to mixtures with similar acting components because it leads to an underestimation of the overall toxicity (Faust et al., 2001; Junghans et al., 2006).
Two main difficulties still remains. First, chemical with and without the same mode of action are very often found in the same mixture. Second, components may toxicologically interact. Furthermore, interspecific differences and possible interactions at the ecological levels are not satisfactorily addressed by the available models.
Recently, Zwart and Posthuma (2005) proposed a mixed two-step approach for mixed-model (MM) calculations. The first step requires evaluation of the CA responses to each individual toxic mode of action, the second step consists in evaluating the IA effect of the different toxic modes of action. We have used such a model to assess toxicity of a mixture of wood preservatives. The experimentals, the results and the main conclusions are given in section 5 (see below). In conclusion, one has to remember that the assessment of the predicting values of the available models is still an opened question (Backhaus et al., 2003; Zwart and Posthuma, 2005; Junghans et al., 2006).

4. Interactive chemicals. Different types of toxicological interactions between chemicals

A common concern for evaluating chemical mixtures is the potential for toxicological interactions to occur from co-exposures. Usually, one considers that toxicological interactions occur when the responses observed deviate from those expected under additivity.

4.1 The Different types of toxicological interactions between chemicals.

When two or more chemicals are combined, they may interact in different ways. The most simple toxicological interactions are *synergism* and *antagonism*. Other interactions, such *potentiation, inhibition* or *masking* may also modulate possible adverse effects. Different types of toxicological interactions between chemicals are briefly summarized in Table 6.

synergism	The combined effect of several chemicals is greater than expected on the basis of the simple summation of the toxicity of each of the individual substances
potentiation	When one substance does not have a toxic effect on a system, but when added to a toxic chemical, it makes the latter more toxic
antagonism	The combined effect of several chemicals is smaller than the solitary effect of any one of those chemicals
inhibition	When one substance does not have a toxic effect on a system, but when added to a toxic chemical, it makes the latter less toxic
masking	When the compounds produce opposite or functionally competing effects at the same site or sites, so that the effects produces by the combination are less than suggested by the component toxic effects.
no influence	When one substance does not have a toxic effect on a system, and but when added to a toxic chemical, it has no influence on the toxicity of the latter chemical.

Table 6. Types of toxicological interactions (Duffus et al., 2009; US EPA, 2000; ATSDR, 2004)

The relations between additivity, similarity of the modes of action, and interactions are listed in Table 7, which gives a theoretical overview of the relations between toxicological interactions and similar or dissimilar joint actions.

	Toxicological interaction	Joint action
No interaction	Dose additivity	Simple similar action
	Response additivity	Simple dissimilar action Independent action
Interaction	Synergism	Complex similar action Effect > additivity
	Potentiation	Complex dissimilar action Effect > additivity
	Antagonism	Complex similar action Effect < additivity
	Inhibition	Complex dissimilar action Effect < additivity

Table 7. Relations between toxicological interactions and similar or dissimilar joint actions

4.2 Interactions between chemicals and newly developed methods for assessment of mixture toxicity

Besides additivity models, there are very few available methods to take into account the toxicological interactions possibly occurring between the components of a mixture (WHO IPCS, 2009). Among these methods, one has to cite qualitative *binary weight of evidence* (BINWOE) proposed from ATSDR (2007). BINWOE evaluates strength of interactions data, mechanism of action, influence of exposure duration and route, and sequence of exposure for each pair of chemicals. For instance, a method has been developed to quantitatively modify the hazard index (HI), using factors that account for interaction weight of evidence, interaction magnitude, fraction of toxic hazard of each interacting chemical pair and relative proportions of the chemicals (Teuschler, 2009; USEPA, 2000). Among the methods currently in development, one has to list Physiologically Based Pharmacokinetic (PBPK) models. Such methods have been used (Haddad et al., 2001) to compare an interaction-based HI for central nervous system effects with an additive HI for different exposure to mixtures of several hydrocarbons showing greater than additive effects at the higher total dose levels of the mixture.

The *Whole Mixture Approach* (Mumtaz et al., 1993) uses effects data from exposure to the mixture of concern. These data are treated as if the mixture behaves like a single substance. Lastly, the *Threshold of Toxicological Concern (TTC)* has been proposed for use with complex mixtures where no effects data are available (Kroes et al., 2005). This method is based on structure–activity relationships and assigns exposure thresholds for comparison with the potential exposure level.

Species differ in their sensitivity toward a single chemical as a result of differences in biological traits (De Zwart & Posthuma, 2005). At the ecosystem level, the risk of chemical exposure to a single compound may be characterized by the proportion of species from a generic species pool that is likely affected by a toxicant at a certain concentration. The *potentially affected fraction* of species (PAF) is used to quantify the risk for species assemblages. Using this concept together with the mixture toxicity models (CA and IA models), De Zwart & Posthuma (2005) have proposed a method to address the risk on direct effects on the composition of species assemblages and biodiversity. This method has still to be validated.

5. Several outcomes from a real world case-study: wood preservatives

5.1 Possible impacts of wood preservatives on aquatic organisms

Wood, especially from coniferous trees is very frequently treated with various pesticides, commonly called wood preservatives (essentially insecticides and fungicides), to prevent attacks by pathogenic agents such as xylophagous insects or lignivorous fungi. Treatments avoid alterations of the wood mechanical qualities, and consequently economic loss or lifespan reduction. Treatment occurs at different stages of the production in tree nurseries, during wood storage, or at sawmills.

Historically, sawmills were established very close to the forests in basin heads along the rivers to get easy energy from water. Consequently, the risk of contaminations of aquatic environment with wood preservative mixture is considered as very high (Gifford et al., 1996; Lyytikaïnen et al., 2001; Hingston et al., 2002, 2006). After accidental or routine releases, wood preservatives exert marked adverse effects on macroinvertebrates and fish populations, and in a more general way in aquatic communities. Moreover, one has to keep

in mind that basin heads may constitute an invaluable resource for drinking water and biodiversity.

5.2 investigations on mixture toxicity of wood preservatives

Very often, wood preservatives, as other pesticides are used as commercial solutions. These commercial solutions of wood preservatives contain mixture of several active chemicals. Therefore, in case of accidental (acute) or routine (chronic) releases in the natural environment, aquatic organisms are exposed to several chemicals at the same time (Helson & Surgeoner, 1986; Green & Abdelghani, 2004).

	mixture 0 (M0)	mixture 1 (M1)			mixture 2 (M2)		
Chemicals	Concentrations in the commercial mixture EX 2002 E.S.E.® (mM)	Concentrations (mM)	Pesticide ratios in mixture 1	Toxic Units (%)	Concentrations (mM)	Pesticide ratios in mixture 2	Toxic Units (%)
Propiconazole	3.62	3.62	45.8%	<0.01%	9888**	76.2%	23.0%
Tebuconazole	1.36	1.36	17.2%	<0.01%	2612**	20.1%	11.5%
IPBC	1.49	1.49	18.8%	0.01%	480**	3.7%	30.5%
Cypermethrin	1.44	1.44	18.2%	99.98%	0.226*	0.0017%	35.0%

Table 8. Concentrations of active substances in the commercial mixture EX 2002 E.S.E.© (M0). Pesticide ratios (%) calculated for mixture 1 (M1), and for mixture 2 (m2) based on *G. pulex* 96-h LC_{50} (*) of cypermethrin (mM) and on *G. pulex* 96-h LC_5 (**) of fungicides (mM). Toxic units ratio (%) based on respective *G. pulex* 96-h LC_{50} are indicated for mixture 1 and mixture 2.

A study was undertaken to mimick the effects of a commercial mixture containing four different pesticides with various mode of action. The results exposed thereafter have been already published in a previous paper, where experimental details can be found (Adam et al., 2009). Freshwater amphipods *Gammarus pulex* (L.) were exposed to propiconazole, tebuconazole, IPBC, and cypermethrin given separately or in mixtures. First, we assess the environmental toxicity of wood preservatives on aquatic biota starting from single chemical exposures. Then, mixture toxicities were modelled using concentration addition (dose additivity, CA), response additivity (independence of action, IA), and mixed model (MM). The modelled toxicity was compared with the measured mixture toxicity. To do that, two experiments were done, *G. pulex* were exposed to (i) a real world commercial mixture (M0, Table 8) and (ii) a laboratory-made mixture (M1, Table 8) containing exactly the same ratio of active substances than the real world commercial mixture. The only difference between these mixtures is that the commercial mixture contained unknown additives and solvents.

Acute toxicity tests were performed. *G. pulex* (L.) free from parasites were collected from an unpolluted stream (Ruisseau de la Fontaine des Ermites, France, N4712404300 E00610303200). Individuals were acclimated in freshwater to laboratory conditions at least 10 days prior to testing. Ten *G. pulex* adults (46 mm) were randomly chosen and inserted into a test chamber (a 100 mL glass container) that was maintained at 15 °C. For each acute

test concentration, six replicates were used. The mortality was observed after 24, 48, 72, and 96 h of exposure.

5.3 Rationales for the choice of the test-organism

The freshwater amphipod *Gammarus pulex* (L.) (*Crustacea, Amphipoda*) has been chosen as test-organism because of its ecological and ecotoxicological importance. This crustacean species is one of the most widespread invertebrates in European streams and it is a major component of the biomass of many streams (Welton, 1979). As a detritus feeder, *G. pulex* plays a key role in nutrient cycling in freshwater systems (Welton, 1979) and *Gammarus* species are among the most eaten prey for many fish species (Bollache et al., 2006). *G. pulex* is known to be sensitive to a wide range of pollutants and to be among the most sensitive aquatic invertebrates (Helson & Surgeoner, 1986; Mian & Mulla, 1992; Schulz & Liess, 1999; Wogram & Liess, 2001; Cold & Forbes, 2004; Van Wijngaarden et al., 2004; Bloor et al., 2005). This amphipod species can be easily grown in the laboratory and has been recommended for use in toxicity tests (McCahon & Pascoe, 1988a, b; Adam et al., 2010). Moreover, we have also investigated the impact of wood preservatives in *Gammarus pulex* L. and *Gammarus fossarum* K. (*Crustacea, Amphipoda*) populations. Results show that populations were highly impaired by treatment areas at very low pesticide contaminations. Densities and age structure of the populations were particularly modified. Results also suggested an active drift of adults from the most contaminated sites. The impact was observed throughout the year but it was higher in summer and after repeated rainfall events.

5.4 Modes of action of the tested chemicals

Propiconazole, tebuconazole, 3-iodo-2-propinyl butyl carbamate (IPBC), and cypermethrin are among the most frequently used chemicals to protect wood. Two of these pesticides, propiconazole and tebuconazole are triazole fungicides, displaying similar physiological effects: they are 14α-demethylase inhibitors and also referred to as ergosterol biosynthesis inhibitors via cytochrome P450 inhibition (Egaas et al., 1999; Iwasa et al., 2004). Tebuconazole is frequently used in agricultural areas (Berenzen et al., 2005) and propiconazole is one of the most widely distributed pesticides in the world (Kronvang et al., 2003). IPBC is a halogenated unsaturated carbamate fungicide mainly used as wood preservative (Bailey et al., 1999). Juergensen et al. (2000) hypothesized that its fungicidal property was related to the terminal iodine, whereas Jarrad et al. (2004) proposed that carbamate pesticides could act on different physiological targets by disturbing the acetylcholine esterase activity. Another commonly used pesticide in commercial mixture is cypermethrin, a pyrethroid insecticide which exerts very severe toxic effects on aquatic invertebrates. Synthetic pyrethroids are among the most widely used insecticides around the world (Hill et al., 1994; Amweg et al., 2005). Pyrethroids act by slowing the gating of the voltage-dependent sodium channels, thus leading to a sustained membrane depolarization of motor neurons (Bradbury & Coats, 1989).

5.5 Single chemical toxicity data

Dose response curves were fitted with the help of Hill's model for the single-contaminant experiment. LC_{50} were calculated by the Regtox macro (open source) running with Microsoft Excel© software. Results are given in Figures 2 & 3. Mortality response curves of the four tested substances followed sigmoid curves. LC_{50}s with their 95% confidence intervals

obtained from the Hill's model are given in Table 9. LC_{50} decreased with increasing exposure duration for the four tested pesticides.

G. pulex exposed to propiconazole displayed 96-h LC_5 and 96-h LC_{50} which respectively occurred at 3384 and 4703 µg.L^{-1} (Fig. 2A). The main part of *G. pulex* response to propiconazole was observed during the first 24 h of exposure, then, the LC_{50} decrease was very low between 24 and 96 h of exposure (Table 9). As for propiconazole, tebuconazole lethality on *G. pulex* displayed a threshold concentration: 96-h LC_5 and 96-h LC_{50} occurred respectively at 804 and 1643 µg.L^{-1} (Fig. 2B). Again, as for propiconazole, the main part of tebuconazole lethality on *G. pulex* is expressed in the first 24 h of exposure (Table 9).

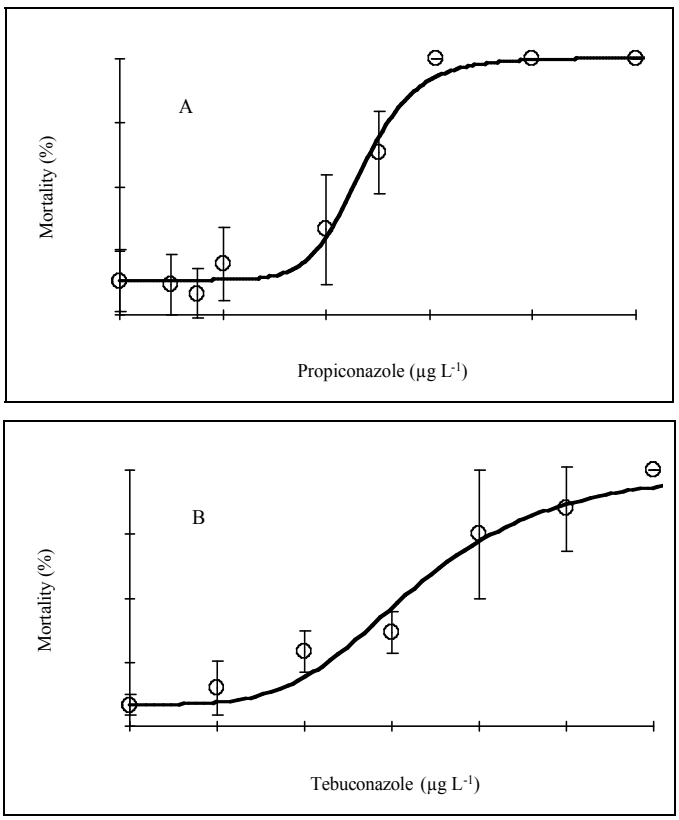

Fig. 2. Dose-response curves obtained by Hill's model after 96 h of exposure of *G. pulex* to triazole fungicides: propiconazole (A) and tebuconazole (B). Percentages correspond to *G. pulex* mortality (%). Pesticide concentrations are expressed in µg L^{-1}. Plots represent the mean (+/-95% CI) of 6 replicates.

Lethality provoked by IPBC on *G. pulex* was observed at very low concentrations. IPBC 96-h LC_5 and 96-h LC_{50} occurred respectively at 135 and 604 µg.L^{-1} (Fig. 3A). Contrary to triazole fungicides, the lethality caused by IPBC on *G. pulex* was low in the first hours of exposure, but strongly increased between 24 and 48 h of exposure. The IPBC LC_{50} decrease was higher than 90 % between 24 and 96 h of exposure (Fig. 3A). As for IPBC, mortality caused by cypermethrin on *G. pulex* was observed at the lowest concentrations. Cypermethrin 96-h LC_5 and 96-h LC_{50} occurred respectively at 0.03 and 0.09 µg.L^{-1} (Fig. 3B). As for triazole

fungicides, and contrary to IPBC, the lethality caused by cypermethrin on *G. pulex* appeared mainly during the first 24 h of exposure (Table 9).

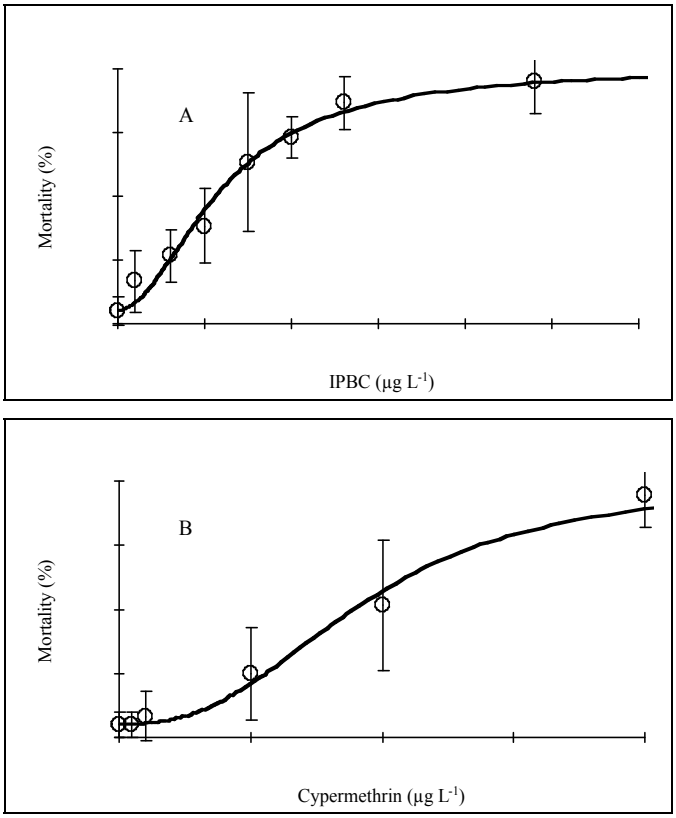

Fig. 3. Dose-response curves obtained by Hill's model after 96 h of exposure of *G. pulex* to a carbamate fungicide IPBC (A), and a pyrethroid insecticide cypermethrin (B). Percentages correspond to *G. pulex* mortality (%). Pesticide concentrations are expressed in µg L^{-1}. Plots represent the mean (+/-95% CI) of 6 replicates.

Chemicals	24h		48h		72h		96h	
	LC_{50} µg L^{-1}	r	LC_{50} µg L^{-1}	r	LC50 µg L^{-1}	r	LC_{50} µg L^{-1}	r
Propiconazole	5156-5507	0.9988	5037-5331	0.9981	4844-5177	0.9971	4439-4919	0.9947
Tebuconazole	2196-2444	0.9817	1823-2172	0.9848	1541-1903	0.9838	1471-1745	0.9905
IPBC	8438-13712	0.9906	929-1294	0.978	605-742	0.9967	517-661	0.9954
Cypermethrin	0.116-0.135	0.9996	0.098-0.116	0.998	0.084-0.103	0.9933	0.082-0.101	0.9957

Table 9. LC_{50} (95% IC) for propiconazole, tebuconazole, IPBC, and cypermethrin obtained after 24, 48, 72, and 96 h of *G. pulex* exposure, and Pearson's correlation coefficient (r) between observed mortality data and predicted lethality values obtained by Hill's model.

When given independently at environmentally realistic concentrations, propiconazole and tebuconazole (triazoles fungicides) were not toxic for *G. pulex*, 3-iodo-2-propinyl butyl

carbamate (IPBC, fungicide) was moderately toxic, and cypermethrin (pyrethroid insecticide) was extremely toxic. 96-h LC_{50} were respectively 4703, 1643, 604, and 0.09 µg L^{-1}.

5.6 Estimates of the mixture toxicity with the available models

The tested mixtures contain chemicals having similar and dissimilar toxic modes of action. Consequently, such mixtures are not expected to display dose additivity (CA) or response additivity (IA). Assessment of toxicities with these CA and IA models is expected to differ from those measured experimentaly on the whole mixtures (M0, M1). A mixed-model (MM) with a two-step approach according to Zwart & Posthuma (2005) was used to produce estimates of the mixture toxicity based on of single chemical toxicity data. During the first step, concentration addition is used to evaluate the CA responses of triazole fungicides according to Faust et al. (2003) who demonstrated the following relationship:

$$ECx_{mix} = \left(\sum_{i=1}^{n} \frac{p_i}{ECx_i} \right)^{-1} \tag{6}$$

where
ECx_{mix} is the effect concentration (x%) of the mixture,
the individual concentrations c_i are added up to c_{mix},
p_i is the constant proportion of the chemical i in the mixture, i.e. $p_i = c_i / c_{mix}$.
The second step consists in evaluating the IA responses of the different toxic modes of action for triazole fungicides, IPBC and cypermethrin.
The independent action model takes into account the relative relationships between response probabilities in test organisms. The dose relationships can be calculated by multiplying the probabilities of nonresponse (Bliss, 1939):

$$E(c_{mix}) = 1 - \prod_{i=1}^{n} [1 - E(c_i)] \tag{7}$$

where
$E(c_{mix})$ is the overall effect (scaled from 0 to 1) of a mixture of n components at the total concentration c_{mix} ($c_{mix} = c_1 + \ldots + c_n$),
$E(c_i)$ is the effect of the compound i if applied singly at the concentration c_i that corresponds to its concentration in the mixture.
The tested mixtures contain chemicals having similar and dissimilar toxic modes of action. Such mixtures are expected to have an intermediate toxicity between CA and IA toxicity predictions.
We have tested a mixed-model (MM) with a two-step approach, as proposed by Zwart and Posthuma (2005): the first step requires evaluation of the CA responses to each individual toxic mode of action, the second step consists in evaluating the IA effect of the different toxic modes of action for triazole fungicides, IPBC and cypermethrin. Dose-response curves predicted by the three mixture toxicity models (Fig. 4) were superimposed and no significant difference occurred between cypermethrin (Fig. 3B) and the M1 dose-response curves (Fig. 4). The lethal effect of M0 (commercial mixture EX 2002 E.S.E.® from Dyrup©) was significantly higher (Wilcoxon test, p = 0.044) than those observed with M1 which did not contain any commercial additives (Fig. 4).

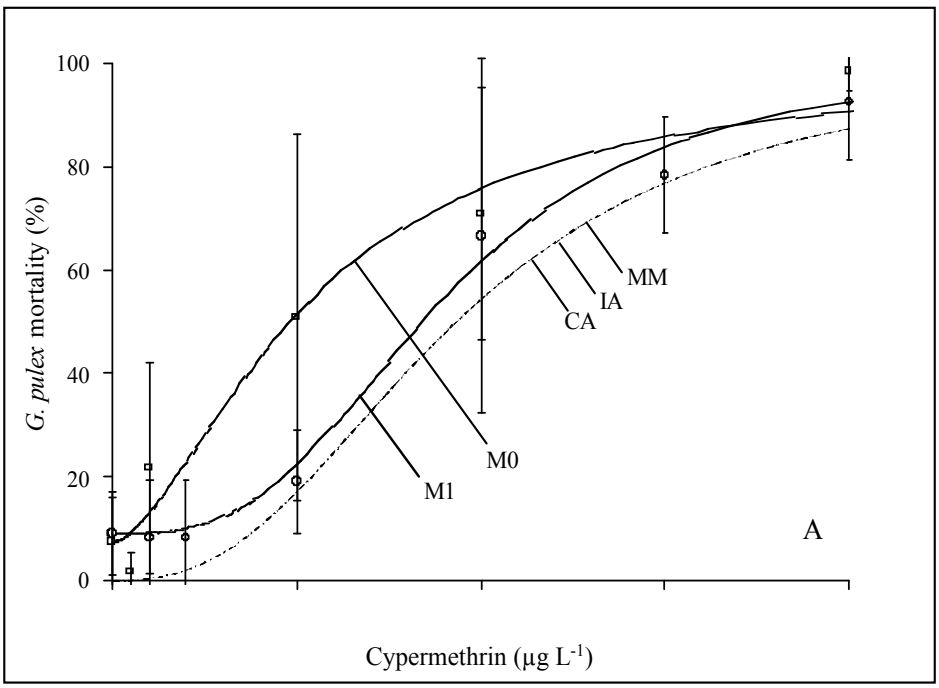

Fig. 4. Dose-response curves obtained for *G. pulex* exposed to M0 or M1 (A) after 96 h of exposure. Percentages correspond to *G. pulex* mortality (%). Pesticide concentrations are expressed in terms of cypermethrin concentration ($\mu g.L^{-1}$) in mixtures. White plots give the mean percentages (+/-Standard Deviation) of 6 replicates. Circle plots correspond to M1. Square plots correspond to M0, commercial solution EX 2002 E.S.E.© (A). Solid lines are the dose-response curves obtained by Hill's model. Predicted dose-response curves calculated by CA, IA models and MM are represented with dotted lines.

CA, IA models and MM have proved to be equally relevant for predicting mixture toxicity of M1. The three predicted dose-response curves were superimposed, and we could not discriminate a better one in this case. The lethality of this mixture for *G. pulex* was mainly caused by cypermethrin. The lethality of fungicides was too low to be observed at the tested concentrations. No synergism or antagonism has been detected between pesticides at the concentration ratio tested in M1. In conclusion, when amphipods were submitted to a mixture mimicking the composition of a commercial solution (18.2% of cypermethrin, 45.8% propiconazole, 17.2% tebuconazole, 18.8% IPBC), the overall toxicity was equal to that of the most toxic component, namely cypermethrin. But, when organisms were submitted to the real commercial mixture containing pesticides, solvents and additives, the toxic effects were markedly higher.

Another mixture (M2) used the same ingredients as M1, but with ratio of pesticides determined on the basis of 96-h LC_{50} for cypermethrin and 96-h LC_5 for the three other components. Cypermethrin represented only 0.002% of the total amount of active substances concentrations in M2, but it still represented 35.0% of the overall mixture toxicity expressed in terms of Toxic Units (Sprague, 1970). Fungicides concentrations in M2 were higher than in M1 (Tab. 9). With M2, the dose-response curves predicted by CA, IA models, and MM were different. Moreover, measured M2 toxicity was higher than toxicities predicted by the

CA, IA, and MM mixture models (Fig. 5). This indicated a synergism occurring between the four pesticides at this ratio-level. M2 toxicity was about 2.5, 17 and 18 fold stronger than predicted by respectively CA, IA models and MM as regards its 96-h LC_{50} (Fig. 5).

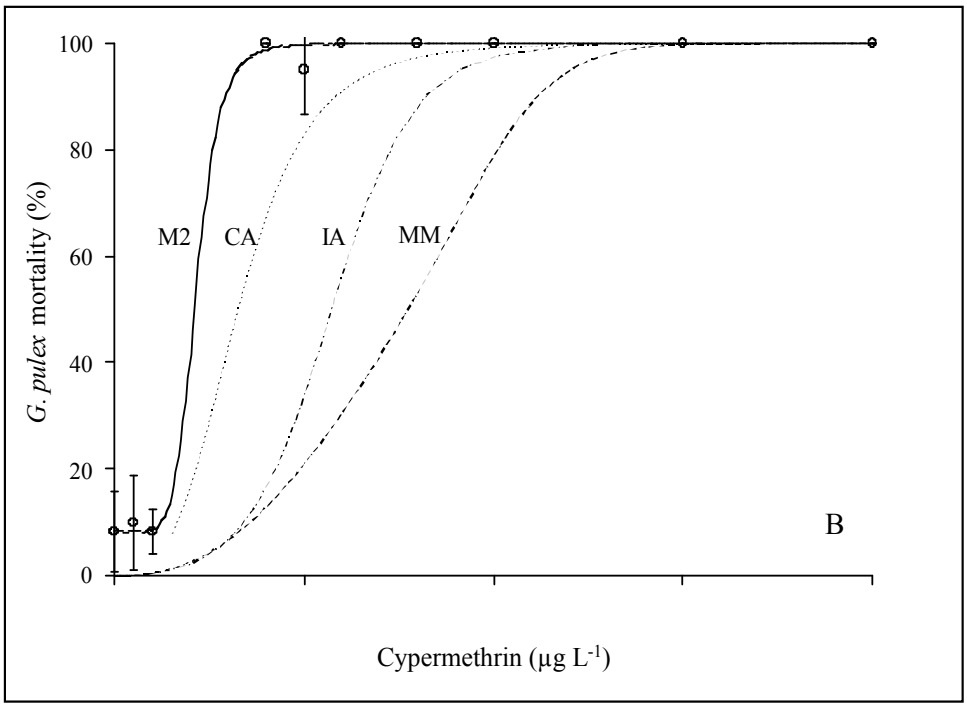

Fig. 5. Dose-response curves obtained for *G. pulex* exposed to M2 (B) after 96 h of exposure. Percentages correspond to *G. pulex* mortality (%). Pesticide concentrations are expressed in terms of cypermethrin concentration (µg.L^{-1}) in mixtures. White plots give the mean percentages (+/-Standard Deviation) of 6 replicates. Solid lines are the dose-response curves obtained by Hill's model. Predicted dose-response curves calculated by CA, IA models and MM are represented with dotted lines.

M2 was designed according to cypermethrin 96-h LC_{50} and fungicides 96-h LC_5. IPBC, cypermethrin and both triazole fungicides acting on three different physiological targets (Coats et al., 1989; Levine et al., 1999; Jackson et al., 2000; Juergensen et al., 2000), we could presume that the MM was the most relevant approach currently available for predicting toxicity of this mixture (Zwart and Posthuma, 2005). The observed M2 mixture toxicity reached up to 18 times the predicted one. This result suggests that a relatively high synergism would occur between active substances in M2. This third mixture with only 0.002% cypermethrin showed lethality 2.5 to 18 fold higher than those predicted by the commonly used models.

5.6 Lessons learnt from the case-study of wood preservative mixture toxicity

The present results (Fig. 4 & 5) show that interactions between active substances would depend on the ratio between chemicals displaying acute toxicity. Consequently, in real world, relevant environmental risk assessment of chemical mixture has also to take into

account changes that may occur in the natural environment. Indeed, pesticide environmental concentrations are known to change at different rates because of differences in degradation rates and transfer properties. The initial pesticide ratio of the commercial solution was likely to be modified between the treatment area and the contaminated aquatic environment. Furthermore, aquatic biota is typically exposed to brief pulses of pesticides in their natural environment (Liess et al., 1999). Then, aquatic organisms are expected to be exposed to fluctuating ratios of pesticides displaying different toxic interactions. Thus, relevant risk assessment should also consider possible patterns of pesticides ratio exposure.
Acute toxicity tests with M0, the corresponding commercial wood preservative mixture, have revealed a higher toxicity on *G. pulex* than observed with M1. In the present study, additives present in M0 commercial solution were likely to modify interactions between active substances and their toxicity expression (Stratton, 1985; Krogh et al., 2003). Additives could also act because they are themselves toxic, or they facilitate pesticides intake, or they reduce activity of detoxification mechanisms (Holloway and Western, 2003; Green and Abdelghani, 2004; Paul et al., 2005). Whatever the mechanism operating, the commercial solution containing additives displayed a higher toxicity than a mixture differing only by the absence of these additives. Therefore, when the composition of the mixture is not known with accuracy, available mixture toxicity models failed to predict ecotoxicity effects even if the accurate contents in active compounds are known. In the present case, toxicity predicted by mixture models was markedly underestimated. Consequently, ecotoxicological risk assessment of wood preservative mixture on aquatic systems have to be based on reliable data obtained by testing the overall commercial mixtures and cannot be calculated from single component toxicity data. CA, IA models, and MM were of limited interest for the environmental risk assessment of wood preservative mixtures especially because the use of additives in the commercial mixtures prevents from predicting toxicity. The present results give evidence that toxicity assessment of wood preservative mixtures should be necessarily based on toxicity experiments performed with real commercial solutions and not be derived from single chemical toxicity data. Furthermore, the present data strongly suggest that the environmental impacts of wood preservative mixtures might be frequently underestimated.

6. Concluding remarks

During the last ten years, mixture toxicology has undergone a remarkable and productive development (University of London, 2009). Because of resource and time limitations, direct toxicological information will never be available on all the possible mixtures to which humans or living organisms are exposed. Single chemical risk assessment has proven to be efficient at its own scale, but fails for the multiple combination of pollutants and various stressors existing in real life. The current methods available to assess mixture toxicity from single chemical toxicity data suffer from severe limitations, except in cases where additivity stands. In other cases, there does not exist any turn-key solution. The responses to health and environmental concerns cannot be only given by laboratory-based approaches and paradigms. The temporality of the exposures and related effects is insufficiently taken into account. Efforts should be made to better estimate exposures. This implies that models of exposure have still to be developed. The effects of low dose are probably insufficiently taken into account. The sensitivities of the various species must be apprehended better. Statiscally based methods may usefully supplement mechanistic approaches. Uncertainties have to be better estimated and taken into account. Biomarkers of effects, environmental monitoring,

biomonitoring, surveillance and population surveys are essential to an accurate exposure assessment. In this context, progress is still to be made to better understand the mechanisms and modes of action of toxicants. The potential of the omic-technics must be investigated. Taking into account interactions between chemicals and between chemicals and the environment remains a very difficult, but compulsory and exciting challenge.

7. References

Adam, O., Badot, P.M., Degiorgi, F. & Crini, G. (2009). Mixture toxicity assessment of wood preservative pesticides in the fresh water amphipod *Gammarus pulex* (L.). *Ecotox. Environ. Saf.* Vol.72, pp. 441–449.

Adam, O., Degiorgi, F., Crini, G. & Badot, P.M. (2010). High sensitivity of *Gammarus sp*. juveniles to deltamethrin: Outcomes for risk assessment. *Ecotox. Environ. Saf.,* Vol. 73, pp. 1402-1407.

Amweg, E.L., Weston, D.P. & Ureda, N.M. (2005). Use and toxicity of pyrethroid pesticides in the Central Valley, California, USA. *Environ. Toxicol. Chem.* Vol.24, pp. 966–972.

Ashford, J.R. (1981). General models for the joint action of mixtures of drugs. *Biometrics* Vol.37, pp. 457-474.

ATSDR (2004). *Guidance manual for the assessment of joint toxic action of chemical mixtures.* U.S. Department of Health and Human Services, Public Health Service, Agency for Toxic Substances and Disease Registry. Atlanta.

ATSDR (2007) *Interaction profiles for toxic substances*. Department of Health and Human Services, Agency for Toxic Substances and Disease Registry, Atlanta, (http://www.atsdr.cdc.gov/interactionprofiles/).

Backhaus, T., Altenburger, R., Boedeker, W., Faust, M., Scholze, M. & Grimme, L.H. (2003). The BEAM-project: prediction and assessment of mixture toxicities in the aquatic environment. *Cont. Shelf Res.* Vol.23, pp. 1757-1769.

Bailey, H.C., Elphick, J.R., Potter, A., Chao, E. & Zak, B. (1999). Acute toxicity of the antisapstain chemicals DDAC and IPBC, alone and in combination, to rainbow trout (*Oncorhynchus mykiss*). *Water Res.* Vol. 33, pp. 2410–2414.

Berenzen, N., Lentzen-Gogging, A., Probst, M., Schulz, H., Schulz, R. & Liess, M. (2005). A comparison of predicted and measured levels of runoff-related pesticides concentrations in small lowland streams on a landscape level. *Chemosphere*, Vol.58, pp. 683–691.

Bliss, C.I. (1939). The toxicity of poisons applied jointly. *Annals of Applied Biology*, Vol.26, pp. 585-615.

Bloor, M.C., Banks, C.J. & Krivtsov, V. (2005). Acute and sublethal toxicity tests to monitor the impact of leachate on an aquatic environment. *Environ. Int.* Vol.31, pp. 269–273.

Bollache, L., Kaldonski, N., Troussard, J.P., Lagrue, C. & Rigaud, T. (2006). Spines and behaviour as defences against fish predators in an invasive freshwater amphipod. *Anim. Behav.* Vol.72, pp. 627–633.

Bradbury, S.P. & Coats, J.R. (1989). Comparative toxicology of the pyrethroid insecticides. *Rev. Environ. Contam. Toxicol.* Vol.108, pp. 133–177.

Coats, J.R., Symonik, D.M., Bradbury, S.P., Dyer, S.D., Timson, L.K. & Atchinson, G.J. (1989). Toxicology of synthetic pyrethroids in aquatic organisms: an overview. *Environ. Toxicol. Chem.* Vol.8. pp. 671-679.

Cold, A. & Forbes, V.E. (2004). Consequences of a short pulse of pesticide exposure for survival and reproduction of *Gammarus pulex*. *Aquat. Toxicol.* Vol.67, pp. 287–299.

De Zwart, D., & Posthuma, L. (2005). Complex mixture toxicity for single and multiple species: proposed methodologies. *Environmental Toxicology and Chemistry*, Vol.24, No.10, pp. 2665-2676, ISSN 0730-7268/05.

Duffus, J.H., Templeton, D.M., & Nordberg, M. (2009). *Concepts in Toxicology*, RSC Publishing, ISBN 978-0-85404-157-2, Cambridge.

Egaas, E., Sandvik, M., Fjeld, E., Kallqvist, T., Goksoyr, A. & Svensen, A. (1999). Some effects of the fungicide propiconazole on cytochrome P_{450} and glutathione-S-transferase in brown trout (*Salmo trutta*). *Comp. Biochem. Physiol. C,* Vol.122, pp. 337–344.

Faust, M., Altenburger, R., Backhaus, T., Blank, H., Boedeker, W., Gramatica, P., Hamer, V., Vighi, M. & Grimme, L.H. (2001). Predicting the joint algal toxicity of multi-component s-triazine mixtures at low-effect concentrations of individual toxicants. *Aquat. Toxicol.* Vol.56, pp. 13-32.

Faust, M., Altenburger, R., Backhaus, T., Blank, H., Boedeker, W., Gramatica, P., Hamer, V., Scholze, M., Vighi, M. & Grimme, L.H. (2003). Joint algal toxicity of 16 dissimilarly acting chemicals is predictable by the concept of independent action. *Aquat. Toxicol.* Vol.63, pp. 43-63.

Gifford, J.S., Buckland, S.S., Judd, M.C., McFarlane, P.N. & Anderson, S.M. (1996).Pentachlorophenol (PCP), PCDD, PCDF and pesticide concentrations in afreshwater lake catchments. *Chemosphere* Vol.32, pp. 2097–2113.

Green, R.M. & Abdelghani, A.A. (2004). Toxicity of a mixture of 2,4-dichlorophenoxyaceticacid and mono-sodium methanearsonate to the red swamp crawfish, *Procambarus clarkii*. *Int. J. Environ. Res. Public Health* Vol.1, pp. 35–38.

Haddad, S, Béliveau, M, Tardif, R. & Krishnan K (2001). A PBPK modeling–based approach to account for interactions in the health risk assessment of chemical mixtures. *Toxicological Sciences*, Vol.63, pp. 125–131.

Helson, B.V. & Surgeoner, G.A. (1986). Efficacy of cypermethrin for the control of mosquito larvae and pupae, and impact on non-target organisms, including fish. *J. Am. Mosquito Control Assoc*. Vol.2, pp. 269–275.

Hill, I.R., Shaw, J.L. & Maund, S.J. (1994). Review of aquatic field tests with pyrethroid insecticides. In: Hill, I.R. (Ed.), *Freshwater Field Tests Hazard Assessment of Chemicals* [Proceedings of the European Workshop on Freshwater Field Tests]. Lewis Publisher, Boca Raton, Fl, pp. 249–271.

Hingston, J.A., Moore, J., Bacon, A., Lester, J.N., Murphy, R.J. & Colins, C.D. (2002). The importance of the short-term leaching dynamics of wood preservatives. *Chemosphere* Vol.47, pp. 517–523.

Hingston, J.A., Murphy, R.J. & Lester, J.N. (2006). Monitoring losses of copper based wood preservatives in the Thames estuary. *Environ. Pollut.* Vol.143, pp. 367–375.

Holloway, P.J. & Western, M. (2003). Tank-mix adjuvants and pesticide residues: some regulatory and quantitative aspects. *Pest. Manag. Sci.* Vol.59, pp. 1237-1244.

Iwasa, T., Motoyama, N., Ambrose, J.T. & Roe, R.M.R.M. (2004). Mechanism for the differential toxicity of neonicotinoid insecticides in the honey bee, *Apis mellifera*. *Crop Protect*. Vol.23, pp. 371–378.

Jackson, C.J., Lamb, D.C., Kelly, D.E. & Kelly, S.L. (2000). Bactericidal and inhibitory effects of azole antifungal compounds on *Mycobacterium smegmatis*. *FEMS Microbiol. Letters* Vol.192, pp. 159-162.

Jarrad, H.E., Delaney, K.R. & Kennedy, C.J. (2004). Impacts of carbamate pesticides on olfactory neurophysiology and cholinesterase activity in coho salmon (*Onchorhynchus kisutch*). *Aquat. Toxicol.* Vol.69, pp. 133–148.

Juergensen, L., Busnarda, J., Caux, P.Y. & Kent, R. (2000). Fate, behavior, and aquatic toxicity of the fungicide IPBC in the Canadian environment. *Environ. Toxicol.* Vol.15, pp. 201–213.

Junghans, M., Backhaus, T., Faust, M., Scholze, M. & Grimme, L.H. (2006). Application and validation of approaches for the predictive hazard assessment of realistic pesticide mixtures. *Aquat. Toxicol.* Vol.76, pp. 93-110.

Kroes, R,. Kleiner, J. & Renwick, A. (2005) The threshold of toxicological concern concept in risk assessment. *Toxicological Sciences*, Vol.86, pp. 226–230.

Krogh, K.A., Halling-Sorensen, B., Mogensen, B.B. & Vejrup, K.V. (2003). Environmental properties and effects on nonionic surfactant adjuvants in pesticides: a review. *Chemosphere* Vol.50, pp. 871-901.

Kronvang, B., Laubel, A., Larsen, S.E. & Friberg, N. (2003). Pesticides and heavy metals in Danish streambed sediment. *Hydrobiologia* Vol.494, pp. 93–101.

Levine, S.L., Oris, J.T. & Denison, M.S. (1999). Modulation of Cyp1A expression in rainbow trout by a technical grade formulation of propiconazole. *Environ. Toxicol. Chem.* Vol.18, pp. 2565-2573.

Liess, M., Schulz, R., Liess, M.H.D., Rother, B. & Kreuzig, R. (1999). Determination of insecticide contamination in agricultural headwater streams. *Wat. Res.* Vol.33, pp. 239-247.

Loewe, S. & Muischnek, H. (1926). Uber kombinationswirkungen. 1. Mitteilung: Hilfsmittel der Fragestellung. *Naunyn Schmiedebergs Arch. Exp. Pathol. Pharmacol.* Vol.11, pp. 313–326.

Lyytikaïnen, M., Sormunen, A., Peraniemi, S. & Kukkonen, J.V.K. (2001). Environmental fate and bioavailability of wood preservatives in freshwater sediments near an old sawmill site. *Chemosphere* Vol.44, pp. 341–350.

McCahon, C.P. & Pascoe, D. (1988a). Culture techniques for three freshwater macroinvertebrate species and their use in toxicity tests. *Chemosphere* Vol.17, pp. 2471–2480.

McCahon, C.P. & Pascoe, D. (1988b). Use of *Gammarus pulex* (L.) in safety evaluation tests: culture and selection of a sensitive life stage. *Ecotoxicol. Environ. Saf.* Vol.15, pp. 245–252.

Mian, L.S. & Mulla, M.S. (1992). Effects of pyrethroid insecticides on nontarget invertebrates in aquatic ecosystems. *J. Agric. Entomol.* Vol.9, pp. 73–98.

Mumtaz, M.M., Sipes, I.G., Clewell, H.J. & Yang, R.S. (1993). Risk assessment of chemical mixtures: Biologic and toxicologic issues. *Fundamental and Applied Toxicology*, Vol.21, pp. 258–269.

Paul, E.A., Simonin, H.A. & Tomajer, T.M. (2005). A comparaison of the toxicity of synergized and technical formulations of permethrin, sumithrin, and resmethrin to trout. *Arch. Environ. Contam. Toxicol.* Vol.48, pp. 251-259.

Plackett, R.L. & Hewlett, P.S. (1952.) Quantal responses to mixtures of poisons. *J. Royal Stat. Soc. B*. 14: 141-163.

Schulz, R. & Liess, M. (1999). A field study of the effects of agriculturally derived insecticide input on stream macroinvertebrate dynamics. *Aquat. Toxicol.* Vol.46, pp. 155–176.

Sprague, J.B. (1970). Measurement of pollutant toxicity to fish. II. Utilizing and applying bioassay results. *Wat. Res.* Vol.4, pp. 3-32.

Stratton, G.W. (1985). The influence of solvent type on solvent-pesticide interactions in bioassays. *Arch. Environ. Contam. Toxicol.* Vol.14, pp. 651-658.

Teuschler, L. Combination toxicity: evaluating chemical mixtures when common mode of action assumptions do not apply. pp. 59-63. In World Health Organization, International Programme on Chemical Safety (2009). *Assessment of combined exposures to multiple chemicals: Report of a WHO/IPCS international workshop on aggregate/cumulative risk assessment.* ISBN 978 92 4 156383 3.

U.S. Environmental Protection Agency (1986). *Guidelines for health risk assessment of chemical mixtures.* Federal Register 51(185): 34014-34025.

U.S. Environmental Protection Agency (1987). *The risk assessment guidelines of 1986*. EPA/600/8-87/045.

U.S. Environmental Protection Agency (2000). *Supplementary guidance for conducting health risk assessment of chemical mixtures.* Washington DC. EPA/630/R-00/002.

University of London, School of Pharmacy (2009). State of the art report on mixture toxicity. http://ec.europa.eu/environment/chemicals/pdf/report_Mixture%20toxicity.pdf

Van den Berg, M., Birnbaum, L., Bosveld, B.T.C., Brunstrom, B., Cook, P., Feeley, M., Giesy, J.P., Hanberg, A., Hasegawa, R., Kennedy, S.W., Kubiak, T., Larsen, J.C., van Leeuwen, F.X.R., Liem, A.K.D., Nolt, C., Peterson, R.E., Poellinger, L., Safe, S., Schrenck, D., Tillitt, D., Tysklind, M., Younes, M., Waern, F. & Zacharewski, T. (1998). Toxic Equivalency Factors (TEFs) for PCBs, PCDDs, PCDFs for Humans and for Wildlife. *Environmental Health Perspectives* Vol.106, pp. 775-787.

Van den Berg, M. Birnbaum, L., Denison, M., DeVito, M. Farland, W., Feeley, M., Fiedler, H., Hakansson, H., Hanberg, A., Haws, L., Rose, M., Safe, S., Schrenk, D., Tohyama, C., Tritscher, A., Tuomisto, J., Tysklind, M., Walker, N. & Peterson, R.E. (2006). The 2005 World Health Organization reevaluation of human and mammalian toxic equivalency factors for dioxins and dioxin-like compounds. *Toxicological sciences*, Vol.93, No.2, pp. 223-241.

Van Wijngaarden, R.P.A., Gappen, J.G.M., Arts, G.H.P., Crum, S.J.H., Van den Hoorn, M.W., Van den Brink, P.J., & Brock, T.C.M. (2004). Aquatic risk assessment of a realistic exposure to pesticides used in bulb crops: a microcosm study. *Environ.Toxicol. Chem*. Vol.23, pp. 1479–1498.

Walker, C.H., Hopkin, S.P., Sibly, R.M., & Peakall, D.B. (2001). *Principles of ecotoxicology (second edition)*, Taylor & Francis, ISBN 0-7484-0939-4, London.

Welton, J.S. (1979). Life-history and production of the amphipod *Gammarus pulex* in a Dorset chalk stream. Freshwater Biol. Vol.9, pp. 263–275.

Wogram, J. & Liess, M. (2001). Rank ordering of macroinvertebrate species sensitivity to toxic compounds by comparison with that of *Daphnia magna. Bull. Environ. Contam. Toxicol*. Vol.67, pp. 360–367

World Health Organization, International Programme on Chemical Safety (2009). Assessment of combined exposures to multiple chemicals: Report of a WHO/IPCS international workshop on aggregate/cumulative risk assessment. ISBN 978 92 4 156383 3.

5

Exposure Factors to Organophosphate and Carbamates Pesticides in the Putumayo Department, 2006

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1. Introduction

One of the main problems faced by the world in the XXI century is the environment degradation. The rapid scientific and technological advances have generated immense developments for the humankind but, also, they have altered in a global way, the ecological balance of the planet.

Among the environmental agents harmful to the health, the chemical products occupy the most important place as a public health problem in the developing countries, due to the inadequate way they are manufactured and the substances used for the same, and also to the way the chemical residues are discarded (Corey, 1988).

The effects of the pesticides in the human and environment contamination is caused by the large quantity and assortment of substances that are applied to the agriculture and to the handling done of the pesticides during the application, transportation, storage and elimination of its residues (Idrovo, 1999).

Among the more than 70,000 chemical substances currently on the market, the synthetic pesticides have been occupying an important place since 1940, and have been converted into the principal strategy for the pest control (OPS, 2004; Henao, 1991).

The agricultural development model in Colombia sustains itself principally in the use of agrochemicals, which most of the times are used without the necessary technical investigation, without knowing the multiplicity of the regional features such as the climate variety, the species diversity and the cultures heterogeneity (Idrovo, 1999).

The World Health Organization (*OMS*, in Spanish) points out that during the first half of the decade of the eighties, about 1,000,000 cases of not deliberate poisoning with pesticides occurred, of which 70 % were originated within the working activity; it is believed that during the same period, close to 2,000,000 poisonings with suicidal purposes happened, and 7, 3 % of all the poisonings were lethal cases (OMS, 1992). The International Labour Organization (*OIT*)

for its part believes that the pesticides can be connected with 14, 0 % of the occupational injuries in the agricultural sector and of 10, 0 % of all the deaths (Córdoba, 2000). In the whole world, 148 epidemic break outs were registered between 1951 and 1990 because of the pesticides, causing 24,731 poisonings and 1065 deaths (Levine R.S, Doull J., 1992).
In the developing countries, the pesticides cause up to one million poisoning cases and up to 20,000 deaths, annually (Durán J.J., Collí J., 2000).
According to the International Organization of Consumers Union, an agricultural worker dies every 4 hours in developing countries due to poisoning by pesticides, which is equivalent to more than 10,000 deaths per year, and another 375,000 poison themselves with these products (United Nations Food and Agricultural Organization, 1986).
The Overseeing System in Public Health (*SIVIGILA,* 2005) informed that during year 2005 in which the study was carried out, the Department of Putumayo was in the country, the region with major poisonings incidence due to pesticides (see chart., 1), 8,777 cases were reported for 2006, 13,179 cases for 2007, 18,105 cases for 2008, and 19,723 cases of poisoning appeared already for pesticides during 2009 (*SIVIGILA*, 2009).

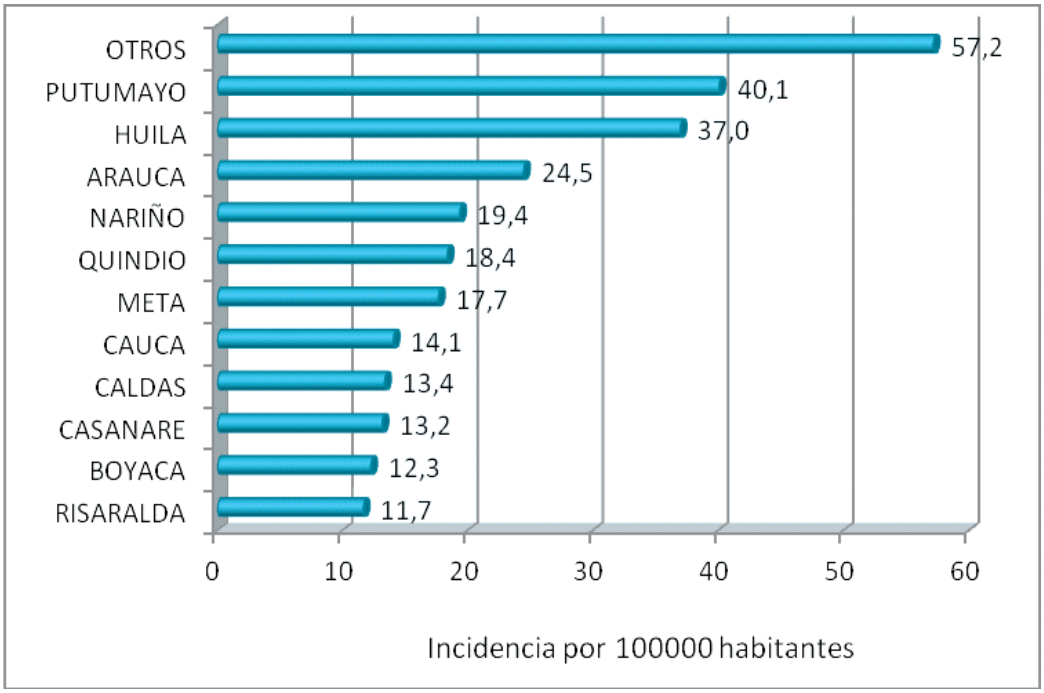

Fig. 1. Incidence by departments, the occurrence of poisonings caused by pesticides, *SIVIGILA*, Colombia, 2005.

The Health Administrative Department of Putumayo showed 248 poisonings due to chemical substances for the year 2005, of which the pesticides were the principal cause in 145 of those (58,4 %); it was reported a major frequency in the municipalities of Puerto Asís (26,9 %), Mocoa (19,3 %), Valle del Guamuez (15,2 %), San Miguel (9,0 %) and Orito (8,3 %). Out of the 145 persons poisoned with pesticides, women presented major number of poisonings (56,5 %) than men (43,4 %); the most affected age group was that from 11 to 20 years (44,1 %), followed by the group from 21 to 30 years (26,9 %) (Revelo D. 2005).

There is a number of types of poisoning, which according to the time passed between the contact with the toxicant and the appearance of the symptoms, the intensity and duration of the symptoms and the quantity of the toxicant, three are classified as follows: acute: for the appearance of a sudden clinical manifestation within 24 hours of exposure to an agent, of short exposure to high doses (cyanide, organophosphate insecticides); chronic: for repeated exposure to very low doses of an agent during long periods of time and with late effects, (organophosphate pesticides, organochlorine pesticides, lead, mercury, white phosphorus) and acute in chronic: acute exposure on a base of chronic exposure to the same agent (Varona M., et al., 2003).

Most of the poisonings reported in our country occur due to organophosphate and carbamates pesticides, which are extensively used as agricultural supplies, domestic pesticides and for the control of epidemic illnesses vectors (Idrovo, 1999; Varona M., et al, 1998). The assessment of the acetylcholinesterase enzyme activity has been used as effect biomarker for these groups of pesticides (Henao, 1990), for which several methodologies have been developed. Among them are those which use colour indicators, such as that of Limperos and Ranta, or the Ellman method, as well as electrometric methodologies, such as that of Michel (Michel, 1949; Limperos G. and Ranta K., 1953).

The colorimetric method of Limperos and Ranta allows the pursuit of large population groups for being a screening technique of low cost and simple to perform. The electrometric method of Michel is based on a quantitative assessment, measuring the fall of the pH as the acetylcholine substratum is hydrolyzed forming acetic acid (Michel, 1949).

The National Health Institute, through the representation of the PanAmerican Health Organization (*OPS*) in Colombia, implemented the methodology *SARAR*, which is an educational strategy that uses visual materials as posters, cards with illustrations, messages suitable and easy to understand to the persons of the rural environment, who can put them into practice to reduce the use of pesticides, the effects on the health, and the avoidance of environmental contamination. With this training, the population acquires the aptitude to recognize situations of risk associated with the handling of pesticides, to identify inconveniences that demonstrate deterioration in the health, to take part in improvement programs of the environmental and work conditions in the localities where they live, to define goals in short and medium term to protect the individual and family health, and to initialize a reflection process on the importance and the profit of the biological control of the handling of pets (*OPS*, 2003; Nutrition Institute of Central America and Panama, 1999).

Likewise, it is clear that the use of pesticides is a direct consequence of the need of improving the harvests and of avoiding the losses caused by the pets. With the use of the pesticides, the food production has increased in 50 %, but unfortunately has generated new risks, which are evident in new pathologies, resultant from the exposure to these toxicants (Garcia, J.E., 1998).

This study was carried out bearing in mind the high use of pesticides in the Department of Putumayo, because in this Department do not exist information sources that allow evaluating the scope of the problem caused by use of pesticides; in order to assess the exposure to organophosphate and carbamates pesticides in the agricultural population, by means of the assessment of the levels of acetylcholinesterase in the blood of the occupationally exposed workers; using Michel's method. There also the use and handling of the pesticides was described with the objective of realizing interventions in the community to minimize the risks associated with the use of these substances.

2. Methodology

A cross sectional study was carried out in a sample of 204 workers occupationally exposed to pesticides, belonging to the municipalities of Puerto Asís, Orito, Valle del Guamuez and San Miguel in the Department of Putumayo, during the years 2005-2006.

The size of the sample was calculated with a power of 80 %, a significance level of 99 %, a predominance expected of 50 % and a percentage loss of 20 %, and a size sample of 198 individuals was obtained, distributed proportionally in every municipality, bearing in mind its agricultural population. Six additional workers were included, who requested to take part and complied with the study criteria of inclusion.

The workers were informed about the targets and the benefits of the investigation and, once they voluntarily accepted their participation, signed a written assent.

The criteria of inclusion of the population under study was determined by the workers who were using organophosphate or carbamates pesticides in their habitual job and they were selected with the help of the Administrative Department of Health of Putumayo. Workers not exposed to these pesticides, as well as pregnant women or who were taking contraceptive oral, workers with history of hepatic illness or diabetes, and all those who did not accept to take part, there were excluded.

A survey was done to them, with which information of demographic type, occupational background, toxicological and clinical precedents, was obtained. A blood sample was gathered for the assessment of the activity of the acetylcholinesterase enzyme within the three days following the exposure to the pesticides; bearing in mind the toxic kinetics of these groups of pesticides (Henao S. &, Corey G., 1991; a Obiols J., 2006; b Obiols J., 2006). A pilot study was carried out in 10 % of the whole force of workers of the sample, in order to conduct the adjustments to the occupational survey. These persons were not part of the population under study.

For the assessment of the acetylcholinesterase enzyme activity of every worker, 10 ml of blood were obtained through venous puncture, in tubes with sodium heparin as anticoagulant. The samples were placed in refrigeration since the moment of the collection until they came to the Environmental and occupational health group, of the National Health Institute, where the analyses were carried out. Once the samples were in the laboratory, they were codified again in order to minimize the analyst's bias.

Later, they were fractioned to be processed and to perform the respective assessment of the activity of the acetylcholinesterase enzyme, using the Michel's method.

For this method it was necessary to carry out a separation of the sample by centrifugation and to work with the plasma and the erythrocytes. The original procedure was validated by the Environmental and occupational health group; the time of the test reaction was modified, which originally was of 60 minutes to 40 minutes for erythrocytes and 45 minutes for plasma (Morato R, Lancheros A, Murcia A. Study of some factors that affect the acetylcholinesterase assessment in erythrocytes and plasma, using the electrometric method of Michel; data without publishing).

The analysis of the results was done using as normality ranges those reported by Henao S. et al. (chart 1) and those found by the Environmental and occupational health group, which values are 0,91-1,64 ΔpH/hour for erythrocytes and 0,71-1,17 ΔpH/hour for plasma.

A simple analysis was carried out of all the variables to assess the descriptive statistics such as the measurement of central tendency and dispersion. Furthermore, the variables were crisscrossed to judge associations statistically noteworthy; for such effect the program Epi-

Info 6.04 and Epidat 3.0 was used. The comparisons between those presenting values of acetylcholinesterase below the established used ranges in each of the methods were carried out in tables of 2x2.

ASSESSMENTS	SEX	RANGE(ΔpH/hour)	AVERAGE(ΔpH/hour)
Erythrocytes	Men	0.58 - 0.95	0.766
	Women	0.56 - 0.94	0.750
Plasma	Men	0.52 - 1.39	0.953
	Women	0.38 - 1.25	0.817

Chart 1. Normal values of acetylcholinesterase (Henao S., et al, 1990)

An analysis of variance ANOVA was carried out between the variable assessment of acetyl cholinesterase and the features of the individuals under the study.
Using an simple random sampling, among the totality of individuals, 51 workers were chosen who were trained using the SARAR methodology of community education in the use and handling of pesticides and, later the acquired knowledge was evaluated.
This study was approved by the Technical Investigation Committee and the Ethics Committee of the National Health Institute.

3. Results

204 workers occupationally exposed to insecticides from the municipalities of Puerto Asís, Orito, Valle del Guamuez and San Miguel, were selected to conform the sample of the study, and an equal number of surveys and biological samples were gathered.
Out of the total participant workers, 94, 1 % (192) belonged to the rural area and the remainder to the urban area.
In terms of sex, 86,8 % (177) were men and 13,2 % (27) women, and their ages were ranging between the 13 and 74 years, with an mean of 34 years for the two sexes. The age of the men was between the 16 and 73 years (mean = 33, 9 years, median = 34 years and DE = 11, 7) and that of the women, between the 13 and 74 years (mean = 34, 8 years, median = 34 years and DE=14, 2). As per statistics there were not significant differences between the ages regarding to sex. As for the enrolment to the General System of Social Security in Health (*SGSSS*), 54,9 % (112) of the individuals of the sample belonged to the contributory regime, 32,8 % (57) were linked and 12,3 % (25) were of the subsidized regime. On the topic of the schooling level, 92, 6 % (188) of the individuals did not finish the secondary school and 3, 9 % (8) are illiterate. At the moment of carrying out the interview, 86,3 % (175) reported themselves as agriculturists.
As for the exposure to pesticides, 53, 0 % (96) of the individuals informed that it was by means of respiratory route and 47, 0 % (109) for direct contact; none reported as exposure route the oral one.
The time of exposure to the pesticides ranged between three months and 30 years, with an average of exposure of nine years, for men (mean =9,6 years, median=10 years and DE=10 years) and in women, between four months and 20 years (mean =5,9 years, median=5 years and DE=10). A difference statistically significant was found in the exposure time to insecticides between men and women (*ANOVA* t=2, 76 p=0,006).
45,6 % (93) of the workers reported to fumigate as minimum of two times a week, 27,5 % (56) fumigates every 15 days and 22,5 % (46) between one and three months. 80, 1 % works full time (8 hours a day) in fumigation and, in general, they work with pesticides an average

of 7, 3 hours per day. The workers recount that the work performed during the no fumigation period is that of preparing the ground (52, 2 %), harvesting (10 %), domestic works (9 %), scraping (5 %) and different activities (5 %), among others.
It is surprising that 91, 2 % (186) of the workers informed that they have not received training on the safe handling of pesticides.
In terms of pesticides use, 100 % of the workers who entered into the study reported the use of organophosphate and carbamates pesticides. 41, 5 % (116) affirmed to have used bi-pyridyl (quaternary ammonium); 19, 6 % (52), phosphonoaminoacid; 29, 1 % (77), 2, 4-D-dichlorophenoxiacetic acid, and 9, 8 % (26) organochlorine pesticides.
The insecticide *tamarón* (organophosphate) was the most used by the workers, with 86, 0 % (177), followed by *furadán* (carbamate), with 56, 4 % (115).
In terms of the toxicological category, 75, 2 % stated to use pesticides category I (extremely toxic); 13, 0 %, category II (highly toxic), and only 11, 8 % uses pesticides category IV (lightly toxic). None of them reported the use of pesticides of toxicological category III. In Colombia the well-known toxicological categories are: category I: extremely toxic, category II: highly toxic, category III: moderately toxic, and category IV: lightly toxic (Department of Health, 1991; ARP Colpatria, 2000).
According to the gathered information, 163 (79, 9 %) of those polled recounted that they use some element of personal protection when they are applying the pesticides (chart 2).
About the elements of personal protection brought by the workers, only a relation was statistically significant between the use of boots of high cane and the minor probability of presenting poisonings (OR=0,11, IC 0,01-0,89, p=0,014).
Upon having evaluated the hygiene measures, it was possible to demonstrate that 96, 1 % (196) of the workers recount that they change clothes at the end of the working day, 82, 4 % (168) change every day, 12, 7 % (26) do it twice a week and 99 % (202) take a shower on having finished the working day. 44, 1 % (90) affirms washing the clothes in the house and 20, 6 % (42) do it in the river. Likewise, 46, 1 % (94) of the polled persons wash the clothes mixed with that of the family.
As for the eating habits, smoking and consuming liquor, it was found that a high percentage of the workers, 85,3 % (174), ingest some food in the farming area and, of these, only 57,1 % (100) informs that always bathes the hands before consuming food. In terms of the habit of smoking, 67 (32, 8 %) workers recount to do it and 19 (28, 4 %) does it in the farming area. They smoke between 1 and 20 cigarettes, with an mean of 6, 4 cigarettes per day. Also, 127 (62, 3 %) individuals consume liquor, of which, 66 (52, 0 %) says to do it occasionally.

ELEMENTS OF PERSONAL PROTECTION	*USAGE FREQUENCY*	*% USAGE*
Street clothes	201	98,5 %
Boots high cane	169	82,8 %
Boots low cane	14	6,9 %
Gloves	6	3,0 %
Disposable mouth cover	5	2,5 %
Respirator	5	2,5 %
Uniform	3	1,5 %
Goggles	2	1,0 %
Bib	1	0,5 %

Chart 2. Elements of personal protection of the polled agricultural workers, Colombia, 2005.

54, 4 % (111) of the workers mention storing the pesticides in an exclusive area, 27, 9 % (57) in a site out of the house and 17, 6 % (36) inside the house. 55,4 % (113) of the workers leave in the farming field the already used packing of the pesticides, 24 % (49) burns them, 18,6 % (38) buries them, 1,5 % (3) leaves them close to the creek and 0,5 % (1) deposits them in the cistern.
Among the symptoms reported by the workers, the most frequent were: cephalic, 51, 3 % (39); dizziness, 43, 4 % (33); ocular burning, 40, 8 % (31); weakness, 30, 3 % (23), and abdominal pain, 28, 9 % (22). The clinical symptoms organized by systems are shown in chat 3, where it is possible to notice that the largest percentage (45, 2 %) appears in the neurological system.

Grouping by system	Nº	%
Neurological system	161	45,2
Digestive	64	18,0
Organs of the senses	58	16,3
Haematopoietic	36	10,1
Skin	19	5,3
Respiratory	18	5,1
TOTAL	356	100,0

Chart 3. Grouping by systems of the clinical symptoms offered by the workers exposed to pesticides in the Department of Putumayo.

46, 6 % (95) of the workers showed to have poisoned themselves with pesticides; of these, 71 % (66) preferred to take home-made medicines and only 17, 2 % (16) consulted a doctor. 76, 1 % (70) poisoned themselves with the insecticide *furadán*.
In terms of assessment of the acetylcholinesterase enzyme activity conducted to 204 workers, the ranges of S. Henao et al. (Henao S., 1990) and those of the Environmental and occupational health group using Michel's method, are shown in chart 4, indicating the frequency found according to sex and the number of persons who presented values below the lower limit, which means enzyme inhibition. Bearing in mind the ranges reported by S. Henao et al., 17,6 % (36) of the individuals of the study presented enzyme inhibition, while, for the ranges obtained by the Environmental and occupational health group, 26,5 % (54) showed abnormal values.
Crosses were made between the levels of acetylcholinesterase and the variables included in the survey. A difference statistically significant was found only between the levels of acetylcholinesterase performed by means of Michel's techniques in red blood cells, of the workers who expressed to have presented poisoning with pesticides and those who did not show it (*ANOVA* t=2, p <0,05), although the mean levels are within the range of normal values. For the plasmatic acetylcholinesterase, no significant differences were found with any variable.
Through this project, 51 agricultural workers of the department of Putumayo were trained and, later the knowledge acquired was evaluated by means of the methodology of community education *SARAR* for the use and suitable handling of pesticides. Out of 17 asked questions, almost half (8) were correctly answered by more than 80% of the interrogated persons.

N Assessment	Sex		Range ΔpH/h Henao S, *et al.*	Range ΔpH/h INS **	Frequency Henao S, *et al.*		Frequency INS **	
					Number	%	Number	%
Erythrocytes	177	Men	0,804-0,992	0,91-1,64	30	16,9	47	26,6
	27	Women	0,822-0,99		2	7,4	2	7,4
Plasma	177	Men	0,799-1,107	0,71-1,14	4	2,3	3	2,3
	27	Women	0,756-0,994		0	0,0	2	7,4

** National Health Institute. Morato R, Lancheros A, Murcia A. Study of some factors affecting the acetylcholinesterase assessment in erythrocytes and plasma, using the electrometrical method of Michel.

Chart 4. Comparison of the acetylcholinesterase enzyme activity with the measurement ranges of Henao S., et al. (Henao S., 1990), and of the Environmental and occupational health group, by genre, using Michel's method.

4. Discussion

Although the incidence of poisonings is not accurately known in the worldwide environment, it is anticipated that every year one million persons dies as a consequence of diverse poisonings.

The World Health Organization informs that the incidence of poisonings caused by pesticides has doubled in the last 10 years in the world; nevertheless, there is not known the entire number of cases that take place annually and the seriousness of the notified cases. That's why it becomes necessary that the countries establish programs and research projects, which allow the identification of the risk factors in order to which preventive measurements be established and, at the same time, be working in the diagnosis of the poisonings and the processing of the subjects poisoned (*OMS*, 1998). This work allows understanding the real dimension of the pesticides problem in the department of Putumayo and using an educational intervention on the community, fundamental pillar in the prevention of risks and in the use and suitable handling of these chemical substances.

Having seen the results, it was observed that, of the whole input of workers who participated in the study, the labour force is mostly of the masculine sex, with a very wide age range, it ranged between the 16 and 73 years; it was found that very young people, as well as of the third age, are farmers exposed to a large variety of pesticides.

Almost a third part of the workers reported not to be affiliated to the General System of Social Security in Health and they do not have the resources to affiliate to the occupational hazards system. The previous matter can be due to the fact that the majority works informally and does not have an employment contract. Neither there exist a Plan of basic attention that values the occupational component of the informal sector and that allows the workers to be trained in the handling of the pesticides.

In terms of the exposure to pesticides, the principal input routes were the respiratory and the dermal, as it is expected for this group of substances. It is important to highlight that the average exposure time to pesticides was nine years for the men and 5,9 for the women, which is considered to be a chronic exposure that can unleash long-term effects. Also, 45,6% of the workers report to fumigate as a minimum of two times a week and in average 7,3 hours a day, which increases the exposure to the pesticides used.

It was defined that is very remarkable the percentage of use of category I pesticides (extremely toxic) and category II (highly toxic), according to the classification given by the Health Department of Colombia (Department of Health, 1991), The evidence continuously accumulates data on alterations of the health due to the pesticides (Cordoba D, 2000). This makes necessary that the workers learn on the effects that can unleash the exposure to the pesticides, it is necessary also to sensitize them in order that they do a rational use of these products and that they reduce in a significant way the use of toxicological pesticides category I and II.

More than the third part of the interrogated persons recalls to have presented a poisoning with the insecticide *furadán*, which is classified among the carbamates, in toxicological category I (Agricultural Colombian Institute, 2002). In case of poisoning, only one small percentage (17,2 %) consults the doctor; they prefer to take home-made medicines, which makes difficult the correct diagnosis and processing of the poisoned patient, as well as the notification of the case to SIVIGILA, thus increasing the sub-registering of cases of poisoning by pesticides.

All the surveyed persons used the organophosphate or carbamates pesticides; the herbicide *paraquat* was the second most used pesticides by the workers, which is classified by the Health Department of Colombia under toxicological category I (Agricultural Colombian Institute, 2002). It was also found, that 9, 8 % of the workers uses organochlorine insecticides, which are currently prohibited in our country. These substances are a permanent risk for the population working in agriculture, as well as for the environment; therefore it is necessary to press hard over the potential adverse effects on the health that these pesticides can produce.

Among the matters of hygiene and industrial safety, most of the questioned persons reported as personal protection equipment only the street clothes and the high cane boots, and very few use ocular, respiratory, and upper members' protection. It is indispensable that the worker be trained in order that he wears light working clothes covering most of the cutaneous surface when mixing or applying pesticides, as well as when cleaning the equipment and emptying receptacles or disposing the remains of the used pesticides. Also, they must wear gloves, boots and masks adapted to the manoeuvring of the pesticides.

46,1 % of the surveyed persons washes the working clothes mixed with the rest of the clothes, which implies that not only the workers but his families are exposed, since they can transport pesticides particles in their clothes.

Another risk factor is the food consumption, being that a high percentage (85, 3 %) does it in the farming field, and a lower percentage smokes in the site; 17, 6 % of the workers stores the pesticides inside the house, which can cause a potential increase of the exposure to pesticides. Likewise, the workers leave the empty packing in the farming field, others leave them close to the creek or bury them, generating contamination in the environment.

Among the clinical symptoms, the predominant complain of the workers was about the neurological system, which coincide with the symptoms of poisoning proper of organophosphate and carbamates pesticides (Toro G. et al, 2002). The organophosphate is still the most used insecticide in the world, particularly in developing countries (Idrovo A.J., 1999). These insecticides and the carbamates are esters of the phosphoric and carbamic acid, they share as common pharmacological feature the inhibition of enzymes with esterasic activity, more specifically, the inhibition of the acetylcholinesterase enzyme. They are easily hydrolyzed and have scarce permanence power in the environment (Henao S., 1991).

The measurement of the acetylcholinesterase levels in blood keeps on being a biomarker extensively used to measure the exposure to these substances. Nevertheless, the interpretations of the results are very variable. There exist genetic, physiological causes and associate pathologies, which can lessen the levels of this enzyme.
Also, there is an important change within the same individual. For such a reason, the medical suveillance of the workers exposed continuously to these two groups of insecticides, must include, in addition to the medical examination, the assessment of the acetylcholinesterase enzyme before to the exposure (basal) and every three months during the time that the exposure lasts (Henao S., 1991).
In this study, the acetylcholinesterase levels in blood were determined using Michel's technique, which is thought of being the master standard of reference (Henao S., 1991). The reference ranges used to account the results in this work are those reported by Henao S., et al. and they were compared with the ones set up by the Environmental and occupational health group. The distribution of inhibition frequencies for the different normality ranges is similar in both cases.
Nevertheless, the data brought by Henao S., et al. (Henao S., 1990) for pseudocholinesterase protect more the worker, likewise with the data brought by the National Institute of Health, but for the real acetylcholinesterase (Henao S., 1991), being this a very subtle difference.
In order to guide the process of the poisoned patient and to establish the inability or redeployment of the worker, it is suggested to bear in mind the value of the real acetylcholinesterase activity, since this activity delays more the regeneration after its inhibition (Henao S., 1991). That's why the management of the poisoned persons is done according to the grade of inhibition of the enzymatic activity. On this matter, the plasmatic acetylcholinesterase has speedier recovery than the erythrocytic; therefore, the exposure withdrawal of the worker will be kept as long as the acetylcholinesterase erythrocytic return to basal levels or come to levels next to these (Idrovo A.J., 1999).
So, the activity ranges found by Michel's method in the Environmental and occupational health group is comparable with those found by Henao et al. (Henao S., 1990) and it is a precise, exact, sensitive and solid alternative to determine the enzymatic activity, being this constituted in a tool for the follow-up and surveillance of the labor exposure to organophosphate and carbamates pesticides in Colombia (b Obiols J., 2006).
Other bibliographical reports on normality ranges of the acetylcholinesterase activity are those of Rider et al. (Rider J.A. et al, 1957), who carried out an evaluation in 800 healthy patients, establishing their ranges, which for the case of our data are very low values, and of M. Siquiera et al. (Siquiera M.E., 1978), who reported normality ranges for plasmatic and erythrocytic acetylcholinesterase with higher values; none of the previous ones matches the conditions of Colombia. Finally, it is important that in the future a follow up of this group of workers be done, in order to identify if chronic effects exist caused by the use of these pesticides.
There becomes necessary a joint effort of the health organizations, educational entities, health secretariats and organizations of environmental protection, in order to develop surveillance programs for the workers of the informal sector and for their families. The The Health Administrative Department of Putumayo must reinforce the implementation of the surveillance protocol in public health set up by the Department of the Social Protection and the National Institute of Health related to poisonings by pesticides.
Likewise, it is necessary to keep on developing training programs for the workers and their families, by means of the use of the SARAR methodology, and in this way to comply with Decree 1843 of 1991 about the sanitary dispositions on the use and handling of pesticides (Department of Health, 1991).

The need of training is sustained also in the high exposure to pesticides in chronic way and in the low coverage grade by the SGSSS, found in the study; this deserves that the education in the use and handling of pesticides keeps being encouraged. The educational component is very important to prevent the labour exposure risks to pesticides, and in this population its implementation is essential, since 91, 2 % of the polled workers stated not to have received training on the safe pesticides handling. The *SARAR* methodology is a tool simple and easy to be implemented, which seeks to promote durable changes in the practices to reach effectiveness in the suitable pesticides handling and which allows the workers, in turn, to serve as information multipliers and to learn good agricultural practices.

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6. Conflict of interests

The authors declare that no conflict of interests exists in this publication. It is reproduced under authorization from the Biomedical Publishing Committee, which was published in "Varona M, Henao GL, Lancheros A, Murcia A, Díaz S, Morato Ro, et al. Exposure factors to organophosphate pesticides and carbamates in the Department of Putumayo. Biomédica 2007; 27:400-9".

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8. References

ARP Colpatria (2000), Control program of protective products for plant growing. Technical document, Bogotá D.C.: ARP Colpatria.

Cordova D. (2000), Toxicology. Fourth edition, Bogotá: Moderno Manual Editor.

Corey G. (1988), Surveillance in Environmental Epidemiology. Center of Human Ecology and Health, Mexico D.F: OPS-OMS.

Durán-Nah JJ. Collí-Quintal J. (2000), Acute Poisoning by Insecticides. Public Health Mex; 42:53-55.

Garcia, J. E. (1998) Acute Poisonings with Insecticides: human and economic costs. Rev Panam Public Health; 4 (6) ISSN 1020-4989.

Henao S, Zapata FM, Restrepo MD, Marín LE, Ramírez H, Corrales R, et al. (1990), Cholinesterase activity in working minors. Antioquia, Colombia. 1989 - 1990. Medellin: Social Security Institute, sectional Antioquia, University of Antioquia.

Henao S, Corey G. (1991) Pesticides Cholinesterase Inhibitors. Series Surveillance 11. PanAmerican Center of Human Ecology and Health. Mexico D.F: OPS-OMS.

Idrovo AJ. (1999) Massive Poisonings with insecticides in Colombia. Biomédica; 19:67-76.

Agricultural Colombian Institute (2002), Regulation and Control group of Chemical Insecticides for Agricultural Use. Insecticides commercialization: production, sales, import and export. Bogota: Produmedios Editor.

Nutrition Institute of Central America and Panama (INCAP/OPS) (1999), Project Plagsalud (MASICA/OPS), Project health and safety in the agriculture (OIT), Educational skills to promote the prevention and protection of poisoning with insecticides. San Jose de Costa Rica: Pan-American Health Organization.

Levine R.S, Doull J. (1992) Global estimates of acute pesticide morbidity and mortality. Rev Environ Contam Toxicol.; 129:29-50.

Limperos G, Ranta KE. (1953) A rapid screening test for the assessment of the approximate cholinesterase activity in human blood. Science; 117:453-5.

Michel HO. (1949) An electrometric method for the cholinesterase activity assessment of red blood cells and plasma. J Lab Clin Med; 34:1564-8.

Department of Health (1991) Decree 1843 of July 22, 1991, sanitary regulations on the use and handling of insecticides. Bogotá: Department of Health; p.1-69.

Obiols J. organophosphate pesticides (II): toxicodynamics and biological control. [Consulted: February, 2006]. Available in: http://www.insht.es/InshtWeb/ Contenidos/Documentacion/FichasTecnicas/NTP/Ficheros/501a600/ntp_513.pdf

Obiols J. Organophosphate pesticides (I): general aspects and toxicokynetics. [Consulted: February, 2006]. Available in: http://www.insht.es/InshtWeb/ Contenidos/Documentacion/FichasTecnicas/NTP/Ficheros/501a600/ntp_512.pdf

United Nations Food and Agriculture Organization, (1986) The feeding and the environment. *Development Coop* (Federal Germany); 1:18-20.

World Health Organization (1992), Sanitary consequences of the employment of pesticides in the agriculture. Ginebra: WHO.

World Health Organization (1998), Guidelines for the fight against poisoning. International Safety Program of Chemical Substances. Malta: World Health Organization.

OPS, Department of the Social Protection, National Health Institute. (2003) Prevent poisonings and avoid the contamination. Health Education Manual for the visiting and facilitating person. Bogotá: Editor New editions Ltda.

PanAmerican Health Organization. (2004) Sanitary pesticides surveillance: Plagsalud experience in Central America. Washington D.C: Pan-American Health Organization.

Revelo D. (2005), Poisoning Behaviour 2005-2006. Final report. Health Administrative Department of Putumayo.

Rider JA, Hodges JL Jr, Swader J, Wiggins AD. (1957) Plasma and red cells cholinesterase in 800 healthy blood donors. J Lab Clin Med; 50; 376-83.

Siquiera ME, Fernicola NA, Borges EL. (1978) Determinação de nevéis normáis of colínesterase plasmatica e eritrocitária. Rev Saúde Pública; 12:340-4.

SIVIGILA. (2009), Poisoning by pesticides Reports. Factors of Environment Risk Group - Subdepartment Surveillance and Control in Public Health.

Toro G, Cárdenas O, Varona M. (2002) Neurotoxicity (III part): insecticides. Record Neurol Colomb; 18:32-50.

Varona M, Morales L, Ortiz J, Sánchez J, Cárdenas O, de la Hoz F. (1998) Epidemiologic outlook of exposure to inhibiting cholinesterase insecticides in 17 departments of the country. Biomédica; 18:22-9.

Varona M., Henao G.L., Botero J.H. (2003) Diagnosis, prevention, control and surveillance of poisoning by insecticides. Health Personnel, Course. Technical document. National Health Institute - Department of the Social Protection.

6

Pesticides and Parkinson's Disease

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1. Introduction

Neurodegenerative diseases form a subset of pathologies that are characterized by a progressive and specific loss of neurons paralleled by the emergence of misfolded proteins in various cell types, the significance of which is still highly debated (Harris et al. 2009). These pathological traits result in mixed impairments of motor, cognitive and psychological functions (Harris et al. 2009). Parkinson's disease (PD), which is characterized by a prominent loss of dopaminergic neurons and the formation of Lewy bodies - nuclear inclusions largely composed of α-synuclein - is the second neurodegenerative disorder in importance after Alzheimer's disease.

The prevalence of PD increases exponentially between 65 and 90 years of age. Approximately 0.3% of PD cases are found in the general population as opposed to 3% in individuals over 65 (Moghal et al. 1994). While a very small fraction of PD is related to monogenic mutations, over 90% of cases are likely linked to environmental causes (referred to as idiopathic PD), suspected to be in part related to well-water consumption, exposure to heavy metals and pesticides (De Michele et al. 1996; Schapira 1996; Tanner et al. 1999) (**Table 1**). Recent work conducted in animal models has suggested that the onset of the

Environmental factors		References
	Rural living	(Morano, 1994)
	Well-water drinking	(Gatto, 2009)
Exposure to:	Heavy metals	(Seidler, 1996)
	Pesticides	(Pryadarshi, 2000)
	Magnetic fields	(Noonan, 2002)
	Herbicides	(Costello, 2009)
Professions related to:	Wood/pulp plants	(Tanner, 1989)
	Orchards	(Hertzman, 1990)
	Planer mills	(Hertzman, 1990)
	Steel/alloy industry	(Rybicki, 1993)
	Railroad and car shop mechanic	(Seidler, 1996)
	Carpentry	(Fall, 1999)
	Cleaning	(Fall, 1999)
	Logging	(Tsui, 1999)
	Mining	(Tsui, 1999)
	Oil and gas	(Tsui, 1999)
	Farming	(Gorell, 2004)
	Forestry	(Park, 2005)

Table 1. Potential environmental risk factors for Parkinson's disease

disease later in life may derive from various non-exclusive scenarios, namely chronic exposure to low levels of neurotoxicants, time-limited exposure early in life with later manifestation due to the decline of certain brain cell populations with advanced aging, and/or increased sensitivity to exposure with advanced age (see (Di Monte et al. 2002)). These hypotheses are particularly relevant to the interpretation of the epidemiological data gathered over the years and which will be reviewed in this chapter.

1.1 Clinical and pathological features of Parkinson's disease

One of the challenging aspects of PD is the heterogeneous nature and variability of the symptomatology and pathology (Lewis and Barker 2009). Two main subtypes of the disease have emerged from clinical observations based on the age of onset and the evolution/progression of the disease. While the disease in younger patients is rather typified by symptoms of resting tremors, older patients are more likely to suffer from "postural imbalance and gait disorders" (Selikhova et al. 2009). These disparate clinical manifestations may reflect various etiologies derived from interactions between genetic and environmental factors. While this disorder has for long been viewed for its predominant motor deficits, a much more complex scheme of the pathophysiology is being unveiled, and includes several non-motor features such as cognitive impairments, depression, anxiety and sleep-related disturbances (see **Figure 1**). The nature and diversity of the handicaps caused

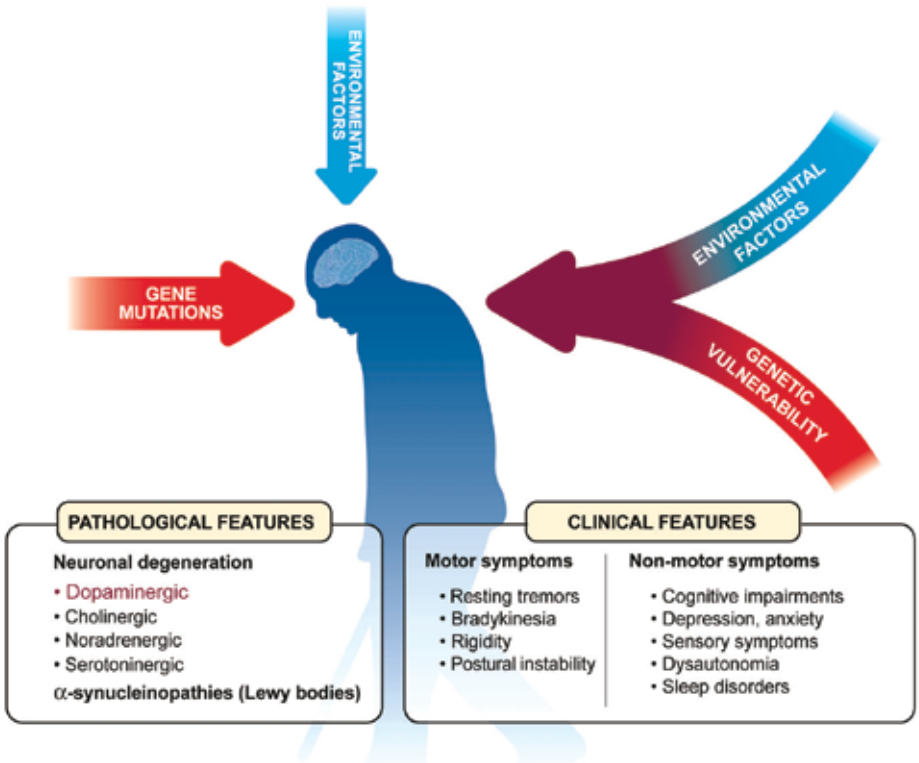

Fig. 1. Current views of the etiology and pathology of Parkinson's disease

by PD make it a very debilitating condition. The long-standing history and research devoted to the motor impairments of the disease have revealed that they result, in large part, from a loss of a specific subpopulation of dopaminergic neurons within the basal ganglia, a subset of brain structures involved in the control of psychomotor behaviors. Conversely, the non-motor alterations observed in PD have been related, for example, to the loss of non-dopaminergic cells, such as the noradrenergic (Zarow et al. 2003), serotoninergic (Braak et al. 2004) and cholinergic neurons of various other brain nuclei (**Figure 1**). PD is also characterized by a synucleinopathy, another pathological hallmark which consists in the entanglement of the mutated form of α-synuclein, which leads to Lewy body formation further postulated to cause cell damage by impairing neuronal functions (Waxman and Giasson 2009).

2. Is pesticide exposure a risk factor for Parkinson's disease?

2.1. Evidence from epidemiological studies

The historical observations, in the late 70's, of the sudden appearance of parkinsonism in seven young individuals exposed to 1-methyl-4-phenyl-4-propionoxy-piperidine (MPPP) contaminated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Langston et al. 1983) gave birth to the notion of a potential link between environmental factors and the development of PD. MPTP itself does not pose a likely exposure risk for the general population. However, MPTP bears striking similarities to other naturally occurring as well as manmade substances, namely the heavily used pesticides rotenone and paraquat. The specific relationship between exposure to pesticides and the development of PD has received increasing attention since the early 80's, when Barbeau discussed the association between manganese or MPTP intoxication and the pathogenesis of this disorder. He proposed that individuals being frequently exposed to environmental compounds bearing chemical conformations similar to MPTP could develop comparable parkinsonism syndromes (Barbeau, 1984). A few years later, he reported his observations of an association between living in an agricultural environment and the risk of developing PD in the province of Quebec, Canada, where pesticide use was strikingly high at the time (Barbeau et al. 1987). While the epidemiological studies published in the last three decades have raised awareness of the potential health issues related to pesticide-promoted agriculture, they have not indisputably demonstrated a specific relationship between these toxins and the development of PD. In the following sections, we will attempt to shed light on the investigations published since 1989, discussing both the positive evidence and the lack of association between pesticide exposure and neurodegeneration.

2.1.1 Pesticides and Parkinson's disease: Positive associations from retrospective studies

Evidence tying pesticide use and PD has surfaced from all over the world, although the identification of specific compounds under this general heading has been more complex. Considering this important limitation, dithiocarbamates and pesticides of the organochlorine family of insecticides have been the targets of the vast majority of retrospective case-control studies conducted to date. Despite small sample sizes, a large number of these inquiries have revealed a positive correlation between pesticide contact and PD. Such observations were made in nursing homes for elderly in two Hong Kong districts, where 3.4% of these residents (> 60 years) suffered from PD (odds ratio (OR) = 3.6; 95%

confidence interval (CI) 1.0-12.9)[1] (Ho et al. 1989). The results of another study involving 106 patients diagnosed with PD (their spouses serving as controls), showed that patients had significantly greater rural experience and were more likely to have routinely sprayed pesticides in comparison to their partners (OR = 7.0, $p < 0.05$) (Golbe et al. 1990). In a population-based case-control study comprising 130 PD subjects living in Calgary and 260 randomly selected age- and sex-matched community controls, herbicide (OR = 3.06; 95% CI 1.34-7.00, $p = 0.006$) and insecticide use (OR = 2.05; 95% CI 1.03-4.07, $p = 0.042$) (but not fungicide) were found to be significant predictors of PD, after controlling for confounding factors or interactions between the exposure variables (Semchuk et al. 1992). A link between herbicide exposure and PD (OR = 3.22, $p = 0.033$) was also found in young onset PD patients (i.e. diagnosed before the age 50) as compared to controls diagnosed with rheumatoid arthritis (Butterfield et al. 1993) (see **Table 2** for details).

In the late 90's, three additional studies reported a connection between the risk of developing PD and exposure to pesticides. In a Hong Kong hospital-based, case-control study regrouping 215 PD cases and 313 controls, the duration of exposure to farm pesticides correlated with increased PD risk (multivariate analysis). Pesticide exposure in women conducting farming activities (OR = 6.84; 95% CI 1.90-24.7; $p = 0.003$) was also found, although this was not the case for men. It should be noted that sample sizes were very small (6 men and 13 women with PD; 13 control men and 3 women) (Chan et al. 1998). In a population-based case-control study referring to a cohort of men and women over 50 pooled from medical centers of metropolitan Detroit (144 PD cases and 464 controls), a significant association between occupational exposure to herbicides (OR = 4.10; 95% CI 1.37-12.24) or insecticides (OR = 3.55; 95% CI 1.75-7.18) was reported. However, no relation was found again with regard to fungicide exposure (Gorell et al. 1998). In 1999, a case-control study conducted in southeastern Sweden and involving the participation of 113 idiopathic PD cases and 263 control subjects reported an increased risk of idiopathic PD in men (10 men with PD and 10 men in controls had a history of handling pesticides), which was associated with agricultural labor and pesticide contact (OR = 2.8; 95% CI 0.89–8.7) (Fall et al. 1999).

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[1] A relative risk (RR) is a comparative measure of the observed risk of developing PD in individuals who are exposed to pesticides vs. the observed risk of developing PD in a group of "equivalent" subjects who were not exposed. A RR of 1.0 indicates that there is no increased risk. Using 1.0 as the benchmark, a reported RR of, for example, 1.34 may indicate that the PD risk from pesticide exposure is 0.34 or 34% higher. However, for the result to be taken into consideration, the RR has to achieve statistical significance, using confidence interval (CI) levels. The generally agreed upon confidence level is 95%, where there is a 5% chance that the significant result is due to the random luck of draw. The CI implies that there is a 95% probability that the "real" RR lies anywhere in the range between the numbers of the interval. The CI is affected by sample size and by variability among subjects. This signifies that the findings are statistically significant only when the lower number of the interval exceeds 1.0. Additionally, the narrower is the interval, the more statistical power there is to the result. When the higher number of the interval is below 1.0, pesticide exposition is either not associated to PD, or is protective against PD. Any RR rating of less than 2 is very weak, difficult to interpret and very likely to be due to either bias, confounding factors or chance. Case-control studies often prevent you from evaluating a RR, but the odd ratio (OR) can always be calculated and interpreted. A case-control design usually involves the selection of research subjects on the basis of having PD rather than on the basis of having been exposed to pesticide. The probability of developing PD in pesticide-exposed subjects can be estimated, but not the probability of being exposed to pesticides when you have PD. The OR offers a reasonable interpretation, as long as the outcome event is rare and its interpretation rely strongly on how the controls were recruited, and is usually higher than the RR.

An additional number of retrospective studies reporting a relationship between pesticide exposure and PD were conducted after 2000. Using a cohort of 310 men, mostly orchardists who had previously participated in a cohort study of men occupationally exposed to pesticides in Washington State, Engel *et al.* (2001a) found a significant association for older subjects exposed to pesticides (Prevalence ratio (PR) = 2.0; 95% CI 1.0-4.2). Similar results were obtained for the middle tertile but did not reach statistical significance (PR = 1.9; 95% CI 0.9-4.0). However, no specific pesticide, or classes of pesticides, were associated with an increased risk of having PD (Engel et al. 2001a). In another study in Israel, the second strongest predictor of PD risk in 93 PD patients living in cities and 93 age- and sex- matched controls was exposure to pesticides (OR = 6.34; 95% CI 0.75-53.8, $p = 0.06$) (Herishanu et al. 2001). A subsequent case-control study performed in northeastern Italy and composed of 136 PD cases and 272 controls affected by other neurological diseases reported a positive association between pesticide exposure and PD (crude OR = 2.0; 95% CI 1.1-3.5, $p = 0.0237$). The mean length of exposure to pesticides was also significantly different in these cases, as compared to control subjects (4.1 years, standard deviation (SD) = 10.9 and 2 years, SD = 6.4, respectively; $p < 0.05$) (Zorzon et al. 2002). Gender differences were also outlined in a study involving 113 prevalent cases of PD. Multivariate analyses were independently performed for men and women, and ownership of licenses for pesticide use was positively associated with PD, but only in men (OR = 3.68; 95% CI 1.57–8.64) (Baldereschi et al. 2003). A population-based study in a genetically isolated community in a rural area of Turkey pointed to an increase in the prevalence of parkinsonism (4.1%) in individuals ≥ 65 years of age (36 cases of parkinsonism and 108 age- and sex-matched community controls). In this cohort, pesticide exposure was significantly associated with parkinsonism (OR = 2.96; 95% CI 1.31-6.69, $p = 0.015$) (Duzcan et al. 2003). An increased prevalence in men was also demonstrated (OR = 2.4; 95% CI, 1.1-5.4; $p = 0.04$) in a cohort that included every PD patients in Olmsted County (MN), from 1976 through 1995. Cases were matched to general population controls for age and gender (Frigerio et al. 2006) (see **Table 2** for details and **Figure 2** for geographical mapping of studies conducted).

In counterpart to the positive association of the use of pesticide *per se* and the occurrence of PD, the authors of another case-control study conducted in British Columbia (Canada), and involving 127 PD cases and 245 controls (121 with cardiac disease and 124 randomly selected from electoral registers) established a significant association between idiopathic PD in men practicing a profession in which exposure to pesticides was highly probable (OR = 2.32; 95% CI 1.10-4.88). However, they considered occupational exposure to several chemicals, including organochlorines, organophosphates, carbamates and dithiocarbamates, but none of these chemicals alone were connected with idiopathic PD (Hertzman et al. 1994). The authors concluded that the pathogenesis of PD is more likely to be multifactorial, thus excluding the possibility of a single-agent hit.

2.1.2 Pesticides and Parkinson's disease: Lack of association from retrospective studies

A comparable number of retrospective epidemiological studies have failed to identify a relationship between pesticide exposure and the risk of developing PD. In a case-control study using 150 PD cases from a Kansas movement disorder clinic and 150 age- and sex-matched controls attending other neurological and medical centers, no significant difference in the incidence of PD was detected for exposure to herbicides or pesticides with respect to the number of years of exposure, type of herbicide or pesticide, circumstances of exposure,

surface of land or type of crops on which herbicides/pesticides were employed, except for a marginal significance for exposure to herbicides/pesticides sprayed on corn (Koller et al. 1990). In another case-control study in which 42 PD subjects were matched to 84 controls of the Community Health Department of Valleyfield (Quebec, Canada), pesticide handling did not relate to PD. Oddly, other factors frequently identified as risk factors for PD, such as living in rural (OR = 0.31; 95% CI 0.11-0.91, $p < 0.05$) or industrial areas or working in mines (OR = 0.15; 95% CI 0.04-0.55, $p < 0.05$), were associated with a decreased risk for being struck with the disease (Zayed et al. 1990). Another case-control study using a cohort of 80 patients with late-onset PD (> 60 years old) and 69 early-onset patients (< 40 years old) recruited from various American hospitals, and 149 age- and sex-matched control subjects selected by the case subjects (relatives and spouses were not eligible) or from hospital files failed to implicate exposure to herbicides or pesticides in the incidence of PD (Stern et al. 1991). Moreover, in a case-control study involving 19 families harboring two or more PD cases, and 38 controls, herbicide and pesticide exposure was not a significant risk factor, although statistically significant differences were found with the following factors: rural residence, well-water consumption and farming (Wong et al. 1991). Additionally, in a study realized in a selected urban area of Madrid, among 128 unselected PD patients and 256 age- and sex-matched controls, past exposure to pesticides (for at least one year) and duration of exposure was apparently not associated with an increased risk of developing PD (Jimenez-Jimenez et al. 1992) (see **Table 2** for details and **Figure 2** for geographical mapping of studies conducted).

In South East Queensland and Central West New South Wales in Australia, a case-control study involving 224 PD cases and 310 control subjects reported no significant difference between patients and controls for exposure to herbicides and pesticides, but rural residence emerged as a significant risk factor for PD (McCann et al. 1998). Furthermore, exposure to pesticides and herbicides was similar between 86 PD cases and 86 matched controls, which were all outpatients from the same hospital (Smargiassi et al. 1998), and between 140 PD cases who were recruited from the Boston University Medical Center, where 147 friends and in-laws served as control subjects (Taylor et al. 1999). One other study undertaken in 1999 failed to detect an association between herbicide and pesticide exposure and PD, which consisted in a community-based case-control study in rural municipalities of the southwestern part of Finland using 123 PD cases and 246 matched control subjects (Kuopio et al. 1999). Additionally, no risk association was found with pesticide and/or herbicide contact in a case-control study in the Limousin region of France, using a cohort composed of 140 PD patients and 280 age-matched control subjects. The duration of exposure to pesticides or herbicides, however, was not determined (Preux et al. 2000).

More recently, a population-based case-control study using a cohort of 250 idiopathic PD cases and 388 healthy control subjects derived from a health care system database in western Washington State and the University of Washington, observed no significant association between occupational exposure and PD, but suggested a gradient of risk for occupational titles that paralleled the predicted level of pesticide exposure (e.g. pesticide worker > crop farmer > combined animal and crop farmer > dairy farmer) (OR = 2.07; 95% CI 0.67-6.38). ORs were also elevated for herbicides (OR = 1.41; 95% CI 0.51-3.88) and particularly paraquat (OR = 1.67; 95% CI 0.22-12.76), but there was no evidence of risk for exposure to pesticides used on a home basis (Firestone et al. 2005). The same group reported that the risk of PD was not significantly associated with exposure to pesticides in general. When

exploring specific pesticides, the only increased risk trend was for men exposed to parathion, the most potent organophosphate known, although this was not statistically significant. The cohort was composed of 404 idiopathic PD cases and 526 controls (Firestone et al. 2010). In New Delhi, India, a case-control study involving 377 PD patients attending a movement disorder clinic and an equal number of outpatients with other neurological diseases, did not report any significant correlation between the occurrence of PD and exposure to insecticides, herbicides and rodenticides. Nevertheless, exposure to herbicides was increased among control subjects (Behari et al. 2001). This is, to our knowledge, the only report of a trend towards a negative correlation between pesticide use and PD, although it did not reach statistical significance.

Taken together, the results of the retrospective epidemiological analyses are inconsistent, which reflects the great variability in the methodologies employed and the increased bias inherent to self-report studies. In addition, most of the studies described above used a relatively small number of subjects, with an even smaller number of subjects presenting a past history of pesticide exposure. This drawback considerably decreases statistical power and therefore limits the relevant analyses. Overall, despite the large number of retrospective studies conducted in the past 20 years, the association between pesticide/herbicide exposure and the increased risk of PD remains inconclusive, although the available evidence tends to suggest that pesticide exposure plays a role in some idiopathic forms of the disease.

2.1.3 From the angle of prospective studies and meta-analysis

In an attempt to eliminate recall bias, some epidemiological studies have used a prospective approach. The first study conducted in such a manner analyzed the relationship between exposure to pesticides and an increased risk of developing PD 30 years following the determined moment of initial pesticide exposure. Among the 7 986 participants in a cohort of Hawaiian plantation workers, 116 men were diagnosed with PD during the 30-year follow-up, with a significantly increased incidence among men who worked for more than 10 years on a plantation. Despite the fact that age-adjusted incidence of PD was higher in men exposed to pesticides, in comparison with those spared from pesticide exposure, this analysis did not reach statistical significance (Petrovitch et al. 2002). Another prospective cohort study - of 1 507 French elderly - used a job exposure matrix to assess occupational exposure and revealed that subjects who had been occupationally exposed to pesticides exhibited lower cognitive performance. The authors of this study further reported that exposure to pesticides increased the relative risk of developing PD in men (OR = 5.63; 95% CI 1.47-21.58), but not in women, after confounding factors (smoking and education level) were taken into account (Baldi et al. 2003b). In a subsequent study focusing specifically on PD and using 84 PD cases and 252 population-based controls belonging to the same French elderly cohort, a positive association was observed with occupational pesticide exposure (OR = 2.2; 95% CI 1.1-4.3). However, no clear dose-response relationship was found (Baldi et al. 2003a). Using participants enrolled in the Cancer Prevention Study II Nutrition Cohort, Ascherio *et al.* (2006) reexamined whether individuals exposed to pesticides expressed a higher risk for PD. In 1982, participants completed a survey concerning occupation and exposure to selected chemicals or dusts, including pesticides. After follow-up surveys in 1997, 1999, and 2001, 7 864 participants reported exposure to pesticides, of which 1 956 were farmers, ranchers, or fishermen, thereby revealing a 70% higher incidence of PD in individuals exposed to pesticides (adjusted relative risk (RR), 1.7; 95% CI 1.2-2.3; $p = 0.002$).

The RR for pesticide exposure was similar in farmers and non-farmers. No relation was found between risk for PD and any of the other occupational exposures surveyed (e.g. asbestos, chemicals/acid solvents, coal and stone dust, dyes, gasoline exhaust) (Ascherio et al. 2006) (see **Table 2** for details and **Figure 2** for geographical mapping of studies conducted).

In 2000, Priyadarshi and colleagues conducted a meta-analysis encompassing 19 studies published between 1989 and 1999. When all studies were combined, the OR for PD risk in association with pesticide exposure was 1.94 and 2.15 for studies performed in the United States alone, corresponding to a 2-fold risk increase. The risk of PD increased with duration of exposure, but no significant dose-response relationship was established and no specific type of pesticides was identified (Priyadarshi et al. 2000). The consistency of the results obtained in the studies selected for this meta-analysis allowed the authors to conclude that exposure to pesticides may be a risk factor for PD, independently of the place where the study was conducted.

2.1.4 Additional types of analyses

Other methodologies have been employed to assess the possible link between pesticide exposure and PD. For example, one study used a proportional odds model for survival data comparing all PD cases that were recorded as underlying or associated causes of death occurring in California, with all deaths from ischemic heart disease during the same period. They further classified Californian counties into several pesticide use categories based on data from pesticide use reports. Results showed that mortality from PD as the underlying cause of death was higher in counties in the category of agricultural pesticide use. Moreover, a dose-response relationship was reported for insecticide use per area of county land treated, but not for the amounts of restricted pesticides used or length of residency in a county prior to death (Ritz and Yu 2000).

2.1.5 Analyses of quantitative measures of pesticides

Finally, a few studies have assessed the link between pesticide exposure and PD by quantifying pesticide levels in PD patients. This has been tackled by measuring pesticides in both the serum and brain of deceased patients. A case-control study at the University of Texas Southwestern Medical Center collected serum samples from 50 PD patients and 43 controls to quantify the levels of 16 organochlorine pesticides. Hexachlorocyclohexane was detectable in 76% of PD patients and 40% of controls. The higher frequency of detection in PD cases was significant ($p < 0.05$), as was the OR for the presence of this pesticide in serum predicting the diagnosis of PD (OR = 4.39; 95% CI 1.67-11.6). None of the other 15 organochlorine pesticides showed detectable differences between controls and PD patients (Richardson et al. 2009). In addition, a study used organochlorine pesticide exposure data collected several years prior to the onset of PD as a potential biomarker for PD. Forty thousand two hundred and twenty-one serum samples of individuals aged ≥ 15 years were collected between 1968 and 1972 as part of a nested case-control study within the Finnish Mobile Clinic Health Examination Survey, and were analyzed in 2005–2007 for organochlorine pesticides. A total of 196 incident PD cases were identified during the follow-up in 1994 and were matched to 349 controls. Overall, 5 organochlorine pesticides were found at high levels, but only weak association emerged with this analysis. Only

increased dieldrin concentrations were associated with increased odds of PD (OR per interquartile range 1.95; 95% CI 1.26–3.02, p = 0.003) after adjustment for confounding factors (Weisskopf et al. 2010). Although this study presents an interesting design, with data collected *prior* to the development of PD, several limitations have to be taken into account. Serum samples were collected only once and reflect past pesticide exposure, but not exposure during the following decades. Importantly, exposure to pesticides with shorter half-lives that could have contributed to the pathology cannot be ruled out.

The presence of organochlorines was also verified in *post-mortem* brain samples of 20 PD patients and 14 non-neurological control subjects. Of all the organochlorines measured, dieldrin and dichlorodiphenyltrichloroethane (DDT) were the only pesticides detected. Dieldrin was found in 6 out of 20 PD brains and in none of 14 control samples. The association between dieldrin and the diagnosis of PD was significant (p = 0.031) (Fleming et al. 1994). Others have analyzed organochlorine concentrations in brain areas more specifically affected by the pathology (e.g. caudate nucleus). There were indeed significantly higher concentrations of dieldrin in PD tissues as compared to controls (Corrigan et al. 1998). The same group reported significantly higher levels of dieldrin and lindane in the substantia nigra (another structure largely affected by neuronal degeneration in PD) of PD patients as compared to nonparkinsonian controls (Corrigan et al. 2000).

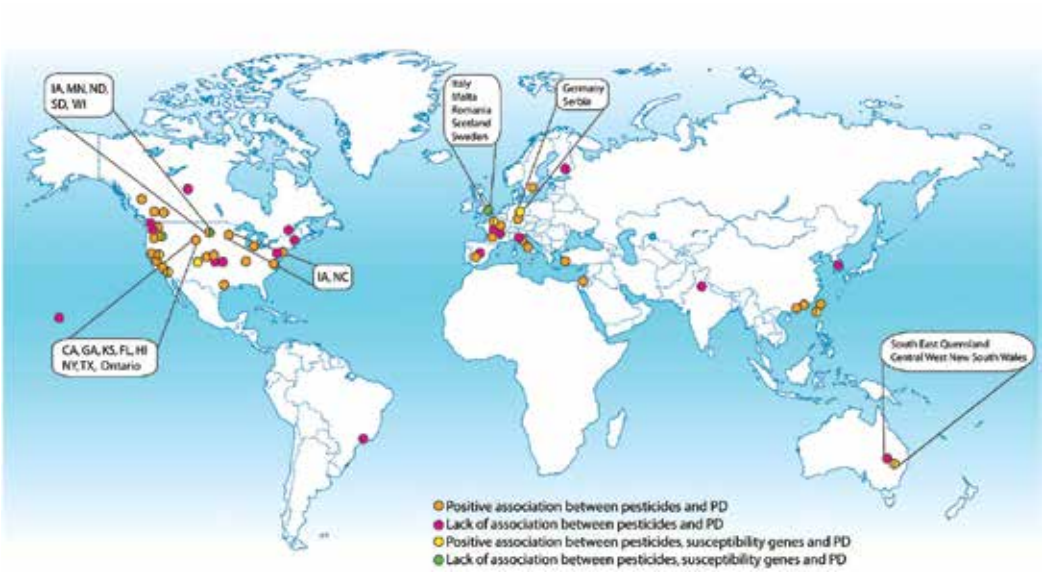

Fig. 2. Mapping of epidemiological studies assessing the relationship between pesticide exposure and the risk of developing Parkinson's disease. *Orange* circles represent studies reporting a positive association between pesticide exposure and PD, whereas *pink* circles illustrate studies reporting a lack of association. Studies assessing the vulnerability of specific gene polymorphisms are represented by a *yellow* circle for a positive association, and a *green* circle for a lack of association. Double-colored circles depict positive or lack of associations in studies assessing both the risk of PD from pesticide exposure alone, or including genetic vulnerability.

2.2 Epidemiological studies targeting specific pesticides

2.2.1 Organochlorines

Organochlorines are cholinesterase inhibiting pesticides that were introduced on a large scale on the world market during the 60's and 70's. These compounds are classified as moderately (e.g. DDT, endosulfan, toxaphene), highly (e.g. aldrin, dieldrin, endrin) or extremely toxic (e.g. hexachlorobenzene) by the International Program of Chemical Safety of the World Health Organization. A case-control study of 380 PD patients recruited from nine German clinics, 379 neighborhood and 376 regional control subjects found a significantly elevated risk of PD for general pesticide use and for organochlorines (OR = 5.8; 95% CI 1.1-30.4) and alkylated phosphates (OR = 2.5; 95% CI 1.3-4.6) in particular (Seidler et al. 1996). More recently, another group reported that organochlorine and organophosphorus pesticides were significantly associated with PD in a family-based case-control study involving 319 cases and 296 relatives and other controls, matched on genetic and demographic factors. Other controls were ascertained as spouses, unrelated controls or as related controls in families where no environmental risk factor data were available. PD patients reported significantly greater direct pesticide application/contact than their unaffected relatives (OR = 1.61; 95% CI 1.13–2.29). Furthermore, PD was associated with the highest frequency of exposure in both genders (OR = 2.15; 95% CI 1.06-4.35 for men and OR = 2.43; 95% CI 1.18-5.01 for women). A dose-response trend (OR = 2.47; 95% CI 1.12-5.44) and an association of PD with the lowest duration (p = 0.0058) were also reported, but only in women. Nevertheless, an association with PD was significant for the highest duration (OR = 2.70; 95% CI 1.35-5.40) and cumulative exposure (OR = 2.34; 95% CI 1.14-4.79), and significant dose-response trends were detected (p = 0.021 for duration and p = 0.036 for cumulative exposure). The latter associations were restricted to individuals without a family history of PD (Hancock et al. 2008). Moreover, a recent community-based case-control study examined the relationship between PD and pesticides in a population characterized by a high prevalence of exposure. Dose-effect analyses were performed using a cohort of 224 PD cases and 557 controls from the French Health Insurance (*Mutualité Sociale Agricole)* database for agricultural workers and related occupations. Twenty-nine pesticide families, based on a chemical classification, were analyzed in men exclusively. PD and overall professional pesticide use were positively associated (OR = 1.8; 95% CI 1.1-3.1), and a dose-effect relationship was found for the number of years of usage (p < 0.01). Insecticides were associated with PD (OR = 2.2; 95% CI 1.1-4.3), and more particularly insecticides belonging to the organochlorine family (OR = 2.4; 95% CI 1.2-5.0). In men with late-onset PD, these associations were more prominent (p < 0.01) and were characterized by a dose-effect relationship (Elbaz et al. 2009) (see **Table 2** for details and **Figure 2** for geographical mapping of studies conducted).

An association between PD risk and exposure to several specific pesticides was reported in a study that enrolled individuals applying for certification for using restricted pesticides in Iowa and North Carolina. Data were obtained from licensed private pesticide applicators and spouses participating in the Agricultural Health Study Cohort. In this particular study, PD cases were selected based on self-report, thus the diagnosis was not confirmed by a neurologist. PD cases were compared with cohort members who did not report PD. The incidence of the disease was associated with cumulative days of pesticide use, applying pesticides themselves more than half of the time (OR 1/4 = 1.9; 95% CI 0.7-4.7). However, prevalence of PD was not associated with overall pesticide use. The investigators further

observed elevated ORs for the prevalence of PD for the herbicides pendimethalin, paraquat, and cyanazine, and for the fumigants CS_2/CCl_4 and ethylene dibromide. ORs for incident PD were also elevated for the herbicides dicamba, trifuralin, 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), and butylate, the insecticides lindane and phorate, the fungicides chlorothalonil and benomyl, and the fumigant CH_3Br (Kamel et al. 2007). A more recent study employed a geographic information system that integrated data from California pesticide use reports and land-use maps (instead of surveys) to assess the degree and nature of pesticide exposure in PD patients to estimate potential well-water contamination with agricultural pesticides among 368 PD cases and 341 population controls who participated in the Parkinson's Environment and Genes Study. The study investigated six different pesticides (diazinon, chlorpyrifos, propargite, paraquat, dimethoate and methomyl). Elevated levels of possible well-water contamination with methomyl (OR = 1.67; 95% CI 1.00–2.78), chlorpyrifos (OR = 1.87; 95% CI 1.05–3.31), and propargite (OR = 1.92; 95% CI 1.15–3.20) were associated with a 70 to 90% increase in RR of PD. They further showed that exposure to a higher number of water-soluble and organophosphate pesticides also increased the RR of PD in that cohort (Gatto et al. 2009). Another recent study employed a multicenter case-control study design involving 8 movement disorder centers in North America to evaluate the relationships between occupations, specific job tasks, or exposure and the risk of parkinsonism. Five-hundred nineteen PD patients and 511 controls that were primarily non-blood relatives, or acquaintances, participated in the study. Pesticide use was associated with an elevated risk of parkinsonism (OR = 1.90; 95% CI 1.12-3.21, $p = 0.02$). In addition, the use of any of the 8 pesticides selected *a priori* as presenting a particular interest to the development of animal models of PD (2,4-dichlorophenoxyacetic acid (2,4-D), paraquat, permethrin, dieldrin, diquat, maneb, mancozeb and rotenone; cf. section 2.3) also increased the risk of parkinsonism (OR = 2.20; 95% CI 1.02-4.75. $p = 0.04$). 2,4-D was the only pesticide significantly associated *per se* with an increased risk for PD (2.59; 95% CI 1.03-6.48 p = 0.04) (Tanner et al. 2009).

2.2.2 Paraquat, maneb and rotenone

Some epidemiological studies have gone further with their analyses, isolating specific classes of pesticides or individual pesticides to determine their possible influence on the probability of developing PD. One of these pesticides is paraquat, a contact herbicide belonging to the heterocyclic quaternary ammonium family and a very potent, self-regenerating oxidizing agent and photosynthesis inhibitor which is widely used in agriculture, based in part on the fact that it acts quickly and is characterized by a short bioavailability. A first report was based on 57 PD cases and 122 age-matched, randomly selected controls from regional electoral rolls, all < 80 years of age, where four PD patients and no controls reported paraquat contact. Although an OR could not be calculated (no exposed controls), a Fisher's exact test gave a significant probability estimate of 0.01 for the association between paraquat contact and development of PD (Hertzman et al. 1990). Over 120 PD cases recruited from the Movement Disorder Clinic of the National Taiwan University Hospital in Taipei and 240 hospital controls recruited from the neurological or medical outpatient clinics at the same hospital, 28 PD cases and 18 control subjects reported having been previously exposed to paraquat. In the univariate analysis, the use of herbicides and pesticides (OR = 2.89; 95% CI 2.28-3.66, $p < 0.01$), and the use of paraquat (OR = 3.22; 95% CI 2.41-4.31, $p < 0.01$) were associated with an increased PD risk that also followed a

dose-response relationship. The biological gradient between PD and the previous use of herbicides and pesticides, and paraquat specifically, remained significant even after adjusting for multiple risk factors. Of note, there was a greater risk of developing PD for subjects who had used paraquat and other herbicides/pesticides than for those who had used herbicides/pesticides but not paraquat (Liou et al. 1997).

Considering that the geographical distribution of paraquat and maneb overlaps in several areas of the USA, their potentially synergistic neurotoxic effects have been more closely examined. Both compounds are similarly applied, but paraquat has a much longer half-life than maneb. Costello and coll. have reported that exposure to paraquat and maneb within 500 m of the residence increased PD risk by 75% (OR 1/4 = 1.75; 95% CI 1.13-2.73), using the geographic information system that integrated data from the California pesticide use reports and land-use maps reported by Gatto et al. (2009). This study incorporated 368 idiopathic PD cases and 341 population controls from the Central Valley of California. The authors also evaluated PD risk for two separate periods of pesticide exposure (between the years 1974–1989 and 1990–1999). The risk of developing PD was higher in younger subjects or when exposed at a younger age to either maneb or paraquat alone (OR 1/4 = 2.27; 95% CI 0.91-5.70) or to both pesticides in combination (OR 1/4 = 4.17; 95% CI 1.15-15.16) (Costello et al. 2009) (see **Table 2** for details and **Figure 2** for geographical mapping of studies conducted).

More recently, a case-control study in eastern Texas recruited 100 PD cases and 84 controls, and observed a strong association between the risk for PD and the use of organic pesticides such as rotenone within the past year of gardening (OR = 10.9; 95% CI 2.5-48.0, $p < 0.001$) and any rotenone use in the past (OR = 10.0; 95% CI 2.9-34.3, $p < 0.001$). Exposure to several other pesticides was also evaluated, and an elevated risk was associated with domestic use of chlorpyrifos products (OR = 2.0; 95% CI 1.02-3.8, $p = 0.043$). A possible association of increased PD risk with the domestic use of paraquat was also observed, but did not reach statistical significance (Dhillon et al. 2008).

2.3 Evidence from animal studies

2.3.1 Paraquat- and maneb-induced animal models of Parkinson's disease

If epidemiological studies have left a rather confusing picture of the contribution of pesticides to PD, basic research has also addressed the question by attempting to duplicate the clinical and pathological signs of PD in both petri dishes and small laboratory animals. The following section is devoted to some *in vitro,* but particularly *in vivo* work performed in rodents in the hope of shedding light on the role of environmental toxins, such as pesticides, to the development of a syndrome resembling human parkinsonism.

It has been suggested that dopaminergic cell degeneration observed in PD is consequential to the toxic accumulation and aggregation of proteins, mitochondrial dysfunction and oxidative stress. The neurotoxicity of paraquat resides within its strong redox cycling properties that leads to its transformation to the reduced paraquat radical which is then readily reoxidized by O_2, thereby generating reactive oxygen species, including superoxide anions (O_2^-) (Autor 1977; Bus et al. 1974; Jones and Vale 2000). The oxidative stress thus generated is believed to cause lipid peroxidation, inhibition of complex I in the mitochondrial respiratory chain, as well as cell death. Several animal studies have reported that paraquat can cause dopaminergic neuronal degeneration, the vast majority of which used a systemic paraquat administration approach [e.g.: (Brooks et al. 1999; Fredriksson et al. 1993; Kang et al. 2009; Kuter et al. 2007; Li et al. 2005; Peng et al. 2004; Shimizu et al. 2003; Somayajulu-Nitu et al. 2009; Tawara et al.

1996)]. Systemic administration of paraquat can also produce motor deficits such as decreased locomotor activity, reduced spontaneity in gait performance, and impaired pole test performance (Brooks et al. 1999; Li et al. 2005; Somayajulu-Nitu et al. 2009), and can induce the upregulation and aggregation of α-synuclein in the substantia nigra of wild-type mice (Manning-Bog et al. 2002). Although this model – as any animal model of human diseases – does not entirely mimic the human pathology, studies of paraquat-induced parkinsonism in animal models have provided valuable information with regards to the potential mechanisms involved in neurodegenerative processes associated with environmental toxicity. Pathological observations made in animal models thus imply that paraquat is unlikely a single contributor to the etiology of PD.

One of the most compelling findings of animal studies has derived from using combination of paraquat and maneb. Indeed, combined administration of both compounds to rodents has been useful to demonstrate the potential synergistic effects of environmental compounds in reproducing some features of PD in animals. In addition to causing nigrostriatal dopaminergic depletion [e.g. (Cicchetti et al. 2005; Drouin-Ouellet et al. 2007; Saint-Pierre et al. 2006; Thiruchelvam et al. 2000a; Thiruchelvam et al. 2003a; Thiruchelvam et al. 2000b)], the paraquat and maneb combination has also been shown to potentiate α-synuclein-induced toxicity (Norris et al. 2007; Thiruchelvam et al. 2004). Moreover, systemic administration of paraquat and maneb induces motor impairments reminiscent of PD.

Two studies have further explored the effect of a developmental exposure to paraquat and maneb followed by a re-challenge latter in adult life. Early postnatal exposure to the combination of compounds generated a decrease in activity, striatal dopamine depletion and dopaminergic cell loss in the substantia nigra. An adult re-challenge of the paraquat/maneb combination showed an even more striking decrease in locomotor activity, striatal dopamine levels, and dopaminergic cell loss. While postnatal exposure to paraquat or maneb alone produced minimal changes in adulthood, a re-challenge at that time unveiled a quiescent toxicity due to these pesticides (Thiruchelvam et al. 2002). Another study assessed whether *in utero* exposure to paraquat and maneb would interfere with the development of the nigrostriatal dopaminergic pathway and enhance its vulnerability to dopaminergic neurotoxicant exposures in adulthood. Only males exposed to maneb prenatally and to paraquat in adulthood displayed significant decrease in locomotor activity, changes in striatal dopamine and selective dopaminergic neuronal loss in the substantia nigra (Barlow et al. 2004). The results obtained with the paraquat- and maneb-induced animal model have provided support for a multi-hit hypothesis in PD pathogenesis and recapitulate most epidemiological studies assessing this particular hypothesis, although the routes of delivery employed (mainly intraperitoneal (i.p.) and subcutaneous (s.c)) remain unrepresentative of human exposure. Taken together, research shows 1) an age-related propensity to incur degeneration of the nigrostriatal pathway in response to toxin (herbicide, pesticide, fungicide) exposure (Thiruchelvam et al. 2003b; Thiruchelvam et al. 2002), and 2) an exacerbation of nigrostriatal pathology by double-exposure whereby early (prenatal, postnatal) contact with these toxins predispose older animals to the effects of re-exposure to the toxins (Carvey et al. 2003; Ling et al. 2002; Thiruchelvam et al. 2002). The mechanism for the increased sensitivity to toxins in adults and/or re-exposed animals is to date unknown.

2.3.2 Rotenone-induced animal models of Parkinson's disease

Unlike quaternary amines like paraquat or diquat, rotenone crosses the blood-brain barrier due to its lipophilic attributes. This insecticide has been targeted as a potentially active agent

in PD pathogenesis based on its ability to inhibit complex I of the mitochondrial respiratory chain, which triggers the production of reactive oxygen species and the activation of mitochondria-dependent apoptotic molecular pathways. This subsequently leads to oxidative damage targeting proteins, lipids and DNA, ultimately leading to dopaminergic cell death (Dauer and Przedborski 2003; Vila and Przedborski 2003). Mutations in specific genes linked to mitochondrial proteins have also been associated with some familial forms of PD (Bueler, 2009). However, the specific mechanism involved in the enhanced vulnerability of nigral dopaminergic neurons to rotenone is still undeciphered. In opposition, an *in vitro* study has suggested that complex I inhibition might not be necessary for dopaminergic neuronal death (Choi et al. 2008). Nevertheless, rotenone has been suggested to act as a proteosome inhibitor (Chou et al. 2010; Wang et al. 2006b).

The most studied rotenone-induced animal model of PD involved a chronic mode of intravenous (i.v.) delivery via osmotic minipumps, although other delivery methods have also been explored, including i.p., and s.c. osmotic minipumps as well as intranasal and oral routes. In the vast majority of these studies, the substantia nigra dopaminergic neurons were affected, in addition to other types of neurons within the striatum. Several studies have reported that rotenone administration generated motor deficits reminiscent of several clinical features of PD such as hypokinesia, rigidity, hunched posture, unsteady movements, prolonged descent latency as well as resting tremors [e.g. (Alam et al. 2009; Alam et al. 2004; Alam and Schmidt 2004; Betarbet et al. 2000; Hoglinger et al. 2005; Luo et al. 2007; Pasha et al. 2005; Richter et al. 2007; Sherer et al. 2003; Tapias et al. 2009)]. Moreover, ubiquitin and α-synuclein aggregates were detected in striatal and nigral neurons in animals challenged with rotenone [e.g. (Betarbet et al. 2006; Cannon et al. 2009; Hoglinger et al. 2005; Inden et al. 2007; Luo et al. 2007; Monti et al. 2009; Takeuchi et al. 2009)]. As for paraquat and maneb-induced animal models of PD, rotenone induces peripheral toxicity leading to a high mortality rate, a phenomenon that does not resemble the human form of the disease (see review Cicchetti et al. 2009; Lapointe et al. 2004).

The mode of administration of rotenone employed in most animal studies does not mimic the route of exposure experienced in humans, as it is more likely that rotenone gains access to the brain via direct exposure of neurons in the gut and/or olfactory regions, i.e. the only nervous system structures directly exposed to environmental compounds (Lerner and Bagic 2008). One study administered rotenone intranasally daily for one month at a dose range similar to that used in i.p., i.v., and s.c. delivery studies, but reported no change in the nigrostriatal dopaminergic system and no behavioral alterations. A few studies have also explored the oral route of administration of rotenone and have observed degeneration of dopaminergic cells and their terminals. All of these studies also reported an upregulation or aggregation of α–synuclein accompanied with motor impairments (Inden et al. 2007; Inden et al. 2009; Pan-Montojo et al. 2010; Takeuchi et al. 2009). One of these studies administered rotenone intragastrically and observed α–synuclein accumulation and aggregation first at the periphery, and then in structures of the central nervous system affected in PD (Pan-Montojo et al. 2010). This reflects, to some extent, the course of the PD pathogenesis, where the synucleinopathy is restricted to the peripheral organs at the presymptomatic stages, whereas at latter stages, the substantia nigra and other nuclei of the midbrain and forebrain display similar pathological changes (Braak et al. 2003). The effect of chronic oral administration of rotenone to transgenic mice overexpressing human α–synuclein was also investigated. Despite increased cytoplasmic expression of α–synuclein and PINK1, along with decreased spontaneous locomotor movements induced by rotenone, no change in brain

dopamine levels or nigrostriatal cell loss was observed. The authors concluded that this model could mimic presymptomatic PD features and compensatory changes in early PD stages (George et al. 2010).

2.3.3 Animal model of Parkinson's disease induced by other pesticides

Most of the studies pertaining to the effects of various pesticides, especially organochlorines, have demonstrated some dopaminergic alterations within the nigrostriatal system (Miller et al. 1999; Pittman et al. 2003; Schuh et al. 2009), while others have failed to report such changes (Hatcher et al. 2008; Thiffault et al. 2001). However, results do suggest that developmental or adult exposure to dieldrin increases the vulnerability of nigrostriatal dopaminergic neurons by persistently altering the development of the dopaminergic system, or by inducing oxidative stress (Hatcher et al. 2007; Richardson et al. 2006). When probing other pesticides such as heptachlor, endosulfan and zineb, studies have shown their detrimental effect on the development of the dopaminergic system, subsequently leading to an increased vulnerability in adulthood (Caudle et al. 2005; Jia and Misra 2007; Richardson et al. 2008). Taken together, these studies suggest that developmental pesticide exposure causes long-term alterations of the dopaminergic system thereby rendering it more susceptible to dopaminergic damage in adulthood. Longitudinal epidemiological studies assessing the effect of pesticide exposure during the developmental stages would be of high relevance to evaluate the impact of such exposure on neurological disorder development in adulthood.

3. Genes and pesticide exposure

Although 90-95% of PD cases are of unknown etiology, 5-10% of patients are known to have monogenic forms of the disease. To date, 13 loci and 9 genes are associated with both autosomal dominant (e.g. α-synuclein, ubiquitin C-terminal hydrolase L1 (UCHL1), LRRK2, GIGYF2, Omi/Htra2) and autosomal recessive (e.g. parkin, PINK1, DJ-1, ATP13A2) PD, but additional genes have also been associated with the disease. Genes may also play a role in the sporadic form of PD, given the role of the encoded proteins in various important cellular functions such as in mitochondrial (e.g. α-synuclein, parkin, PINK1, Omi/HtrA2, DJ-1, POLG1) and lysosomal (e.g. α-synuclein, ATP13A2, GBA) functions, protein degradation (e.g. parkin, UCHL1, α-synuclein), developmental regulation (e.g. α-synuclein, parkin, UCHL1, LRRK2, Omi/HtrA2, Nurr1, PITX3, various microRNAs, etc.), and their localization at the synapse (e.g. α-synuclein, parkin, LRRK2, UCHL1, synphilin, etc.) (for a review, see Biskup 2008) (see **Table 3** for details and **Figure 2** for geographical mapping of studies conducted). Alterations in these proteins contribute to the pathological features encountered in the different forms of PD.

3.1 Susceptibility genes involved in the metabolism of pesticides

In recent years, a subset of epidemiological studies has thus focused on investigating a potential association between candidate genes for susceptibility to PD and exposure to pesticides. The first gene polymorphisms that have been studied code for glutathione-*S*-transferases (GSTs), which are a ubiquitous group of detoxification enzymes involved in the metabolism of several toxins, including pesticides, and that can protect cells against oxidative stress (Di Ilio et al. 1995). Using a cohort of 95 PD and an equal number of control

subjects, four *GST* classes were genotyped (*GSTM1*, *GSTT1*, *GSTP1*, and *GSTZ1*). In subjects who had been exposed to pesticides, there was a significant difference in *GSTP1* genotype between PD patients and controls (p = 0.009), but other *GST* polymorphisms did not show any association with PD (Menegon et al. 1998). A second study assessed the relationship between *GST* polymorphisms, PD and pesticide exposure in a multicenter study of paired relatives diagnosed with PD, designed for genetic linkage analyses. Seven single-nucleotide polymorphisms (SNPs) were genotyped in the *GSTP1* class, of which 3 were connected to the age of onset in the group of men occupationally exposed to herbicides. Significant trends were observed in the herbicide exposure group for the association of age of PD onset for three additional SNPs. The authors also reported that herbicide exposure modified the association between *GSTP1* and the age of onset. Furthermore, one haplotype was associated with earlier onset of PD (7.93 years) in the occupationally exposed group (p = 0.008) and a later PD onset (2.82 years) in the non-exposed group (p = 0.048) (Wilk et al. 2006). GSTP1 is expressed at the level of the blood-brain barrier and could influence the response to neurotoxins such as pesticides, by offering protection against the oxidative damage that is hypothesized to play a role in PD pathogenesis. However, another study failed to corroborate these results and reported that seven *GST* polymorphisms were in fact not associated with PD, nor was pesticide use (238 Japanese PD cases and 370 controls). In this particular study, controls were not matched to cases, which led to a significant difference between the age of subjects (controls being younger than cases) and the number of pesticide users was small, which gave negligible power to the analysis (Kiyohara et al. 2010).

Similar analyses were conducted to probe the potential association between PD and the organophosphates diazinon, chlorpyrifos, and parathion, and the influence of a functional polymorphism at position 55 in the coding region of the *PON1* gene (*PON1-55*). This gene codes for paraoxonase, an enzyme which hydrolyzes organophosphates and predicts the susceptibility of an individual to these compounds, more particularly the insecticides diazinon and chlorpyrifos (Costa et al. 2003). Exposure to chlorpyrifos was associated with an increased risk of PD, both at low and high frequency levels of exposure, and more prominently among people over 60 (OR = 2.65; 95% CI 1.19-5.90). An association between PD and high, but not low levels of diazinon exposure, was also reported, but not with parathion. Within subjects exposed to organochlorines, carriers of the variant *MM PON1-55* genotype displayed an increased risk of PD compared with subjects carrying the wild-type or heterozygous genotype and without a history of exposure (diazinon, OR = 2.2; 95% CI 1.1-4.5; chlorpyrifos, OR = 2.6; 95% CI 1.3-5.4). Additionally, the effect estimate for chlorpyrifos was greater in earlier-onset cases and controls (≤ 60 years of age; OR = 5.3; 95% CI 1.7-16), but no increase in PD risk was noted for parathion (Manthripragada et al. 2010).

In a larger case-control study, Dick and colleagues (2007) investigated the interactions between several polymorphic genes that metabolize foreign chemicals, metabolize or transport dopamine and that occur relatively frequently in the European population (*CYP2D6*, *PON1*, *GSTM1*, *GSTT1, GSTM3*, *GSTP1*, *NQO1*, *CYP1B1*, *MAO-A*, *MAO-B*, *SOD2*, *EPHX*, *DAT1*, *DRD2* and *NAT2*), exposure to solvents, pesticides and metals, and risk of PD. Nine hundred and fifty-nine prevalent cases of parkinsonism (767 with PD) and 1 989 control subjects were recruited from five European centers. Parkinsonism was modestly, but significantly associated with *MAO-A* polymorphism in males (G vs. T, OR = 1.30; 95% CI

1.02-1.66, adjusted for confounding factors). Although a possible interaction between a *GSTM1* null genotype and solvent exposure was shown, other gene-environment interactions failed to show any significant association (Dick et al. 2007).

3.2 Susceptibility genes involved in pesticide transport to the brain

The *multidrug resistance protein 1* (*MDR1* or *ABCB1*) gene encodes an integral membrane glycoprotein expressed in various tissues, including the blood-brain barrier, and which regulates brain penetration of a wide range of endogenous molecules and xenobiotics, including several pesticides such as organochlorines (Bain and LeBlanc 1996). In a case-control study, Zschiedrich and coll. (2009) evaluated the potential relationship between *ABCB1* variants and PD in relation to pesticide exposure in 599 PD patients and control subjects. Despite the fact that *ABCB1* was not associated with PD in this particular study, a different genotype distribution was observed between patients exposed to pesticides compared to non-exposed patients (OR = 4.74; 95% CI 1.009-22.306, p = 0.047), suggesting that common *ABCB1* variants may interact with pesticide exposure to influence PD risk (Zschiedrich et al. 2009). A second study evaluated the association between two polymorphisms in *ABCB1*, PD and organochlorine insecticide exposure among 207 cases and 482 matched control subjects enrolled in the French health system for agricultural workers described previously (Elbaz et al. 2009). As for the study of Zschiedrich and coll. (2009), *ABCB1* polymorphisms were not associated with PD. However, the OR for organochlorines was 3.5 (95% CI 0.9-14.5) times higher among homozygous carriers of variant *G2677* (A,T) alleles than noncarriers. The case-only analysis uncovered an association between carrying two variant *G2677* (A,T) alleles and organochlorines (OR = 5.4; 95% CI 1.1-27.5), as well as with the number of cumulative lifetime number of hours of exposure (overall, p = 0.005; analyses restricted to subjects exposed to organochlorines, p = 0.03) (Dutheil et al. 2010).

3.3 Susceptibility genes involved in elimination of toxic compounds derived from pesticides

A case-control study evaluated the role of manganese-containing superoxide dismutase (MnSOD) and NAD(P)H: quinone oxidoreductase 1 (NQO1) genes with PD risk in a southwestern Taiwanese population with a high prevalence of pesticide exposure. MnSOD is an enzyme that converts superoxide anions (O_2^-) into hydrogen peroxide (H_2O_2) and dioxygen (O_2). Suppressing free radicals within mitochondria protects against the detrimental effects of oxidative stress on cell integrity. In Japanese patients with familial PD, the *MnSOD* C allele is significantly related to the disease (Shimoda-Matsubayashi et al. 1997). NQO1 is also an enzyme that reduces several neurotoxic quinonoid compounds, which leads to protection of the cells against reactive oxygen species damage during redox cyclic processes (Chen et al. 2000). The genotypes of *MnSOD (−9 TNC)* and *NQO1 (609 CNT)* genes were determined among the 153 patients with idiopathic PD and 155 matched healthy controls. After adjustment for confounding factors, a significant association was found between pesticide exposure and PD risk (OR = 1.68; 95% CI 1.03-2.76, p = 0.023). In this population, *MnSOD* and *NQO1* polymorphisms were not associated with increased PD risk, but there was a significant difference in genotype distribution among subjects exposed to pesticide for the *MnSOD* C allele (OR = 2.49; 95% CI 1.18-5.26, p = 0.0072) and for the *NQO1*

T allele (OR = 2.42; 95% CI 1.16-4.76, p = 0.0089). Furthermore, a significant association was reported between the combined *MnSOD/NQO1* variant genotype among subjects exposed to pesticides and increased PD risk (OR = 4.09; 95% CI 1.34-10.64, p = 0.0052) (Fong et al. 2007).

3.5 Susceptibility genes targeted in Parkinson's disease

Two studies have examined the possible interaction between the dopamine transporter (DAT) gene (*SLC6A3*), of which eight haplotypes have been identified (grouped into two evolutionary clades (A and B)), and PD risk after pesticide exposure. In a case-control study of 293 cases and 395 controls that were classified by the number of risk alleles, a significant interaction between occupational pesticide exposure in men and the number of risk alleles was reported, the OR for having two or more risk alleles reaching 5.66 (95% CI 1.73–18.53) among subjects exposed to pesticides (Kelada et al. 2006). A subsequent study independently investigated the genetic variability in the DAT locus in 324 incident PD cases and 334 controls from the rural California case-control study using the previously described geographic information system for pesticide exposure evaluation (Costello et al. 2009; Gatto et al. 2009). Two SNPs were genotyped for the *DAT* 5´ A clades and the 3´ variable number of tandem repeats (VNTR), the susceptibility alleles being defined as the 5´ A clade and the 3´ VNTR 9-repeat. Carriers of one susceptibility allele who were highly exposed to paraquat and maneb had an increased PD risk (OR = 2.99; 95% CI 0.88-10.2), and this was more prominent in those with two or more alleles (OR = 4.53; 95% CI, 1.70-12.1). Similar results were also obtained for occupational pesticide analysis (Ritz et al. 2009).

Furthermore, it has been suggested that SNPs might lead to slight alterations in the *PINK1* gene and might play an important role in the development of sporadic late-onset PD (Wang et al. 2006a). In a study with 48 PD cases and 61 controls from Brazil carrying *PINK1* SNPs, 31.3% and 39.4% presented the *PINK1* SNP *IVS1−7* A→G polymorphism, respectively. Exposure to various environmental risk factors (living in rural areas, well-water drinking, and exposure to pesticides, herbicides or organic solvents) in collaboration with *PINK1* SNP *IVS1−7* A→G polymorphism had a significant effect in lowering the age of PD onset, whereas when singling out exposures to the various environmental factors, no association was found between such exposures, *PINK1* SNP *IVS1−7* A→G polymorphism and PD (Godeiro et al. 2010).

A case-control study with 833 case-control pairs further examined the possible interaction between *SNCA* REP1 genotypes (coding for α-synuclein), which have been shown to confer susceptibility to sporadic PD, and pesticide exposure on the risk of PD in human. This epidemiological study did not find any interaction between the *SNCA* REP1 genotype and herbicides, although both *SNCA* REP1 score (OR = 1.18; 95% CI 1.02-1.37; p = 0.03) and pesticide exposure were significantly associated with PD in younger subjects (≤ 59.8 years of age; OR = 1.80; 95% CI 1.12-2.87; p = 0.01 for all pesticides; OR = 2.46; 95% CI 1.34–4.52; p = 0.004 for herbicides) (Brighina et al. 2008).

Taken together, the results of the gene-environment epidemiological studies converge toward an influence of certain gene polymorphisms on the effect of exposure to at least some pesticides on PD and its onset. They further suggest that individual genetic susceptibility may affect the outcome of epidemiological studies. This is a very likely explanation for the inconsistent results reported thus far and needs to be taken into account when reviewing past studies on the effect of pesticide exposure and PD risk.

4. Conclusion

Numerous challenges must be faced in interpreting epidemiological studies, as illustrated in this chapter. Data gathered thus far originate from various approaches, which include case reports, mortality studies (geographical analysis of death certification), ecological studies (e.g. analyzing pesticide exposure from levels detected in the environment), case-control and cohort studies. The discrepancies in the results obtained with epidemiological studies are largely due to issues related to the methodological approaches utilized, case ascertainment, selection of controls and diagnostic criteria, all factors likely to introduce bias. Most methodologies employed are based on self-surveys and recall of chemical usage, which can be particularly precarious when collected from patients suffering from neurodegenerative disorders. Furthermore, questionnaires used to determine the type and length of pesticide exposure may vary significantly among studies - with the accuracy for self-reported pesticide exposure being high for broad categories and commonly employed pesticides, but not for specific pesticides (Engel et al. 2001b). Unbiased selection of controls is also of utmost importance but may be challenging. Controls and cases may be recruited from the general population or be hospital-based, which can result in selection bias for both cases and controls if participation is influenced by factors such as disease severity, personal income, cultural differences and geographic location. Control subjects are very often relatives or friends of the subjects, allowing for the possibility of similar exposure history and thus invalidating the risk estimate due to bias.

Misdiagnosis remains frequent in PD, especially in the early stages of the disease, and can thus have a significant impact on the outcomes of clinical and epidemiological studies (Litvan et al. 2003). A single causative factor might also be difficult to identify, because such a factor may differ among patients with different clinical manifestations. Stratifications of clinical subsets (e.g. age of onset, progression, motor symptoms, etc.) may help identify environmental causes of PD. Several elements, such as age, family history of PD, earlier head trauma due to accidents, smoking habits, caffeine consumption and infectious diseases, as well as environmental factors including well-water drinking and farming, have all been suggested to play a role in the incidence of PD, and can be sources of confounding factors. All of these potential biases may pose a significant hurdle in evaluating the actual contribution of pesticide exposure to PD. Despite accumulating evidence supporting the hypothesis that pesticide exposure may be responsible for the etiology of PD, at least in a subset of cases, the overall picture remains inconclusive but at least convincing enough to justify the debate to be pursued and the issue to be clarified.

Future research should focus on understanding how combining compounds that target different cellular functions might work cooperatively to also cause neuronal damage. As challenging as this task might be, pesticide properties, such as biological availability, persistence in the environment as well as application methods that can lead to widespread exposure, should be taken into account when evaluating the potential of a pesticide to participate to PD pathogenesis. In addition, genetic vulnerability is likely a key player in the outcome of pesticide exposure. In that regard, more studies are clearly needed to target specific polymorphisms. Finally, epidemiological studies have thus far provided rather disparate and somehow incoherent results, and segregation of different PD subtypes, as well as better methodologies with regard to the evaluation of pesticide exposure, might help in pinpointing the involvement of specific compounds in PD incidence and provide us with tools to design and develop drug targets to prevent PD pathogenesis.

	Study	Country	Exposed/ Total cases	Exposed/ Total controls	OR (95% CI)	*p* value	Specifications
Pesticides	(Ho, 1989)	China	7/34	7/105	3.6 (1.0-12.9)		Pesticide and herbicide use combined
	(Golbe, 1990)	USA	14/106	2/106	7.0	$p < 0.05$	
	(Hertzman, 1990)	Canada	31/57	57/121	1.34	$p = 0.184$	Analysis includes glyphosate, picloram, formaldehyde, malathion, 2,4-D, tebuthiuron, paraquat, diazinon, atrazine, pyrethrum, diquat and bromacil
	(Koller, 1990)	USA	NA/150	NA/150	1.1	$p = 0.82$	Pesticide and herbicide use combined
	(Zayed, 1990)	Canada	23/42 6/42 4/42 5/42 8/42	43/84 16/84 8/84 10/84 9/84	1.23 (0.46-3.29) 0.81 (0.24-2.68) 1.08 (0.24-4.66) 1.23 (0.24-4.12) 1.23 (0.56-6.57)	$p = 0.35$	Pesticide use 1-10 years of pesticide use 11-20 years of pesticide use 21-30 years of pesticide use >30 years of pesticide use
	(Wong, 1991)	USA	NA/38	NA/38	1.0 (0.33-3.06)	$p = 1.00$	Pesticide and herbicide use combined
	(Jimenez-Jimenez, 1992)	Spain	43/128	70/256		NS	
	(Semchuck, 1992)	Canada	NA/130	NA/260	2.25 (1.27-3.99) 1.41 (0.73-2.73) 2.27 (1.08-4.76) 2.21 (0.99-4.94) 2.25 (0.91-4.72)	$p = 0.005$ NS $p = 0.03$ NS NS	Pesticide use 16-25 years of pesticide use 26-35 years of pesticide use 36-45 years of pesticide use 46-55 years of pesticide use
	(Hubble, 1993)	USA	NA/63	NA/76	3.42 (1.27-7.32)	$p = 0.004$	
	(Hertzman, 1994)*	Canada	33/71	22 /80 16/60	2.03 (1.00-4.12) 2.32 (1.10-4.88)		♂/Vs. controls with cardiac disease ♂/Vs. controls randomly selected
			9/56	5/41 8/64	1.11 (0.32-3.80) 1.36 (0.48-3.85)		♀/Vs. controls with cardiac disease ♀/Vs. controls randomly selected
	(Morano, 1994)	Spain	40/74	60/148		$p = 0.056$	Chi Square = 3.64
	(Chaturvedi, 1995)	Canada	22/87	323/2070	1.81 (0.92-3.36)	NS	Occupational exposure to pesticides and fertilizers combined
			12/87	178/2070	1.67 (0.67-3.63)	NS	Exposure to pesticides and herbicides combined as a hobby
	(Liou, 1997)	Taiwan	46/120	41/240	2.89 (2.28-3.66)	$p < 0.01$	Pesticide and herbicide exposure combined
			14/120	21/240	1.41 (0.52-3.85)	NS	1-19 years of combined pesticide and herbicide exposure
			32/120	20/240	6.72 (2.62-17.21)	$p < 0.01$	≥20 years of combined pesticide and herbicide exposure
	(Chan, 1998)	China	19/215	16/313	0.75 (0.26-2.22) 1.05 (0.992-1.11)	$p = 0.608$ $p = 0.090$	Pesticide exposure in farming Number of years exposed to pesticides
	(McCann, 1998)	Australia	NA/224	NA/310	1.2 (0.8-1.5)	$p = 0.5$	Pesticide and herbicide exposure combined
	(Menegon, 1998)	Australia	39/95	26/95	2.3 (1.2-4.4)	$p = 0.02$	

Study	Country	Exposed/ Total cases	Exposed/ Total controls	OR (95% CI)	*p* value	Specifications
(Smargiassi, 1998)	Italy	25/86	20/86	1.15 (0.56-2.36)	NS	Pesticide and herbicide exposure combined
(Fall, 1999)	Sweden	10/NA	10/NA	2.8 (0.89-8.7)	$p = 0.081$	♂/Handling pesticides within any occupation
		6/NA	8/NA	1.9 (0.46-7.3)	$p = 0.45$	♂/Handling pesticides within agriculture
(Kuopio, 1999)	Finland	16/123	42/246	0.65 (0.33-1.29)	$p = 0.221$	Regular use of pesticides
		32/123	54/246	1.23 (0.74-2.04)	$p = 0.431$	Occasional use of pesticides
		48/123	96/246	1.02 (0.63-1.65)	$p = 0.935$	Regular and occasional use of pesticides
(Taylor, 1999)	USA	NA/140	NA/147	1.02 (0.90-1.17)	$p = 0.73$	
(Werneck, 1999)	Brazil	6/92	3/110	2.49 (0.53-13.14)		Pesticide, herbicide and insecticide use combined
(Preux, 2000)	France	42/140	68/280		$p = 0.21$	Pesticide and herbicide exposure combined
(Engel, 2001)*	USA	48/65				PR = 0.8 (0.5-1.2)
(Herishanu, 2001)	Israel	6/93	1/93	6.81 (0.75-64.89)	$p < 0.1$	
(Zorzon, 2002)	Italy	25/136	28/272	1.6 (1.0-2.4)	$p = 0.035$	
(Baldereschi, 2003)	Italy	7/113	82/4383	3.68 (1.57-8.64)		Pesticide-use licence
		7/58	51/2247	4.41 (1.84-10.56)		♂/Pesticide-use licence
(Baldi, 2003a)	France	8/24			$p = 0.07$	♂/RR = 5.63 (1.47-21.58)
					NS	♀/RR = 1.02 (1.47-21.58)
(Baldi, 2003b)*	France	19/84	38/252	2.20 (1.11-4.34)	$p = 0.02$	
(Duzcan, 2003)	Turkey	15/36	21/108	2.96 (1.31-6.69)	$p = 0.015$	
(Firestone, 2005)	USA	19/156	28/241	1.01 (0.53-1.92)	NS	♂/Occupational exposure
		178/250	280/388	0.95 (0.66-1.37)	NS	Home-based exposure
(Ascherio, 2006)*	USA	43/413	NA		$p = 0.0003$	Pesticide and herbicide use combined, RR 1.8 (1.3-2.5)
(Frigerio, 2006)*	USA	24/90	10/78	2.4 (1.1-5.4)	$p = 0.04$	♂/Pesticides (including herbicides, insecticides and others)
		6/59	8/51	0.6 (0.2-1.9)	$p = 0.4$	♀/Pesticides (including herbicides, insecticides and others)
(Kedala, 2006)	USA	47/178	55/239	1.30 (0.81-2.06)	NS	
(Fong, 2007)	Taiwan	85/153	66/155	1.68 (1.03-2.76)	$p = 0.023$	
(Kamel, 2007)*	USA	67/82	65116/78938	0.5 (0.2-1.1)		Prevalent PD
		68/75	45325/54744	1.3 (0.5-3.3)		Incident PD
(Brighina, 2008)*	USA	303/833	278/833	1.11 (0.89-1.38)	$p = 0.37$	
(Cho, 2008)	Korea	44/230	8/75	1.105 (0.999-1.221)	$p = 0.091$	
(Dhillon, 2008)*	USA	70/100	59/84	1.0 (0.5-1.9)	$p = 0.972$	
(Hancock, 2008)*	USA	200/319	147/296	1.61 (1.13-2.29)		
		143/228	102/215	1.80 (1.20-2.70)		Negative family history
		57/91	45/81	1.20 (0.58-2.50)		Positive family history
(Costello, 2009)*	USA	110/368	75/341	1.52 (1.08-2.14)		
(Elbaz, 2009)*	France	48/224	121/557	1.4 (0.9-2.3)	$p = 0.18$	Gardening exposure
		107/224	225/557	1.8 (1.1-3.1)	$p = 0.02$	Professional exposure

	Study	Country	Exposed/ Total cases	Exposed/ Total controls	OR (95% CI)	*p* value	Specifications
Fungicides	(Ritz, 2009)	USA	93/324	74/334	1.44 (1.01-2.06)		
	(Tanner, 2009)	Canada/USA	44/519	27/511	1.90 (1.12-3.21)	$p = 0.02$	Pesticides
			21/519	11/511	2.20 (1.02-4.75)	$p = 0.04$	Any of 8 specific pesticides
	(Dutheil, 2010)	France	41/207	97/482	1.4 (0.8-2.4)	$p = 0.22$	Gardening exposure
			96/207	207/482	1.8 (1.1-3.3)	$p = 0.03$	Professional exposure
	(Firestone, 2010)	USA	12/252	24/326	0.6 (0.30-1.29)	NS	♂
			3/152	1/200	3.9 (0.39-39.4)	NS	♀
	(Semchuck, 1992)	Canada	NA/130	NA/260	1.63 (0.81-3.29)	NS	
	(Hertzman, 1994)*	Canada	20/71	20 /80	1.04 (0.49-2.24)		♂/Vs. controls with cardiac disease
				26/60	0.52 (0.25-1.08)		♂/Vs. controls randomly selected
			5/56	6/41	0.53 (0.13-2.18)		♀/Vs. controls with cardiac disease
				12/64	0.44 (0.14-1.33)		♀/Vs. controls randomly selected
	(Gorell, 1998)*	USA	NA/144	9/464	1.50 (0.43-5.26)	$p = 0.526$	
	(Engel, 2001)*	USA	35/65				PR = 0.8 (0.6-1.3)
	(Firestone, 2005)	USA	2/156	6/241	0.38 (0.07-2.05)	NS	♂/Occupational exposure
			14/250	39/388	0.55 (0.29-1.05)	NS	Home-based exposure
	(Brighina, 2008)* (Dhillon, 2008)*	USA	NA/833	NA/833	0.83 (0.44-1.59)	$p = 0.58$	
		USA	4/100	6/84	0.5 (0.2-2.0)	$p = 0.349$	
	(Elbaz, 2009)*	France	NA/118	NA/291	1.5 (0.8-3.0)	$p = 0.22$	♂
			NA/106	NA/266	3.5 (1.2-10.3)	$p = 0.02$	♀
	(Tanner, 2009)	Canada/USA	1/519	1/511	1.01 (0.06-16.28)	$p > 0.99$	Mancozeb
Herbicides			81/149	77/149	0.9 (0.6-1.5)	$p = 0.73$	
	(Stern, 1991)	USA	NA/149	NA/149	0.9 (0.5-1.7)	NS	Early onset
					1.3 (0.7-2.4)	NS	Late onset
	(Semchuck, 1992)	Canada	NA/130	NA/260	3.06 (1.34-7.00)	$p = 0.006$	Herbicide use
					1.40 (0.46-4.30)	NS	16-25 years of herbicide use
					4.82 (1.51-15.35)	$p = 0.004$	26-35 years of herbicide use
					3.84 (1.16-12.70)	$p = 0.021$	36-45 years of herbicide use
					4.88 (1.28-18.60)	$p = 0.013$	46-55 years of herbicide use
	(Butterfield, 1993)	USA	18/63	6/68	3.22	$p = 0.033$	
	(Hertzman, 1994)*	Canada	47/71	55 /80	1.02 (0.50-2.07)		♂/Vs. controls with cardiac disease
				38/60	1.19 (0.57-2.45)		♂/Vs. controls randomly selected
			12/56	13/41	0.55 (0.21-1.48)		♀/Vs. controls with cardiac disease
				19/64	0.67 (0.29-1.56)		♀/Vs. controls randomly selected
			6/127	5 /121	1.11 (0.32-3.87)		♂/Vs. controls with cardiac disease/Paraquat
				4/124	1.25 (0.34-4.63)		♂/Vs. controls randomly selected/Paraquat

Study	Country	Exposed/ Total cases	Exposed/ Total controls	OR (95% CI)	*p* value	Specifications
		61/380	46/379	1.7 (1.0-2.7)		
		59/380	44/376	1.7 (1.0-2.6)		Dose-years=1-40/Vs. Neighboring controls
		34/380	27/379	1.4 (0.8-2.5)		Dose-years=1-40/Vs. Regional controls
(Seidler, 1996)	Germany	34/380	15/376	3.0 (1.5-6.0)		Dose-years=41-80/Vs. Neighboring controls
		20/380	11/379	2.2 (0.9-5.2)		Dose-years=41-80/Vs. Regional controls
		20/380	10/376	2.4 (1.0-6.0)		Dose-years>80/Vs. Neighboring controls
						Dose-years>80/Vs. Regional controls
		31/120	21/240	3.22 (2.41-4.31)	$p < 0.01$	Paraquat
(Liou, 1997)	Taiwan	7/120	13/240	0.96 (0.24-3.83)	NS	1-19 years of paraquat exposure
		24/120	9/240	6.44 (2.41-17.2)	$p < 0.01$	≥20 years of paraquat exposure
(Gorell, 1998)	USA	NA/144	7/464	3.36 (1.09-10.33)	$p = 0.034$	
		16/123	37/246	0.79 (0.38-1.66)	$p = 0.539$	Regular use of herbicides
(Kuopio, 1999)	Finland	25/123	34/246	1.71 (0.90-3.23)	$p = 0.101$	Occasional use of herbicides
		41/123	71/246	1.40 (0.79-2.48)	$p = 0.245$	Regular and occesional use of herbicides
(Taylor, 1999)	USA	NA/140	NA/147	1.06 (0.68-1.65)	$p = 0.81$	
(Behari, 2001)	India	20/377	40/377		$p = 0.01$ NS	Chi square = 6.67
(Engel, 2001)*	USA	39/65				PR = 0.9 (0.6-1.3)
		9/156	8/241	1.41 (0.51-3.88)	NS	♂/Occupational exposure
(Firestone, 2005)	USA	116/250	175/388	1.09 (0.77-1.53)	NS	Home-based exposure
		2/156	2/241	1.67 (0.22-12.76)	NS	♂/Paraquat
(Frigerio., 2006)*	USA	7/90	5/78	1.2 (0.4-3.9)	$p = 0.8$	♂
(Brighina, 2008)*	USA	NA/833	NA/833	1.25 (0.94-1.66)	$p = 0.12$	
(Dhillon, 2008)*	USA	23/100	22/84	0.8 (0.4-1.7)	$p = 0.616$	
		15/319	8/296	2.07 (0.69-6.23)		Chlorophenoxy acid/ester
(Hancock, 2008)*	USA	57/319	40/296	1.53 (0.92-2.53)		Phosphonoglycine
		5/319	7/296	1.08 (0.32-3.59)		Triazine
		149/368	152/341	1.01 (0.71-1.43)		Exposure 1974-1999/Paraquat
(Costello, 2009)*	USA	3/368	1/341	3.04 (0.30-30.86)		Exposure 1974-1999/Maneb
		88/368	49/341	1.75 (1.13-2.73)		Exposure 1974-1999/Paraquat+Maneb
		NA/118	NA/291	1.4 (0.7-2.6)	$p = 0.35$	♂
(Elbaz, 2009)*	France	NA/106	NA/266	1.2 (0.4-3.8)	$p = 0.72$	♀
(Gatto, 2009)*	USA	79/368	60/341	1.10 (0.75-1.63)		Paraquat
(Ritz, 2009)	USA	38/324	15/324	2.80 (1.52-5.25)		Paraquat+Maneb
		16/519	7/511	2.59 (1.03-6.48)	$p = 0.04$	2,4-D
(Tanner, 2009)	Canada/USA	9/519	4/511	2.80 (0.81-9.72)	$p = 0.10$	Paraquat
		1/519	1/511	1.02 (0.06-16.60)	$p = 0.99$	Diquat

	Study	Country	Exposed/ Total cases	Exposed/ Total controls	OR (95% CI)	*p* value	Specifications
Insecticides	(Stern, 1991)	USA	130/149	136/149	0.5 (0.2-1.1)	$p = 0.10$	
			NA/149	NA/149	0.6 (0.2-1.7)	NS	Early onset
			NA/149	NA/149	0.8 (0.3-2.1)	NS	Late onset
	(Semchuck, 1992)	Canada	NA/130	NA/260	2.05 (1.03-4.07)	$p = 0.042$	Insecticide use
					1.49 (0.58-3.81)	NS	16-25 years of insecticide use
					2.33 (0.78-6.94)	NS	26-35 years of insecticide use
					1.75 (0.63-4.83)	NS	36-45 years of insecticide use
					3.50 (1.03-11.96)	$p = 0.040$	46-55 years of insecticide use
	(Butterfield, 1993)	USA	24/63	8/68	5.75	$p < 0.001$	
	(Hertzman, 1994)*	Canada	53/71	65/80	0.62 (0.28-1.38)		♂/Vs. controls with cardiac disease
				54/60	0.33 (0.12-0.90)		♂/Vs. controls randomly selected
			29/56	24/41	0.65 (0.27-1.57)		♀/Vs. controls with cardiac disease
				47/64	0.41 (0.19-0.88)		♀/Vs. controls randomly selected
	(Seidler, 1996)	Germany	46/380	38/379	1.4 (0.9-2.1)		Dose-years=1-40/Vs. Neighboring controls
			70/380	55/376	1.8 (1.1-2.7)		Dose-years=1-40/Vs. Regional controls
			21/380	16/379	1.5 (0.9-2.5)		Dose-years=41-80/Vs. Neighboring controls
			46/380	25/376	2.5 (1.4-4.5)		Dose-years=41-80/Vs. Regional controls
			26/380	24/379	1.6 (0.07-3.4)		Dose-years>80/Vs. Neighboring controls
			21/380	14/376	2.1 (0.9-4.8)		Dose-years>80/Vs. Regional controls
	(Gorell, 1998)	USA	NA/144	19/464	3.15 (1.54-6.49)	$p = 0.002$	
	(Fall, 1999)	Sweden	5/NA	7/NA	2.2 (0.48-9.0)	$p = 0.40$	♂/Handling pesticides within agriculture
	(Behari, 2001)	India	NA/377	NA/377		$p = 0.169$	Chi square = 1.89
	(Engel, 2001)*	USA	51/65				PR = 0.9 (0.6-1.5)
	(Firestone, 2005)	USA	15/156	25/241	0.88 (0.44-1.76)	NS	♂/Occupational exposure
			141/250	236/388	0.82 (0.58-1.14)	NS	Home-based exposure
			5/156	1/241	8.08 (0.92-70.85)	NS	♂/Parathion
			6/156	9/241	1.04 (0.35-3.06)	NS	♂/Malathion
			8/156	10/241	1.67 (0.22-12.76)	NS	♂/Diazinon
	(Frigerio, 2006)*	USA	8/90	3/78	2.5 (0.6-9.8)	$p = 0.2$	♂
	(Brighina, 2008)*	USA	NA/833	NA/833	0.95 (0.74-1.22)	$p = 0.69$	
	(Dhillon, 2008)*	USA	27/100	3/84	10.0 (2.9-34.3)	$p < 0.001$	Rotenone
	(Hancock, 2008)*	USA	7/319	1/296	5.93 (0.63-56.10)		Botanical insecticides
			38/319	32/296	1.31 (0.75-2.28)		*N*-Methyl carbamate
			53/319	21/296	1.89 (1.11-3.25)		Organophosphorus
	(Elbaz, 2009)*	France	NA/118	NA/291	2.2 (1.1-4.3)	$p = 0.03$	♂
			NA/106	NA/266	1.4 (0.5-3.8)	$p = 0.49$	♀

	Study	Country	Exposed/ Total cases	Exposed/ Total controls	OR (95% CI)	*p* value	Specifications
	(Gatto, 2009)*	USA	73/368	41/341	1.58 (1.03-2.43)		Diazinon
			78/368	51/341	1.41 (0.94-2.11)		Dimethoate
			78/368	53/341	1.28 (0.85-1.91)		Methomyl
			67/368	41/341	1.45 (0.94-2.24)		Chlorpyrifos
			77/368	53/341	1.31 (0.88-1.96)		Propargite
	(Tanner, 2009)	Canada/USA	7/519	2/511	3.21 (0.65-15.80)	$p = 0.15$	Permethrin
			3/519	2/511	1.30 (0.21-7.94)	$p = 0.77$	Dieldrin
			1/519	1/511	0.82 (0.05-13.34)	$p = 0.89$	Rotenone
	(Firestone, 2010)	USA	5/252	1/326	5.8 (0.66-50.79)	NS	♂/Parathion
			10/252	12/326	1.0 (0.39-2.30)	NS	♂/Malathion
			7/252	11/326	0.8 (0.30-2.15)	NS	♂/Diazinon
	(Manthripragada, 2010)*	USA	125/351	89/363	1.55 (1.05-2.30)		Diazinon
			88/351	74/363	1.56 (1.02-2.40)		Chloropyrifos
			90/351	83/363	0.98 (0.65-1.48)		Parathion
Organochlorines	(Hertzman, 1994)*	Canada	29/71	33/80	0.89 (0.45-1.76)		♂/Vs. controls with cardiac disease
				28/60	0.80 (0.40-1.63)		♂/Vs. controls randomly selected
			16/56	12/41	0.75 (0.34-1.64)		♀/Vs. controls with cardiac disease
				23/64	0.75 (0.29-1.96)		♀/Vs. controls randomly selected
	(Seidler, 1996)	Germany	7/380	5/379	1.6 (0.4-6.2)		Vs. Neighboring controls
				2/376	5.8 (1.1-30.4)		Vs. Regional controls
	(Kuopio, 1999)	Finland	54/123	53/246	1.04 (0.68-1.60)	$p = 0.855$	DDT
	(Engel, 2001)*	USA	45/65				PR = 0.8 (0.5-1.3)
	(Hancock, 2008)*	USA	42/319	21/296	1.99 (1.09-3.64)		
	(Elbaz, 2009)*	France	NA/118	NA/291	1.9 (1.1-3.5)	$p < 0.05$	♂
			NA/118	NA/291	3.0 (1.2-7.9)	$p < 0.05$	♂/> 65 years at onset
	(Dutheil, 2010)	France	42/101	71/234	2.2 (1.1-4.5)	$p = 0.02$	♂
	(Firestone, 2010)	USA	14/252	22/326	0.8 (0.40-1.64)	NS	♂/DDT

* Summary of selected data only

Abbreviations: 2,4-D: 2,4-Dichlorophenoxyacetic acid
DDT: dichlorodiphenyltrichloroethane
NA: Not available
NS: Not significant
PD: Parkinson's disease
OR: Odds ratio
PR: Prevalence ratio
RR: Relative risk

Table 2. Summary of case-control studies investigating pesticide exposure and the risk of developing Parkinson's disease

Study	Country	Carriers/ Exposed cases	Carriers/ Exposed controls	OR (95% CI)	*p* value	Specifications
(Menegon, 1998)	Australia	32/39	12/26		$p = 0.009$	*GSTP1* (*AB*, *BB*, *AC*)
(Kedala, 2006)	USA	14/47	23/55	1.63 (0.52-5.15)		*DAT*, 1 risk allele
		26/47	15/55	5.66 (1.73-18.53)	S	*DAT*, 2 or more risk alleles
(Wilk, 2006)*	USA	104/278			$p = 0.04$	*GSTP1* SNP *rs749174*
					$p = 0.04$	*GSTP1* SNP *rs1871042*
					$p = 0.009$	*GSTP1* SNP *rs947895*
(Dick, 2007)*	Scotland, Italy, Sweden, Romania and Malta				NS	*CYP2D6, PON1, GSTM1,GSTT1, GSTM3, GSTP1, NQO1, CYP1B1, NAT2* analyzed
(Fong, 2007)*	Taiwan	31/153	18/155	2.49 (1.18-5.26)	$p = 0.0072$	*MnSOD C* allele
		55/153	41/155	2.42 (1.16-4.76)	$p = 0.0089$	*NQO1 T* allele
		20/153	11/155	4.09 (1.34-10.64)	$p = 0.0052$	combined *MnSOD* (*T/C* and *C/C*)/*NQO1* (*C/T* and *T/T*)
(Brighina, 2008)*	USA	NA	NA	1.18 (1.02-1.37)	$p = 0.03$	Rep1 score (α-synuclein gene (*SNCA*))
				1.28 (0.95-1.72)	$p = 0.10$	Herbicides
				0.91 (0.69-1.19)	$p = 0.49$	Insecticides
				0.92 (0.46-1.84)	$p = 0.81$	Fungicides
						No significant pairwise interaction (multivariate analysis)
(Ritz, 2009)	USA	10/38	4/15	2.99 (0.88-10.21)	p for trends =	*DAT*, 1 risk allele, Paraquat+Maneb
		24/38	6/15	4.53 (1.70-12.09)	0.006	*DAT*, 2 or more risk alleles, Paraquat+Maneb
		28/77	18/53	2.00 (0.71-5.67)	p for trends =	*DAT*, 1 risk allele, pesticides
		36/77	17/53	2.83 (1.01-7.92)	0.05	*DAT*, 2 or more risk alleles, pesticides
(Zschiedrich, 2009)	Serbia, Germany	17/19		4.74 (1.01-22.31)	$p = 0.047$	*c.3435C/T* SNP of the *ABCB1* gene
(Dutheil, 2010)	France			5.4 (1.1-27.5)	$p = 0.04$	Carriers of 2 variant G2677 (A,T) alleles (ABCB1 gene) and organochlorines
				4.1 (1.0-17.0)	$p = 0.05$	Carriers of 2 variant *C3435T* alleles (*ABCB1* gene) and organochlorines
(Kiyohara, 2010)*	Japan	187/NA	194/NA	0.77 (0.39-1.52)	$p = 0.449$	*GSTO1 rs4925 Ala/Ala* genotype (*GST* gene)
		151/NA	75/NA	1.96 (0.51-7.46)	$p = 0.323$	*GSTO1 rs4925 Ala/Asp+Asp/Asp* genotype (*GST* gene)
(Manthripragada, 2010)*	USA	48/32	35/15	2.24 (1.12-4.48)		*PON1-55 MM*/Diazinon
		48/27	35/13	2.61 (1.25-5.44)		*PON1-55 MM*/Chloropyrifos
		48/20	35/14	1.21 (0.57-2.60)		*PON1-55 MM*/Parathion

* Summary of selected data only

Abbreviations: DAT: Dopamine transporter; GST: glutathione-S transferase; NA: Not available; NS: Not significant; S: significant; SNP: single-nucleotide polymorphisms

Table 3. Summary of studies investigating genetic vulnerability, pesticide exposure and the risk of developing Parkinson's disease

5. References

Alam M, Danysz W, Schmidt WJ, Dekundy A. 2009. Effects of glutamate and alpha2-noradrenergic receptor antagonists on the development of neurotoxicity produced by chronic rotenone in rats. Toxicol Appl Pharmacol 240(2):198-207.

Alam M, Mayerhofer A, Schmidt WJ. 2004. The neurobehavioral changes induced by bilateral rotenone lesion in medial forebrain bundle of rats are reversed by L-DOPA. Behav Brain Res 151(1-2):117-24.

Alam M, Schmidt WJ. 2004. L-DOPA reverses the hypokinetic behaviour and rigidity in rotenone-treated rats. Behav Brain Res 153(2):439-46.

Ascherio A, Chen H, Weisskopf MG, O'Reilly E, McCullough ML, Calle EE, Schwarzschild MA, Thun MJ. 2006. Pesticide exposure and risk for Parkinson's disease. Ann Neurol 60(2):197-203.

Autor AP. 1977. Biochemical Mechanisms of paraquat Toxicity. New York: Academic Press. 240 p.

Bain LJ, LeBlanc GA. 1996. Interaction of structurally diverse pesticides with the human MDR1 gene product P-glycoprotein. Toxicol Appl Pharmacol 141(1):288-98.

Baldereschi M, Di Carlo A, Vanni P, Ghetti A, Carbonin P, Amaducci L, Inzitari D. 2003. Lifestyle-related risk factors for Parkinson's disease: a population-based study. Acta Neurol Scand 108(4):239-44.

Baldi I, Cantagrel A, Lebailly P, Tison F, Dubroca B, Chrysostome V, Dartigues JF, Brochard P. 2003a. Association between Parkinson's disease and exposure to pesticides in southwestern France. Neuroepidemiology 22(5):305-10.

Baldi I, Lebailly P, Mohammed-Brahim B, Letenneur L, Dartigues JF, Brochard P. 2003b. Neurodegenerative diseases and exposure to pesticides in the elderly. Am J Epidemiol 157(5):409-14.

Barbeau A. 1984. Manganese and extrapyramidal disorders (a critical review and tribute to Dr. George C. Cotzias). Neurotoxicology 5(1):13-35.

Barbeau A, Roy M, Bernier G, Campanella G, Paris S. 1987. Ecogenetics of Parkinson's disease: prevalence and environmental aspects in rural areas. Can J Neurol Sci 14(1):36-41.

Barlow BK, Richfield EK, Cory-Slechta DA, Thiruchelvam M. 2004. A fetal risk factor for Parkinson's disease. Dev Neurosci 26(1):11-23.

Behari M, Srivastava AK, Das RR, Pandey RM. 2001. Risk factors of Parkinson's disease in Indian patients. J Neurol Sci 190(1-2):49-55.

Betarbet R, Canet-Aviles RM, Sherer TB, Mastroberardino PG, McLendon C, Kim JH, Lund S, Na HM, Taylor G, Bence NF et al. 2006. Intersecting pathways to neurodegeneration in Parkinson's disease: effects of the pesticide rotenone on DJ-1, alpha-synuclein, and the ubiquitin-proteasome system. Neurobiol Dis 22(2):404-20.

Betarbet R, Sherer TB, MacKenzie G, Garcia-Osuna M, Panov AV, Greenamyre JT. 2000. Chronic systemic pesticide exposure reproduces features of Parkinson's disease. Nat Neurosci 3(12):1301-6.

Braak H, Del Tredici K, Rub U, de Vos RA, Jansen Steur EN, Braak E. 2003. Staging of brain pathology related to sporadic Parkinson's disease. Neurobiol Aging 24(2):197-211.

Braak H, Ghebremedhin E, Rub U, Bratzke H, Del Tredici K. 2004. Stages in the development of Parkinson's disease-related pathology. Cell Tissue Res 318(1):121-34.

Brighina L, Frigerio R, Schneider NK, Lesnick TG, de Andrade M, Cunningham JM, Farrer MJ, Lincoln SJ, Checkoway H, Rocca WA et al. 2008. Alpha-synuclein, pesticides, and Parkinson disease: a case-control study. Neurology 70(16 Pt 2):1461-9.

Brooks A, Chadwick C, Gelbard H, Cory-Slechta DA, Federoff H. 1999. Paraquat elicited neurobehavioral syndrome caused by dopminergic neuron loss. Brain Res 823:1-10.

Bueler H. 2009. Impaired mitochondrial dynamics and function in the pathogenesis of Parkinson's disease. Exp Neurol 218(2):235-46.

Bus JS, Aust SD, Gibson JE. 1974. Superoxide- and singlet oxygen-catalyzed lipid peroxidation as a possible mechanism for paraquat (methyl viologen) toxicity. Biochem Biophys Res Commun 58(3):749-55.

Butterfield PG, Valanis BG, Spencer PS, Lindeman CA, Nutt JG. 1993. Environmental antecedents of young-onset Parkinson's disease. Neurology 43(6):1150-8.

Cannon JR, Tapias V, Na HM, Honick AS, Drolet RE, Greenamyre JT. 2009. A highly reproducible rotenone model of Parkinson's disease. Neurobiol Dis 34(2):279-90.

Carvey PM, Chang Q, Lipton JW, Ling Z. 2003. Prenatal exposure to the bacteriotoxin lipopolysaccharide leads to long-term losses of dopamine neurons in offspring: a potential, new model of Parkinson's disease. Front Biosci 8:s826-37.

Caudle WM, Richardson JR, Wang M, Miller GW. 2005. Perinatal heptachlor exposure increases expression of presynaptic dopaminergic markers in mouse striatum. Neurotoxicology 26(4):721-8.

Chan DK, Woo J, Ho SC, Pang CP, Law LK, Ng PW, Hung WT, Kwok T, Hui E, Orr K et al. 1998. Genetic and environmental risk factors for Parkinson's disease in a Chinese population. J Neurol Neurosurg Psychiatry 65(5):781-4.

Chen S, Wu K, Knox R. 2000. Structure-function studies of DT-diaphorase (NQO1) and NRH: quinone oxidoreductase (NQO2). Free Radic Biol Med 29(3-4):276-84.

Choi WS, Kruse SE, Palmiter RD, Xia Z. 2008. Mitochondrial complex I inhibition is not required for dopaminergic neuron death induced by rotenone, MPP+, or paraquat. Proc Natl Acad Sci U S A 105(39):15136-41.

Chou AP, Li S, Fitzmaurice AG, Bronstein JM. 2010. Mechanisms of rotenone-induced proteasome inhibition. Neurotoxicology 31(4):367-72.

Cicchetti F, Drouin-Ouellet J, Gross RE. 2009. Environmental toxins and Parkinson's disease: what have we learned from pesticide-induced animal models? Trends Pharmacol Sci 30(9):475-83.

Cicchetti F, Lapointe N, Roberge-Tremblay A, Saint-Pierre M, Jimenez L, Ficke BW, Gross RE. 2005. Systemic exposure to paraquat and maneb models early Parkinson's disease in young adult rats. Neurobiol Dis 20(2):360-71.

Corrigan FM, Murray L, Wyatt CL, Shore RF. 1998. Diorthosubstituted polychlorinated biphenyls in caudate nucleus in Parkinson's disease. Exp Neurol 150(2):339-42.

Corrigan FM, Wienburg CL, Shore RF, Daniel SE, Mann D. 2000. Organochlorine insecticides in substantia nigra in Parkinson's disease. J Toxicol Environ Health A 59(4):229-34.

Costa LG, Cole TB, Jarvik GP, Furlong CE. 2003. Functional genomic of the paraoxonase (PON1) polymorphisms: effects on pesticide sensitivity, cardiovascular disease, and drug metabolism. Annu Rev Med 54:371-92.

Costello S, Cockburn M, Bronstein J, Zhang X, Ritz B. 2009. Parkinson's disease and residential exposure to maneb and paraquat from agricultural applications in the central valley of California. Am J Epidemiol 169(8):919-26.

Dauer W, Przedborski S. 2003. Parkinson's disease: mechanisms and models. Neuron 39(6):889-909.

De Michele G, Filla A, Volpe G, Gogliettino A, Ambrosio G, Campanella G. 1996. Etiology of Parkinson's disease. The role of environment and heredity. Adv Neurol 69:19-24.

Dhillon AS, Tarbutton GL, Levin JL, Plotkin GM, Lowry LK, Nalbone JT, Shepherd S. 2008. Pesticide/environmental exposures and Parkinson's disease in East Texas. J Agromedicine 13(1):37-48.

Di Ilio C, Sacchetta P, Iannarelli V, Aceto A. 1995. Binding of pesticides to alpha, mu and pi class glutathione transferase. Toxicol Lett 76(2):173-7.

Di Monte DA, Lavasani M, Manning-Bog AB. 2002. Environmental factors in Parkinson's disease. Neurotoxicology 23:487-502.

Dick FD, De Palma G, Ahmadi A, Osborne A, Scott NW, Prescott GJ, Bennett J, Semple S, Dick S, Mozzoni P et al. 2007. Gene-environment interactions in parkinsonism and Parkinson's disease: the Geoparkinson study. Occup Environ Med 64(10):673-80.

Drouin-Ouellet J, Lafontaine-Lacasse M, Saint-Pierre M, Cicchetti F. 2007. Short-term effects of paraquat and maneb treatment in mice: evidence of brain and lung injuries. J Environ Neurosci Biomed 1:78-90.

Dutheil F, Beaune P, Tzourio C, Loriot MA, Elbaz A. 2010. Interaction between ABCB1 and professional exposure to organochlorine insecticides in Parkinson disease. Arch Neurol 67(6):739-45.

Duzcan F, Zencir M, Ozdemir F, Cetin GO, Bagci H, Heutink P, Bonifati V, Sahiner T. 2003. Familial influence on parkinsonism in a rural area of Turkey (Kizilcaboluk-Denizli): a community-based case-control study. Mov Disord 18(7):799-804.

Elbaz A, Clavel J, Rathouz PJ, Moisan F, Galanaud JP, Delemotte B, Alperovitch A, Tzourio C. 2009. Professional exposure to pesticides and Parkinson disease. Ann Neurol 66(4):494-504.

Engel LS, Checkoway H, Keifer MC, Seixas NS, Longstreth WT, Jr., Scott KC, Hudnell K, Anger WK, Camicioli R. 2001a. Parkinsonism and occupational exposure to pesticides. Occup Environ Med 58(9):582-9.

Engel LS, Seixas NS, Keifer MC, Longstreth WT, Jr., Checkoway H. 2001b. Validity study of self-reported pesticide exposure among orchardists. J Expo Anal Environ Epidemiol 11(5):359-68.

Fall PA, Fredrikson M, Axelson O, Granerus AK. 1999. Nutritional and occupational factors influencing the risk of Parkinson's disease: a case-control study in southeastern Sweden. Mov Disord 14(1):28-37.

Firestone JA, Lundin JI, Powers KM, Smith-Weller T, Franklin GM, Swanson PD, Longstreth WT, Jr., Checkoway H. 2010. Occupational factors and risk of Parkinson's disease: A population-based case-control study. Am J Ind Med 53(3):217-23.

Firestone JA, Smith-Weller T, Franklin G, Swanson P, Longstreth WT, Jr., Checkoway H. 2005. Pesticides and risk of Parkinson disease: a population-based case-control study. Arch Neurol 62(1):91-5.

Fleming L, Mann JB, Bean J, Briggle T, Sanchez-Ramos JR. 1994. Parkinson's disease and brain levels of organochlorine pesticides. Ann Neurol 36(1):100-3.

Fong CS, Wu RM, Shieh JC, Chao YT, Fu YP, Kuao CL, Cheng CW. 2007. Pesticide exposure on southwestern Taiwanese with MnSOD and NQO1 polymorphisms is associated with increased risk of Parkinson's disease. Clin Chim Acta 378(1-2):136-41.

Fredriksson A, Fredriksson M, Eriksson P. 1993. Neonatal exposure to paraquat or MPTP induces permanent changes in striatum dopamine and behavior in adult mice. Toxicol Appl Pharmacol 122(2):258-64.

Frigerio R, Sanft KR, Grossardt BR, Peterson BJ, Elbaz A, Bower JH, Ahlskog JE, de Andrade M, Maraganore DM, Rocca WA. 2006. Chemical exposures and Parkinson's disease: a population-based case-control study. Mov Disord 21(10):1688-92.

Gatto NM, Cockburn M, Bronstein J, Manthripragada AD, Ritz B. 2009. Well-water consumption and Parkinson's disease in rural California. Environ Health Perspect 117(12):1912-8.

George S, Mok SS, Nurjono M, Ayton S, Finkelstein DI, Masters CL, Li QX, Culvenor JG. 2010. alpha-Synuclein Transgenic Mice Reveal Compensatory Increases in Parkinson's Disease-Associated Proteins DJ-1 and Parkin and Have Enhanced alpha-Synuclein and PINK1 Levels After Rotenone Treatment. J Mol Neurosci 42(2):243-54.

Godeiro C, Jr., Aguiar PM, Felicio AC, Barsottini OG, Silva SM, Borges V, Andrade LA, Ferraz HB. 2010. PINK1 polymorphism IVS1-7 A-->G, exposure to environmental risk factors and anticipation of disease onset in Brazilian patients with early-onset Parkinson's Disease. Neurosci Lett 469(1):155-8.

Golbe LI, Farrell TM, Davis PH. 1990. Follow-up study of early-life protective and risk factors in Parkinson's disease. Mov Disord 5(1):66-70.

Gorell JM, Johnson CC, Rybicki BA, Peterson EL, Richardson RJ. 1998. The risk of Parkinson's disease with exposure to pesticides, farming, well water, and rural living. Neurology 50(5):1346-50.

Gorell JM, Peterson EL, Rybicki BA, Johnson CC. 2004. Multiple risk factors for Parkinson's disease. J Neurol Sci 217(2):169-74.

Hancock DB, Martin ER, Mayhew GM, Stajich JM, Jewett R, Stacy MA, Scott BL, Vance JM, Scott WK. 2008. Pesticide exposure and risk of Parkinson's disease: a family-based case-control study. BMC Neurol 8:6.

Harris MK, Shneyder N, Borazanci A, Korniychuk E, Kelley RE, Minagar A. 2009. Movement disorders. Med Clin North Am 93(2):371-88, viii.

Hatcher JM, Delea KC, Richardson JR, Pennell KD, Miller GW. 2008. Disruption of dopamine transport by DDT and its metabolites. Neurotoxicology 29(4):682-90.

Hatcher JM, Richardson JR, Guillot TS, McCormack AL, Di Monte DA, Jones DP, Pennell KD, Miller GW. 2007. Dieldrin exposure induces oxidative damage in the mouse nigrostriatal dopamine system. Exp Neurol 204(2):619-30.

Herishanu YO, Medvedovski M, Goldsmith JR, Kordysh E. 2001. A case-control study of Parkinson's disease in urban population of southern Israel. Can J Neurol Sci 28(2):144-7.

Hertzman C, Wiens M, Bowering D, Snow B, Calne D. 1990. Parkinson's disease: a case-control study of occupational and environmental risk factors. Am J Ind Med 17(3):349-55.

Hertzman C, Wiens M, Snow B, Kelly S, Calne D. 1994. A case-control study of Parkinson's disease in a horticultural region of British Columbia. Mov Disord 9(1):69-75.

Ho SC, Woo J, Lee CM. 1989. Epidemiologic study of Parkinson's disease in Hong Kong. Neurology 39(10):1314-8.

Hoglinger GU, Lannuzel A, Khondiker ME, Michel PP, Duyckaerts C, Feger J, Champy P, Prigent A, Medja F, Lombes A et al. 2005. The mitochondrial complex I inhibitor rotenone triggers a cerebral tauopathy. J Neurochem 95(4):930-9.

Inden M, Kitamura Y, Takeuchi H, Yanagida T, Takata K, Kobayashi Y, Taniguchi T, Yoshimoto K, Kaneko M, Okuma Y et al. 2007. Neurodegeneration of mouse nigrostriatal dopaminergic system induced by repeated oral administration of rotenone is prevented by 4-phenylbutyrate, a chemical chaperone. J Neurochem 101(6):1491-1504.

Inden M, Kitamura Y, Tamaki A, Yanagida T, Shibaike T, Yamamoto A, Takata K, Yasui H, Taira T, Ariga H et al. 2009. Neuroprotective effect of the antiparkinsonian drug pramipexole against nigrostriatal dopaminergic degeneration in rotenone-treated mice. Neurochem Int 55(8):760-7.

Jia Z, Misra HP. 2007. Developmental exposure to pesticides zineb and/or endosulfan renders the nigrostriatal dopamine system more susceptible to these environmental chemicals later in life. Neurotoxicology 28(4):727-35.

Jimenez-Jimenez FJ, Mateo D, Gimenez-Roldan S. 1992. Exposure to well water and pesticides in Parkinson's disease: a case-control study in the Madrid area. Mov Disord 7(2):149-52.

Jones GM, Vale JA. 2000. Mechanisms of toxicity, clinical features, and management of diquat poisoning: a review. J Toxicol Clin Toxicol 38(2):123-8.

Kamel F, Tanner C, Umbach D, Hoppin J, Alavanja M, Blair A, Comyns K, Goldman S, Korell M, Langston J et al. 2007. Pesticide exposure and self-reported Parkinson's disease in the agricultural health study. Am J Epidemiol 165(4):364-74.

Kang MJ, Gil SJ, Koh HC. 2009. Paraquat induces alternation of the dopamine catabolic pathways and glutathione levels in the substantia nigra of mice. Toxicol Lett 188(2):148-52.

Kelada SN, Checkoway H, Kardia SL, Carlson CS, Costa-Mallen P, Eaton DL, Firestone J, Powers KM, Swanson PD, Franklin GM et al. 2006. 5' and 3' region variability in the dopamine transporter gene (SLC6A3), pesticide exposure and Parkinson's disease risk: a hypothesis-generating study. Hum Mol Genet 15(20):3055-62.

Kiyohara C, Miyake Y, Koyanagi M, Fujimoto T, Shirasawa S, Tanaka K, Fukushima W, Sasaki S, Tsuboi Y, Yamada T et al. 2010. GST polymorphisms, interaction with smoking and pesticide use, and risk for Parkinson's disease in a Japanese population. Parkinsonism Relat Disord 16(7):447-52.

Koller W, Vetere-Overfield B, Gray C, Alexander C, Chin T, Dolezal J, Hassanein R, Tanner C. 1990. Environmental risk factors in Parkinson's disease. Neurology 40(8):1218-21.

Kuopio AM, Marttila RJ, Helenius H, Rinne UK. 1999. Environmental risk factors in Parkinson's disease. Mov Disord 14(6):928-39.

Kuter K, Smialowska M, Wieronska J, Zieba B, Wardas J, Pietraszek M, Nowak P, Biedka I, Roczniak W, Konieczny J et al. 2007. Toxic influence of subchronic paraquat administration on dopaminergic neurons in rats. Brain Res 1155:196-207.

Langston JW, Ballard P, Tetrud JW, Irwin I. 1983. Chronic Parkinsonism in humans due to a product of meperidine-analog synthesis. Science 219(4587):979-80.

Lapointe N, St-Hilaire M, Martinoli MG, Blanchet J, Gould P, Rouillard C, Cicchetti F. 2004. Rotenone induces non-specific central nervous system and systemic toxicity. Faseb J 18(6):717-9.

Lerner A, Bagic A. 2008. Olfactory pathogenesis of idiopathic Parkinson disease revisited. Mov Disord 23(8):1076-84.

Lewis SJ, Barker RA. 2009. Understanding the dopaminergic deficits in Parkinson's disease: insights into disease heterogeneity. J Clin Neurosci 16(5):620-5.

Li X, Yin J, Cheng CM, Sun JL, Li Z, Wu YL. 2005. Paraquat induces selective dopaminergic nigrostriatal degeneration in aging C57BL/6 mice. Chin Med J (Engl) 118(16):1357-61.

Ling Z, Gayle DA, Ma SY, Lipton JW, Tong CW, Hong JS, Carvey PM. 2002. In utero bacterial endotoxin exposure causes loss of tyrosine hydroxylase neurons in the postnatal rat midbrain. Mov Disord 17(1):116-24.

Liou HH, Tsai MC, Chen CJ, Jeng JS, Chang YC, Chen SY, Chen RC. 1997. Environmental risk factors and Parkinson's disease: a case-control study in Taiwan. Neurology 48(6):1583-8.

Litvan I, Bhatia KP, Burn DJ, Goetz CG, Lang AE, McKeith I, Quinn N, Sethi KD, Shults C, Wenning GK. 2003. Movement Disorders Society Scientific Issues Committee report: SIC Task Force appraisal of clinical diagnostic criteria for Parkinsonian disorders. Mov Disord 18(5):467-86.

Luo C, Rajput AH, Akhtar S, Rajput A. 2007. Alpha-synuclein and tyrosine hydroxylase expression in acute rotenone toxicity. Int J Mol Med 19(3):517-21.

Manning-Bog AB, McCormack AL, Li J, Uversky VN, Fink AL, Di Monte DA. 2002. The herbicide paraquat causes up-regulation and aggregation of alpha-synuclein in mice: paraquat and alpha-synuclein. J Biol Chem 277(3):1641-4.

Manthripragada AD, Costello S, Cockburn MG, Bronstein JM, Ritz B. 2010. Paraoxonase 1, agricultural organophosphate exposure, and Parkinson disease. Epidemiology 21(1):87-94.

McCann SJ, LeCouteur DG, Green AC, Brayne C, Johnson AG, Chan D, McManus ME, Pond SM. 1998. The epidemiology of Parkinson's disease in an Australian population. Neuroepidemiology 17(6):310-7.

Menegon A, Board PG, Blackburn AC, Mellick GD, Le Couteur DG. 1998. Parkinson's disease, pesticides, and glutathione transferase polymorphisms. Lancet 352(9137):1344-6.

Miller GW, Kirby ML, Levey AI, Bloomquist JR. 1999. Heptachlor alters expression and function of dopamine transporters. Neurotoxicology 20(4):631-7.

Moghal S, Rajput AH, D'Arcy C, Rajput R. 1994. Prevalence of movement disorders in elderly community residents. Neuroepidemiology 13(4):175-8.

Monti B, Gatta V, Piretti F, Raffaelli SS, Virgili M, Contestabile A. 2009. Valproic Acid is Neuroprotective in the Rotenone Rat Model of Parkinson's Disease: Involvement of alpha-Synuclein. Neurotox Res.

Morano A, Jimenez-Jimenez FJ, Molina JA, Antolin MA. 1994. Risk-factors for Parkinson's disease: case-control study in the province of Caceres, Spain. Acta Neurol Scand 89(3):164-70.

Noonan CW, Reif JS, Yost M, Touchstone J. 2002. Occupational exposure to magnetic fields in case-referent studies of neurodegenerative diseases. Scand J Work Environ Health 28(1):42-8.

Norris EH, Uryu K, Leight S, Giasson BI, Trojanowski JQ, Lee VM. 2007. Pesticide exposure exacerbates alpha-synucleinopathy in an A53T transgenic mouse model. Am J Pathol 170(2):658-66.

Pan-Montojo F, Anichtchik O, Dening Y, Knels L, Pursche S, Jung R, Jackson S, Gille G, Spillantini MG, Reichmann H et al. 2010. Progression of Parkinson's disease pathology is reproduced by intragastric administration of rotenone in mice. PLoS One 5(1):e8762.

Park J, Yoo CI, Sim CS, Kim HK, Kim JW, Jeon BS, Kim KR, Bang OY, Lee WY, Yi Y et al. 2005. Occupations and Parkinson's disease: a multi-center case-control study in South Korea. Neurotoxicology 26(1):99-105.

Pasha MK, Sharma RK, Rajput AH. 2005. Increased myocardial N-myristoyltransferase activity in rotenone model of Parkinsonism. Int J Mol Med 15(6):987-91.

Peng J, Mao XO, Stevenson FF, Hsu M, Andersen JK. 2004. The herbicide paraquat induces dopaminergic nigral apoptosis through sustained activation of the JNK pathway. J Biol Chem 279(31):32626-32.

Petrovitch H, Ross GW, Abbott RD, Sanderson WT, Sharp DS, Tanner CM, Masaki KH, Blanchette PL, Popper JS, Foley D et al. 2002. Plantation work and risk of Parkinson disease in a population-based longitudinal study. Arch Neurol 59(11):1787-92.

Pittman JT, Dodd CA, Klein BG. 2003. Immunohistochemical changes in the mouse striatum induced by the pyrethroid insecticide permethrin. Int J Toxicol 22(5):359-70.

Preux PM, Condet A, Anglade C, Druet-Cabanac M, Debrock C, Macharia W, Couratier P, Boutros-Toni F, Dumas M. 2000. Parkinson's disease and environmental factors. Matched case-control study in the Limousin region, France. Neuroepidemiology 19(6):333-7.

Priyadarshi A, Khuder SA, Schaub EA, Shrivastava S. 2000. A meta-analysis of Parkinson's disease and exposure to pesticides. Neurotoxicology 21(4):435-40.

Richardson JR, Caudle WM, Wang M, Dean ED, Pennell KD, Miller GW. 2006. Developmental exposure to the pesticide dieldrin alters the dopamine system and increases neurotoxicity in an animal model of Parkinson's disease. Faseb J 20(10):1695-7.

Richardson JR, Caudle WM, Wang MZ, Dean ED, Pennell KD, Miller GW. 2008. Developmental heptachlor exposure increases susceptibility of dopamine neurons to N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)in a gender-specific manner. Neurotoxicology 29(5):855-63.

Richardson JR, Shalat SL, Buckley B, Winnik B, O'Suilleabhain P, Diaz-Arrastia R, Reisch J, German DC. 2009. Elevated serum pesticide levels and risk of Parkinson disease. Arch Neurol 66(7):870-5.

Richter F, Hamann M, Richter A. 2007. Chronic rotenone treatment induces behavioral effects but no pathological signs of parkinsonism in mice. J Neurosci Res 85(3):681-91.

Ritz B, Yu F. 2000. Parkinson's disease mortality and pesticide exposure in California 1984-1994. Int J Epidemiol 29(2):323-9.

Ritz BR, Manthripragada AD, Costello S, Lincoln SJ, Farrer MJ, Cockburn M, Bronstein J. 2009. Dopamine transporter genetic variants and pesticides in Parkinson's disease. Environ Health Perspect 117(6):964-9.

Rybicki BA, Johnson CC, Uman J, Gorell JM. 1993. Parkinson's disease mortality and the industrial use of heavy metals in Michigan. Mov Disord 8(1):87-92.

Saint-Pierre M, Tremblay ME, Sik A, Gross RE, Cicchetti F. 2006. Temporal effects of paraquat/maneb on microglial activation and dopamine neuronal loss in older rats. J Neurochem 98(3):760-72.

Schapira AH. 1996. Neurotoxicity and the mechanisms of cell death in Parkinson's disease. Adv Neurol 69:161-5.

Schuh RA, Richardson JR, Gupta RK, Flaws JA, Fiskum G. 2009. Effects of the organochlorine pesticide methoxychlor on dopamine metabolites and transporters in the mouse brain. Neurotoxicology 30(2):274-80.

Seidler A, Hellenbrand W, Robra BP, Vieregge P, Nischan P, Joerg J, Oertel WH, Ulm G, Schneider E. 1996. Possible environmental, occupational, and other etiologic factors for Parkinson's disease: a case-control study in Germany. Neurology 46(5):1275-84.

Selikhova M, Williams DR, Kempster PA, Holton JL, Revesz T, Lees AJ. 2009. A clinico-pathological study of subtypes in Parkinson's disease. Brain 132(Pt 11):2947-57.

Semchuk KM, Love EJ, Lee RG. 1992. Parkinson's disease and exposure to agricultural work and pesticide chemicals. Neurology 42(7):1328-35.

Sherer TB, Kim JH, Betarbet R, Greenamyre JT. 2003. Subcutaneous rotenone exposure causes highly selective dopaminergic degeneration and alpha-synuclein aggregation. Exp Neurol 179(1):9-16.

Shimizu K, Matsubara K, Ohtaki K, Fujimaru S, Saito O, Shiono H. 2003. Paraquat induces long-lasting dopamine overflow through the excitotoxic pathway in the striatum of freely moving rats. Brain Res 976(2):243-52.

Shimoda-Matsubayashi S, Hattori T, Matsumine H, Shinohara A, Yoritaka A, Mori H, Kondo T, Chiba M, Mizuno Y. 1997. Mn SOD activity and protein in a patient with chromosome 6-linked autosomal recessive parkinsonism in comparison with Parkinson's disease and control. Neurology 49(5):1257-62.

Smargiassi A, Mutti A, De Rosa A, De Palma G, Negrotti A, Calzetti S. 1998. A case-control study of occupational and environmental risk factors for Parkinson's disease in the Emilia-Romagna region of Italy. Neurotoxicology 19(4-5):709-12.

Somayajulu-Nitu M, Sandhu JK, Cohen J, Sikorska M, Sridhar TS, Matei A, Borowy-Borowski H, Pandey S. 2009. Paraquat induces oxidative stress, neuronal loss in substantia nigra region and Parkinsonism in adult rats: neuroprotection and amelioration of symptoms by water-soluble formulation of Coenzyme Q10. BMC Neurosci 10:88.

Stern M, Dulaney E, Gruber SB, Golbe L, Bergen M, Hurtig H, Gollomp S, Stolley P. 1991. The epidemiology of Parkinson's disease. A case-control study of young-onset and old-onset patients. Arch Neurol 48(9):903-7.

Takeuchi H, Yanagida T, Inden M, Takata K, Kitamura Y, Yamakawa K, Sawada H, Izumi Y, Yamamoto N, Kihara T et al. 2009. Nicotinic receptor stimulation protects nigral dopaminergic neurons in rotenone-induced Parkinson's disease models. J Neurosci Res 87(2):576-85.

Tanner CM. 1989. The role of environmental toxins in the etiology of Parkinson's disease. Trends Neurosci 12(2):49-54.

Tanner CM, Ottman R, Goldman SM, Ellenberg J, Chan P, Mayeux R, Langston JW. 1999. Parkinson disease in twins: an etiologic study. Jama 281(4):341-6.

Tanner CM, Ross GW, Jewell SA, Hauser RA, Jankovic J, Factor SA, Bressman S, Deligtisch A, Marras C, Lyons KE et al. 2009. Occupation and risk of parkinsonism: a multicenter case-control study. Arch Neurol 66(9):1106-13.

Tapias V, Cannon JR, Greenamyre JT. 2009. Melatonin treatment potentiates neurodegeneration in a rat rotenone Parkinson's disease model. J Neurosci Res.

Tawara T, Fukushima T, Hojo N, Isobe A, Shiwaku K, Setogawa T, Yamane Y. 1996. Effects of paraquat on mitochondrial electron transport system and catecholamine contents in rat brain. Arch Toxicol 70(9):585-9.

Thiffault C, Langston WJ, Di Monte DA. 2001. Acute exposure to organochlorine pesticides does not affect striatal dopamine in mice. Neurotox Res 3(6):537-43.

Thiruchelvam M, Brockel BJ, Richfield EK, Baggs RB, Cory-Slechta DA. 2000a. Potentiated and preferential effects of combined paraquat and maneb on nigrostriatal dopamine systems: environmental risk factors for Parkinson's disease? Brain Res 873(2):225-34.

Thiruchelvam M, McCormack A, Richfield EK, Baggs RB, Tank AW, Di Monte DA, Cory-Slechta DA. 2003a. Age-related irreversible progressive nigrostriatal dopaminergic neurotoxicity in the paraquat and maneb model of the Parkinson's disease phenotype. Eur J Neurosci 18(3):589-600.

Thiruchelvam M, McCormack AL, Richfield EK, Baggs RB, Tank AW, Di Monte DA, Cory-Slechta DA. 2003b. Age-related irreversible progressive nigrostriatal dopaminergic neurotoxicity in the paraquat and maneb model of Parkinson's disease phenotype. European Journal of Neuroscience 18:589-600.

Thiruchelvam M, Richfield EK, Baggs RB, Tank AW, Cory-Slechta DA. 2000b. The nigrostriatal dopaminergic system as a preferential target of repeated exposures to combined paraquat and maneb: implications for Parkinson's disease. J Neurosci 20(24):9207-14.

Thiruchelvam M, Richfield EK, Goodman BM, Baggs RB, Cory-Slechta DA. 2002. Developmental exposure to the pesticides paraquat and maneb and the Parkinson's disease phenotype. Neurotoxicology 23(4-5):621-33.

Thiruchelvam MJ, Powers JM, Cory-Slechta DA, Richfield EK. 2004. Risk factors for dopaminergic neuron loss in human alpha-synuclein transgenic mice. Eur J Neurosci 19(4):845-54.

Tsui JK, Calne DB, Wang Y, Schulzer M, Marion SA. 1999. Occupational risk factors in Parkinson's disease. Can J Public Health 90(5):334-7.

Vila M, Przedborski S. 2003. Targeting programmed cell death in neurodegenerative diseases. Nat Rev Neurosci 4(5):365-75.

Wang F, Feng X, Ma J, Zou H, Chan P. 2006a. A common A340T variant in PINK1 gene associated with late-onset Parkinson's disease in Chinese. Neurosci Lett 410(2):121-5.

Wang XF, Li S, Chou AP, Bronstein JM. 2006b. Inhibitory effects of pesticides on proteasome activity: implication in Parkinson's disease. Neurobiol Dis 23(1):198-205.

Waxman EA, Giasson BI. 2009. Molecular mechanisms of alpha-synuclein neurodegeneration. Biochim Biophys Acta 1792(7):616-24.

Weisskopf MG, Knekt P, O'Reilly EJ, Lyytinen J, Reunanen A, Laden F, Altshul L, Ascherio A. 2010. Persistent organochlorine pesticides in serum and risk of Parkinson disease. Neurology 74(13):1055-61.

Wilk JB, Tobin JE, Suchowersky O, Shill HA, Klein C, Wooten GF, Lew MF, Mark MH, Guttman M, Watts RL et al. 2006. Herbicide exposure modifies GSTP1 haplotype association to Parkinson onset age: the GenePD Study. Neurology 67(12):2206-10.

Wong GF, Gray CS, Hassanein RS, Koller WC. 1991. Environmental risk factors in siblings with Parkinson's disease. Arch Neurol 48(3):287-9.

Zarow C, Lyness SA, Mortimer JA, Chui HC. 2003. Neuronal loss is greater in the locus coeruleus than nucleus basalis and substantia nigra in Alzheimer and Parkinson diseases. Arch Neurol 60(3):337-41.

Zayed J, Ducic S, Campanella G, Panisset JC, Andre P, Masson H, Roy M. 1990. [Environmental factors in the etiology of Parkinson's disease]. Can J Neurol Sci 17(3):286-91.

Zorzon M, Capus L, Pellegrino A, Cazzato G, Zivadinov R. 2002. Familial and environmental risk factors in Parkinson's disease: a case-control study in north-east Italy. Acta Neurol Scand 105(2):77-82.

Zschiedrich K, Konig IR, Bruggemann N, Kock N, Kasten M, Leenders KL, Kostic V, Vieregge P, Ziegler A, Klein C et al. 2009. MDR1 variants and risk of Parkinson disease. Association with pesticide exposure? J Neurol 256(1):115-20.

7

Pesticides Exposure and Risk of Hypospadias

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1. Introduction

Hypospadias is a common congenital anomaly, the cause of which is still unknown. The term hypospadias is derived from the Greek prefix "hypo" meaning bellow and the term "spadon", meaning rent or defect (Dorland's Medical History, 1981).
The defect is readily observed, and hence it has been recognised since ancient times (Aristotle. Generation of animals, 1943). In both males and females the urethra is a perfect canal, but in the congenital anomaly of hypospadias, the canal becomes a gutter. This phenomenon is easily seen in males but not in females. In females anomalies of the urethral meatus are considered anomalies of the urogenital sinus (J.E. Skandalakis & S.W.Gray, 1994). The term "hypospadias" refers to an opening on the ventral side of the penis, while "epispadias" is an opening on the dorsal side. Hypospadias denotes a condition in which the urethra has failed to completely form and is often associated with a ventral curvature of the penis (chordee). Hypospadias is classified according to severity. The first degree is the mildest form, and the urethra opens on the anterior portion of the penis (glandular and subcoronal). The second degree is more severe and involves openings on the midshaft of the penis. The third degree is the most severe and involves posterior penile, penoscrotal, scrotal, and perineal openings. First-degree hypospadias accounts for approximately 50% of cases, second-degree for 30%, and third-degree for 20% (Duckett et al, 1996; Levitt SB & Reda, 1988).

2. Embryology of hypospadias

The development and elongation of the phallus occurs between weeks 8 and 14 of prenatal life under the influence of the androgens testosterone (T) and 5α dihydrotestosterone (DHT), which are synthesized in response to a surge of luteinizing hormone (LH) from the fetal pituitary (Moore KL, 1988; Bingol N & Wasserman, 1990).
Testosterone is a key hormonal agent that influences the undifferentiated fetal sex organs, including internal and external genitalia, to develop into the male phenotype. The placenta regulates the production of fetal testosterone by the stimulatory effect of human chorionic gonadotropin-(hCG)- on Leydig cells at an early stage of gestational age, including the critical 8th–12th weeks for male genitalia development (Winter *et al.,1977*).

The fetal pituitary begins to regulate the production of testosterone at midgestation by the secretion of luteinizing hormone (LH). The growth and development of the testis and accessory sex structures in the human, are unequivocally dependent upon the continuous support of the hypothalamo-hypophyseal-testicular axis (Grumbach, M.M., 1980; Claude Desjardins 1981).

At the end of the third month, the urethral folds close over the urethral plate to form the penile urethra. The distal part of the glanular urethra, develops by one of the following mechanisms (Gunha GR & Baskin LS., 2004). The classic theory states that the distal portion of the urethra develops as an in growth from the tip of the penis until it joins the proximal tubular urethra (Moore KL et al., 2003; Kurzrock EA et al. 1999) Recent evidence, however, suggests that the entire urethra from base to tip, is formed by continuous extension and fusion of the endodermal urethral groove (Hynes PJ & Fraher JP, 2004; Belman AB., 2002). The penile urethra forms as a result of the medial edges of the endodermal urethral folds fusing to form the median raphe (Baskin LS., 2000).

Undescended testes (cryptorchidism) and inguinal hernias are urogenital anomalies that are most commonly associated with hypospadias. In one series, 9.3% of hypospadias patients had undescended testes (Khuri FJ et al., 1981). There was a 5% incidence of undescended testes with first-degree, 6% with second-degree, and 32% with third-degree hypospadias. The overall occurrence of inguinal hernia associated with hypospadias is estimated to be 9%, but with third degree hypospadias the occurrence can be as high as 17% (Khuri FJ et al., 1981). Congenital anomalies not involving the urogenital tract have been found in 6.7% of all patients with hypospadias, and primarily involve the craniofacial, cardiothoracic, and gastrointestinal systems and the extremities. With increasing severity of hypospadias, the occurrence of associated non-urogenital anomalies can reach 12.7% (Latifoglu et al., 1998).

3. Epidemiology

Recent evidence shows an increase in the prevalence of hypospadias in Europe and US without certain aetiology (Paulozzi LJ et al., 1997; Paulozzi LJ, 1999). Classical textbooks of embryology and Paediatric Surgery report that the incidence of hypospadias is ranging from 1-8.2 per 1000 live male births (J.E. Skandalakis & S.W.Gray, 1994; Ashcraft Murphy, 2000; O'Neil, J, et al, 2006). Hypospadias is a relatively common congenital anomaly, with a birth prevalence ranging from 0.3% to 0.8% of Caucasian male live births in the United States, and 0.05% to 0.4% for other racial groups (Bingol N & Wasserman, 1990). A greater frequency of hypospadias has been found in Caucasians than in other races (Chavez GF et al., 1988; Gallentine ML et al., 2001). In the 1970s and 1980s, birth defect surveillance systems reported transient 1.5- to 2-fold increases in the prevalence of hypospadias in Norway, (Bjerkedal T & Bakketeig LS., 1975) Sweden, (Kallen B & Winberg J., 1982) Denmark, (Kallen B et al., 1986) England, (Matlai P & Beral V, 1985) and Hungary (Czeizel A., 1985).

An international study of hypospadias from seven birth defect surveillance programs (Denmark, Hungary, Italy, Mexico, South America, Spain and Sweden) included data from over 7,400 cases of nonsyndromic hypospadias (Kallen B et al., 1986). Differences in prevalence rates between countries were found that could not be explained completely by variations in case definition or different levels of ascertainment. The inclusion of less severe forms of hypospadias did not explain the higher rates in some programs compared to others. In some countries there was a 5–21% level of over estimation due to misdiagnosis of

normal infants. Counteracting this was a 30–64% under estimation among cases severe enough to require surgery. However, geographic variability in prevalence rates could not be explained solely on the basis of ascertainment problems.

Rates based on Swedish registry data from 1973–1974 (incomplete ascertainment) were 40% higher than rates from 1965–1968 based on hospital records and registry data (more complete ascertainment) (Kallen B & Winberg J., 1982). Consequently, hypospadias is difficult to assess in studies utilizing data from geographically diverse surveillance programs (Kallen B et al., 1986).

A Centers for Disease Control (CDC) study evaluated the birth prevalence of hypospadias in the United States from two birth defects surveillance systems: the Metropolitan Atlanta Congenital Defects Program (MACDP) and the Birth Defects Monitoring Program (BDMP) (Paulozzi LJ et al., 1999). The MACDP is a hospital-based registry that was initiated in 1968 and is based on case ascertainment in 22 hospitals and clinics in the Atlanta, Georgia area. The BDMP is a population-based registry that was initiated in 1970, in which diagnoses from newborn discharge summaries are collected from a nationwide sample of hospitals (Paulozzi LJ et al., 1999). The total hypospadias rate (mild and severe) doubled in the MACDP between 1968 and 1993 from approximately 1.5 to 3.0/1000 births, and the overall trend was statistically significant. The annual rate of increase was 2.9%, and the overall increase occurred at a rate of 1.4% per year among Caucasians and 5.7% per year among non-Caucasians. For severe hypospadias, the rate increased three- to five-fold (0.11–0.55/1000 births) from 1968 to 1993, and this trend was also statistically significant. Overall, however, approximately 60% of cases could not be classified by severity. In the BDMP, the total hypospadias rate also doubled (2.02 to 3.97/1000 births) from 1970 to 1993, and this trend was also statistically significant. The increase occurred nationwide, and was highest in the Southeast and lowest in the West (Paulozzi LJ et al., 1999).

The prevalence of hypospadias was assessed in The Netherlands by prospective examination of newborns in Rotterdam over a 2-year period. A total of 7292 consecutive male births were examined for the presence of hypospadias (excluding glandular cases), and rates were increased four-fold compared to earlier time periods studied (Pierik FH et al.,2002).

Consequently, comparable rates of increases in the birth prevalence of hypospadias have been seen in both the United States as well as in The Netherlands in studies utilizing population-based as well as hospital-based epidemiologic approaches (Paulozzi LJ et al., 1999). A recent study from Denmark reports a rate of hypospadias of about 1% in a population of 1072 boys studied at birth which increased to almost 5% when followed for 3 years (K.A.Boisen, et al, 2005).

On the other hand studies reporting prevalence rates of hypospadias in Washington State and California do not support the idea of increased prevalence during the periods of 1987-2002 and 1989-1997 respectively (Susan L. Carmichael et al., 2003; Michael P. Porter et al, 2005).

Reviewing the international literature on the epidemiology of hypospadias one cannot stress enough the difficulties that arise when comparing different studies with variations in inclusion criteria and even definition of the different forms of the disease. The differences in ascertainment and in the descriptive epidemiology of hypospadias pose a question as to whether the reported increase in prevalence by some authors is true. However increasing trends are being reported by recent careful studies concerning some populations (Michael Michalakis, et al. 2008; A.M.Tsatsakis et al., 2008).

4. Etiology

Reports of increasing prevalence of hypospadias have raised questions concerning aetiology, treatment, and prevention. To date, there is no comprehensive understanding of the aetiology of hypospadias that can inform primary prevention efforts and improve therapeutics. The aetiology of many hypospadias is often assumed to be multifactorial, implicating some combination of genes and environment in the development of the anomaly. Efforts to define a clear aetiology have been unsuccessful (Baskin LS et al., 2001). In a report, 33 patients with severe (scrotal or penoscrotal) hypospadias were evaluated with a range of diagnostic techniques including clinical assessment, ultrasonography, karyotyping, endocrine evaluation, and molecular genetic analysis of the androgen receptor (AR) and 5α-reductase genes to classify and determine the cause of the hypospadias (Albers N, 1997). In 12 patients, diagnoses were determined. The remaining 64% of patients were classified as hypospadias of unknown aetiology.

5. Genetic predisposition forhypospadias

There is a well recognized familial clustering of hypospadias, and male relatives of boys with hypospadias are more likely to have this condition than would be expected by chance. In a study by Sorenson, (Sorenson HR., 1953) male relatives of 103 index cases with nonsyndromic hypospadias were evaluated. It was found that 28% had at least one other family member with hypospadias. The more severe the hypospadias in the index case, the higher the incidence of hypospadias in first-degree relatives. With the mildest form of hypospadias, 3.5% of relatives were affected; with second-degree, 9.1%; and with third-degree, 16.7%. The overall risk for a brother of an affected infant to also have hypospadias was 9.6%. Chen and Woolley (Chen YC, 1971) identified a similar figure of 9.7%. The risk of hypospadias in sibs of affected infants was found to be 4.2% in the international study, with a range of 0–11.3% (Kallen B et al, 1986). These sib occurrence risks are compatible with a multifactorial mode of inheritance for hypospadias. An increased risk for hypospadias among twins has been described. The prevalence of hypospadias is higher among members of male–male pairs and lower among males in male–female pairs. Concordance among twins of the same sex was 18% for both mild and severe forms, with increased risk evident in both monozygotic and dizygotic twins (Kallen B et al, 1986). When monozygotic twins discordant for hypospadias were evaluated, the twin with the lowest birth weight had hypospadias in 16 out of 18 pairs, suggesting a gene-environment interaction (Fredell L et al., 1988).

6. Genetic impairment

Theoretically, genetic alterations in any of the genes involved in development of the male urogenital system could result in hypospadias. However, currently only a small percentage of hypospadias has been linked to genetic or chromosomal damage (Bentvelsen FM et al., 1995; Aaronson IA et al., 1997; Alléra A. et al., 1995).

One in nine patients with severe hypospadias had a single amino acid replacement of the Androgen receptor gene (AR) (Alléra A. et al., 1995). Single-strand conformational polymorphism analysis revealed a missense mutation of exon 2 of the AR gene in 1 of 40 patients with distal hypospadias (Sutherland RW et al., 1996). Several other authors

concluded that mutations in the AR gene are rarely associated with hypospadias, (Wilson JD et al., 1981; McPhaul MJ et al., 1993; McPhaul MJ et al., 1993;– Hiort O et al., 1994) implying that other factors are responsible.

6.1 Homeobox (HOX) genes

Homeobox (*HOX*) genes are transcription factors that play a role in embryonic organization and patterning. Genes of the *Hoxa* and *Hoxd* clusters are expressed in regionalized domains along the axis of the urogenital tract.

Transgenic mice with loss of function of single *Hoxa* or *Hoxd* genes exhibit homeotic transformations and impaired morphogenesis of the urogenital tract (Dollé P et al., 1991; Benson GV et al., 1996; Hsieh-Li HM et al., 1995; Podlasek CA et al., 1997). Human males with hand–foot–genital syndrome, an autosomal dominant disorder characterized by mutations in *HOXA13,* exhibit hypospadias of variable severity, suggesting that *HOXA13* may be important in normal patterning of the penis (Mortlock DP & Innis JW, 1997; Donnenfeld AE et al., 1991; Fryns JP et al., 1993).

6.2 Fibroblast growth factor (FGF) genes

Fibroblast growth factor (*FGF*) genes have been demonstrated to play a role in genital tubercle development. As with Hoxa-13, Fgf-10 and insulin-like growth factor receptor (Igfr) knockout mice have been shown to develop hypospadias. More specifically, the condition of the external genitalia Fgf-10 knockout mice suggests impairment in the development of the glans penis (Haraguchi R et al., 2000).

6.3 The Sonic hedgehog (Shh) gene

Genetic mutations might also interfere with epithelial–mesenchymal interactions necessary for normal embryogenesis (Kurzrock EA et al., 1999). The Sonic hedgehog (*Shh*) gene is expressed in the epithelium of the male urogenital sinus and is not regulated by testosterone. *Shh* has also been shown to be critical for prostate development; however, it has not been studied in relation to hypospadias (Podlasek CA et al., 1999). Genetic impairment of *Shh* during development may be involved in hypospadias and is consistent with the well-established role of *Shh* in limb development (Cohn MJ & Bright PE, 1999). Genetic mutations could theoretically interfere indirectly with fetal testis and adrenal testosterone production and with the adequate virilization of the urogenital sinus and external genitalia during embryogenesis if the conversion of testosterone to DHT by 5α-reductase is interrupted. In addition, any errors in the activity of enzymes involved in converting cholesterol to testosterone could indirectly affect urogenital virilization. Aaronson et al. determined the incidence of defects in three major enzymes in the biosynthetic pathway leading to the production of testosterone (3β-hydroxysteroid dehydrogenase, 17α-hydroxylase, and 17,20-lyase) in 30 boys with fully descended testes but with penoscrotal or proximal shaft hypospadias. One-half of the boys had evidence of impaired function of one or more of these enzymes, suggesting that there was an underlying defect in the biosynthesis of testosterone (Aaronson IA et al., 1997).

6.4 Steroid 5-alpha reductase type 2 (SRD5A2)

The differentiation of the male external genitalia (penis, scrotum and urethra) depends upon conversion of testosterone (T) to dihydrotestosterone (DHT) via the enzyme steroid 5 alpha-

reductase (SRD5A) located in the urogenital tubercle. DHT also initiates differentiation of the urogenital sinus into the prostate while it inhibits the development of the vesico-vaginal septum. T and DHT bind to the same intracellular androgen receptor and interact with a cognate DNA response element to regulate gene expression. Even though T and DHT are active via the same androgen receptor, these hormones produce distinct biological responses. The reasons for this are not clear at the molecular level; however, DHT binds to the androgen receptor more avidly than T, and the DHT-receptor complex is more efficiently transformed to the DNA-binding state than is the T-receptor complex (Siiteri P & Wilson J., 1974; Russell DW &Wilson JD., 1994; Zhu Y-S et al., 1998).

The role of T and DHT in formation of the male genital tract is vividly illustrated in men with congenital SRD5A enzyme deficiency, who are known as male pseudohermaphrodites (Zhu Y-S et al., 1998; Griffin J, & Wilson J., 1989; Imperato-McGinley J et al., 1987). They have a normal XY karyotype but ambiguous external genitalia, and thus are reared as females in many cases. T and estrogen (E) levels are normal to elevated, but DHT levels are reduced. These individuals lack Mullerian duct derivatives, while T-dependent Wolffian duct derivatives (the epididymides, seminal vesicles, vas deferens) are present. The DHT-dependent external genitalia are abnormal, however, and a small phallus, bifid scrotum, blind vaginal pouch, and varying degrees of hypospadias are present. Two different isozymes of SRD5A have been identified in humans: one is located in genital tissue and the prostate, with an optimum pH of 5.5 (type 2; SRD5A2), and the other is found in nongenital skin and the liver, with an optimal pH of 6–9 (type 1; SRD5A1) (Moore R, & Wilson J, 1976). Male pseudohermaphrodites have deficiencies only in the SRD5A2 enzyme, the gene for which is located on the short arm of chromosome 2 (2p23). The gene for SRD5A1 is located on the short arm of chromosome 5 (5p15), and is normal in male pseudohermaphrodites (Andersson S et al., 1991; Thigpen A et al., 1992). In children, SRD5A1 expression is localized to the sebaceous gland in nongenital skin and is markedly elevated at puberty (Thigpen A et al., 1993). The virilization of male pseudohermaphrodites at puberty may be influenced by synthesis of DHT in nongenital skin viaSRD5A1 activity. As a means of diagnosing SRD5A2 deficiency in children, T/DHT ratios have been measured in serum after hCG stimulation. Excretion of 5α/5β steroid metabolites, and enzyme activity in cultured fibroblasts from genital skin have also been examined (Peterson R et al., 1985). These parameters are highly variable and difficult to interpret in prepubertal children, and T/DHT ratios are increased only in the most severely affected patients (Hiort O et al., 1996; Sinnecker GH et al., 1996). More recently, direct evaluation of allelic variants in the SRD5A2 gene has been used to diagnose SRD5A2 deficiency (Hiort O et al., 1996).

6.5 Androgen receptor gene

The AR gene plays a critical role in male sexual differentiation by mediating the biological effects of gonadal androgens. There are more than 150 mutations described in the AR gene that give rise to androgen insensitivity syndrome (MacLean HE et al., 1995). Most reports of AR gene mutations in individuals with hypospadias have included patients with additional genitourinary malformations, and the disorder analyzed was partial androgen insensitivity syndrome rather than nonsyndromic hypospadias. Mutations of the androgen receptor coding sequence are infrequent in patients with isolated hypospadias (Allera A et al., 1995; Klocker H et al., 1995; Sutherland RW et al., 1996).

In a study of 63 cases of severe hypospadias obtained from a single centre, cases were evaluated for a spectrum of known risk factors, including patient history, physical

examination, karyotyping, hormonal evaluation, and assay of genes involved in androgen action and metabolism. In 31% of cases, the underlying aetiology was identified. Of these, 17% were due to complex genetic syndromes, such as Smith-Lemli-Opitz and Opitz-Frias . Sex chromosomal anomalies, such as XY mosaicisms, accounted for 9.5% of cases. Isolated causes in one case each were the androgen insensitivity syndrome and 5α-reductase type 2 deficiency Also, in patients with elevated T to DHT ratios, no mutations were found in the SRD5A2 gene. These findings indicate that gene mutations in the SRD5A2, and AR genes may not be common in patients drawn from the general population with nonsyndromic hypospadias (Boehmer AL et al., 1999). Until larger studies are undertaken, however, the role of genetic factors in genes controlling androgen action and metabolism will not be clearly understood. Evaluations of genotype, as well as exposure to endocrine disrupting chemicals, are likely to be important in explaining the majority of cases of nonsyndromic hypospadias.

7. Risk factors for hypospadias

Several studies have found that male infants with hypospadias have lower birth weight, shorter length of gestation and/or evidence of growth retardation in utero (Sweet RA et al., 1974; Akre O et al., 1999; Hussain N et al.. 2002).

The recent study by Hussain et al contains a systematic evaluation of all these risk factors. A retrospective cohort study of 6,746 male infants admitted to neonatal intensive care units (NICU) at the University of Connecticut from 1987–2000 was conducted. Overall, 1.66% of male infants had a diagnosis of nonsyndromic hypospadias, and there was a 10-fold increase in the birth prevalence over the 13-year period of the study. Hypospadias was significantly more common in infants who had poor intrauterine growth (10th percentile) as measured by birth weight, length, and head circumference. The proportionate decrease in all of these growth parameters suggests that the primary insult occurred early, during the first trimester of pregnancy (Hussain N et al. 2002). There were no differences between singletons and multiple-gestation births, but the frequency was significantly higher among firstborn infants (1.9%) compared to all other infants (0.9%). There was a higher occurrence (35%) of the more severe forms of hypospadias (second- and third-degree) in this study compared with other studies, (Avellan L, 1975) indicating that both the incidence and severity were increasing over time (Hussain N et al. 2002). Common environmental factors that had an impact on intrauterine growth and morphogenesis of the genital tract were considered to be the cause. Exposure to EDC (Endocrine Disrupting Chemicals) was implicated, as significant levels were found in aquatic life in proximity to the study site in Connecticut (Hussain N et al. 2002). While these findings are notable, and diagnostic criteria for hypospadias were rigorously controlled, the prevalence of hypospadias among infants in an NICU environment cannot be extrapolated to the general population. An additional risk factor for hypospadias is parental subfertility, which has been identified as delayed childbearing in several studies (Fisch H et al., 2001; Fritz G, & Czeizel AE, 1996). A 50% increase in severe cases has been demonstrated in children of mothers 35 years old compared to mothers 20 years old (Fisch H et al., 2001; Michael P.Porter et al., 2005). A correlation between paternal subfertility and increased risk of hypospadias has also been found (Fisch H et al., 2001). Abnormalities of the scrotum or testes (e.g., cryptorchidism, varicocele, hydrocele, and atrophic testes) were found in 34% of index fathers whose sons had hypospadias, compared to 3% of control fathers in a study conducted by Fritz and

Czeizel (Sweet RA, 1974; Fritz G, & Czeizel AE, 1996). They also found that 24% of fathers with affected sons had signs of subfertility (such as decreases in sperm density, motility, and morphology) that required medical treatment, compared to 6% of controls (Fritz G, & Czeizel AE, 1996). Subfertile males are now reproducing at a higher rate due to improvements in assisted-reproduction techniques, and this may be contributing to the increased occurrence of hypospadias (Czeizel A, 1985; Fritz G, & Czeizel AE, 1996).

Additional evidence that paternal subfertility is a risk factor for hypospadias has come from studies of birth outcome following *in vitro* fertilization (IVF) procedures. A five-fold increased risk for hypospadias has been found in infants conceived by IVF procedures in the Greater Baltimore Medical Center as compared to state-wide incidence figures in Maryland (Silver RI, 1999). These findings have been confirmed in subsequent studies using the Swedish Medical Birth Registry (Wennerholm UB et al., 2000; Ericson A & Kallen B, 2001). No increased risk for hypospadias was found after standard IVF in Sweden; however, there was an approximately three-fold increased risk (95% CI, 1.4 –5.4) after intracytoplasmic sperm injection (ICSI), a specific IVF procedure that is undertaken when sperm quality is poor (Wennerholm UB et al., 2000; Ericson A & Kallen B, 2001). Women undergoing IVF procedures typically receive treatment with progesterone in the first trimester to support the pregnancy after embryo transfer postulated that this treatment is the cause of increased risk for hypospadias following IVF procedures (Silver RI, 1999). Experimental studies have shown that progestins administered to laboratory animals during pregnancy can cause hypospadias (Goldman AS et al., 1967; Dean HJ, 1984). The epidemiologic evidence, however, does not support an association between increased risk for hypospadias and first trimester exposure to progestins found in oral contraceptives, hormones for pregnancy support, and/or hormone-based pregnancy tests (Sweet RA et al., 1974; Aarskog D, 1979; Czeizel A, 1979; Mau G 1981; Monteleone NR et al., 1981; Calzolari E, 1989; Kallen B et al., 1991). A meta-analysis of human studies also did not find an association between progestin exposures and external genital anomalies in male infants (summary OR, 1.09; 95% CI, 0.90 – 1.32) (Raman-Wilms L et al., 1995). In addition, Wennerholm et al. (Wennerholm UB, 2000) and Ericson and Kallen (Ericson A & Kallen B, 2001) found in the Swedish Medical Birth Registry that an increased risk for hypospadias was specific for ICSI, and not for all the other IVF procedures in which progestin support was administered. These investigators concluded that confounding by paternal subfertility explained the association between ICSI and increased risk for hypospadias. The transfer of genes involving androgen action and metabolism from fathers with poor sperm quality to their sons was considered the most likely cause (Fritz G & Czeizel AE, 19960.

In other reports white race, and pre-existing diabetes were also found to be associated with hypospadias in male offspring (Michael P.Porter et al.,2005). Reports concerning use of maternal drugs during early pregnancy, strongly associate the use of valproic acid with infant hypospadias, amongst other abnormalities (Carla Arpino et al., 2000).

8. Testicular dysgenesis syndrome (TDS) and related male reproductive disorders

Several investigators have reported adverse trends in male reproductive health, including increasing incidence of testicular cancer, cryptorchidism ie undescended testis, hypospadias and low semen quality (Adami et al., 1994; Forman & Moller, 1994; Carlsen et al. 1992; Swan et al., 1997; Andersen et al., 2000; Toppari et al., 1996; Paulozzi et al., 1997). It has been

proposed that all these disorders have common orign in fetal life and thus they all represent different symptoms of the same underlying entity called testicular dysgenesis syndrome (TDS) (Skakkebaek et al., 2001; Sharpe, 2003; Asklund et al., 2004). The observation that the occurrence of one disorder increases the risk of the occurrence of another disorder gives epidemiological evidence for the existence of TDS (Skakkebaek et al., 2001). Additonally the disorders of the male reproductive health share common risk factors. Small for gestational age infants have increased risk of developing undescended testis, hypospadias and testicular cancer (Moller and Weidner, 1999; English et al., 2003). The rapid increase of reproductive disorders and the geographical clustering of these disorders, point to environmental, life style and occupational trends rather than the accumulation of genomic defects, as the most likely cause of TDS (Virtanen HE. Et al., 2005). Animal studies have shown that several chemicals including pesticides, such as vinclozolin, procymidone, DDE, can cause TDS linked disorders (Skakkebaek et al., 2001; Damgaard et al., 2002; Fischer, 2004)

9. Endocrine disrupting chemicals

The familial clustering of hypospadias among first degree relatives has traditionally been perceived as evidence of a genetic component in the aetiology of this anomaly. However, exposure to environmental contaminants is now being considered in familial clusters because of the high probability of shared exposures among first degree relatives (Baskin LS et al., 2001).

As mentioned earlier the risk of developing hypospadias amongst relatives can be as high as 10%. This shows that the cause of hypospadias is mainly genetic for these individuals. The hypothesis of shared exposure on endocrine disrupting chemicals cannot alone justify the high incidence rate of hypospadias in familial clusters. The same hypothesis on the other hand cannot be overlooked since these agents have been shown to interfere with male sexual differentiation.

Several environmental antiandrogens have been identified in rodent models that interfere with male sexual differentiation at environmentally relevant doses (William R. Kelce et al., 1997; Gray LE et al., 1999; Tamura H et al., 2001; Gray LE et al., 2000 Gray LE et al., 2001). At relatively low in utero doses, antiandrogens reduce anogenital distance and induce transient nipple development in the neonatal rat. At mid-doses, hypospadias, agenesis of the sex accessory tissues, and retained nipples occur, while at the highest doses undescended testes and epididymal agenesis are seen. Fetal tissue concentrations of 10–20 ppm of the DDT metabolite, p,p_-DDE, an AR antagonist, are sufficient to produce these antiandrogenic effects in the rat fetus (William R. Kelce et al., 1997). These concentrations are similar to those measured in first-trimester human fetal tissues in the late 1960s. The pesticides vinclozolin, procymidone, linuron, and fenitrothion are also AR antagonists that produce dose-response effects on the developing male reproductive system (Gray LE et al., 1999; Tamura H et al., 2001).

Phthalate esters (PE) inhibit testosterone synthesis during fetal life, and produce dose-related abnormalities of the male reproductive tract similar to those caused by the AR antagonists (Gray LE et al., 2000). The PE have effects at extremely low in utero doses, and no-effect levels could not be found for the most sensitive endpoint, reduction in anogenital distance in male neonates (Gray LE et al., 2000). Prenatal administration of a single low dose of dioxin (50 –1,000 ng TCDD/kg) alters differentiation of androgen-dependent tissues; however, the mechanism of action involves interaction with the hormone-like receptor,

AhR, rather than the AR (Gray LE et al., 2001). Attempts to extrapolate findings from these rodent models to humans have been problematic. It can be difficult to determine whether effects obtained at doses employed in rodent models are relevant to human environmental exposures (Gray LE et al., 2001). An even greater problem is the difficulty of accurately measuring in utero human exposure to environmental agents for comparison. A number of epidemiology studies have been conducted concerning pesticide exposures of fathers employed as gardeners, agricultural pilots, or aerial sprayers (Roan CC et al., 1984; Rupa DS et al., 1991; Smith AH et al.,1982; Garcia AM, 1988). These studies have provided evidence, (some authors claim this evidence as very weak if any), that these types of occupational exposures result in adverse pregnancy outcomes (Roan CC et al., 1984; Rupa DS et al., 1991; Smith AH et al.,1982). A review of epidemiology studies published in 1980–1996 on the effects of agricultural occupation (and presumably pesticide exposure) on congenital malformations has been conducted. Of 34 published studies, few resulted in significant associations between birth defects and pesticide exposure (Garcia AM, 1988). Problems inherent in most of the studies were the use of occupational title or residence as a surrogate for pesticide exposure, the assessment of outcome through parental interview rather than medical records, and small study size. Recommendations from this and other reviews are that exposure to specific active ingredients or at least chemical classes must be quantitatively evaluated before assessment of effects on reproductive function can be accurately determined (Nurimen T, 1995). Regardless of these methodological issues, however, enough positive associations have been reported to warrant further investigation of pesticide effects on human reproduction.

10. Conclusion

There is substantial evidence for increases in the birth prevalence of hypospadias in the United States and in Europe. Several clinical risk factors have been identified, including intrauterine growth reduction and paternal subfertility, particularly when accompanied by assisted-reproduction procedures. Familial clustering has been well documented, and may involve both genetic and environmental risk factors. A number of candidate genes that control androgen action and metabolism are logical choices for genomic evaluation in population-based studies, including the SRD5A2, HSD17B3, and the AR genes.

The role of EDC exposure in the aetiology of hypospadias in human populations is far from clear (C M. Rochelau, 2009). Major improvements in methodologies to measure environmental exposures are needed to determine the role of environmental exposures in the increased rates of hypospadias. The need of a prospective study that will accurately determine the exposure to EDC and quantitavely measure this exposure in a large sample of the population is needed.

11. References

Aaronson IA, Cakmak MA, Key LL. Defects of the testosterone biosynthetic pathway in boys with hypospadias. 1997;J Urol 157(5):1884–1888 .

Aarskog D. Current concepts: maternal progestins as a possible cause of hypospadias. 1979;N Engl J Med 300:75–78.

Adami, H.O., Bergstrom, R., Mohner, M., Zatonski, W., Storm, H.,Ekbom, A., Tretli, S., Teppo, L., Ziegler, H., Rahu, M., et al., 1994.Testicular cancer in nine northern European countries. Int. J. Cancer59, 33– 38.

Akre O, Lipworth L, Cnattingius S, et al. Risk factor patterns for cryptorchidism and hypospadias. 1999Epidemiol;10:364 –369.

Albers N, Ulrichs C, Gliier S, Hiort O, Gernot HG, Sinnecker GHG, Mildenberger H, Brodehl J. 1997Etiologic classification of severe hypospadias: implications for prognosis and management. J Pediatr;131(3):386–392

Alléra A, Herbst MA, Griffin JE, Wilson JD, Schweikert H, McPhaul MJ. Mutations of the androgen receptor coding sequence are infrequent in patients with isolated hypospadias. 1995J Clin Endocrinol Metab;80(9):2697–2699

Allera A, Herbst MA, Griffin JE, Wilson JD, Schweikert HU, McPhaul MJ. among minority groups in the United States, 1981–1986. 1988 ; MMWR 37:17–24

Andersson S, Berman D, Jenkins E, Russell 1991D. Deletion of steroid 5 alpha reductase 2 gene in male pseudohermaphroditism. Nature;354: 159–161.

Aristotle.(1943).*Generation of animals. AL Peck (transl.) The Loeb classical library*. Harvard University Press, Cambridge.

Ashcraft Murphy Pediatric Surgery 3rd ed. W.B.Saunders Co. 2000

Asklund, C., Jorgensen, N., Kold Jensen, T., Skakkebaek, N.E., 2004.Biology and epidemiology of testicular dysgenesis syndrome. BJU Int.93 (Suppl. 3), 6 – 11.

Avellan L. 1975The incidence of hypospadias in Sweden. Scand J Plast Reconstr Surg;9:129 – 139.

Baskin LS, Himes K, Colborn T, 2001 "Hypospadias and Endocrine Disruption: Is There a Connection?", *Environmental Health Perspectives*; 109, pp1175-1183.

Baskin LS. 2000Hypospadias and urethral development. J Urol;163:951–956.

Belman AB. 2002Hypospadias and chordee.In: Belman AB, King LR, Kramer SA, eds.Clinical Pediatric Urology. 4th ed. London, England: Martin Dunitz;.

Benson GV, Lim H, Paria BC, Satokata I, Dey SK, Maas RL. Mechanisms of reduced fertility in Hoxa-10 mutant mice: uterine homeosis and loss of maternal Hoxa-10

Bentvelsen FM, Brinkmann AO, van der Linden JETM, Schröder FH, Nijman JM1995. Decreased immunoreactive androgen receptor levels are not the cause of isolated hypospadias. Br J Urol;76(3):384–388

Bingol N, Wasserman E. 1990Hypospadias. In: Buyse ML, editor. Birth defects encyclopedia.Cambridge, MA: Blackwell Scientific Publications, p 931–932.

Bjerkedal T, Bakketeig LS1975. Surveillance of congenital malformations and other conditions of the newborn. Int J Epidemiol;4:31–36.

Boehmer AL, Brinkmann AO, Sandkuijl LA, et al. 1999 17Beta-hydroxysteroid dehydrogenase-3 deficiency: diagnosis, phenotypic variability, population genetics, and worldwide distribution of ancient and de novo mutations. J Clin Endocrinol Metab;84:4713– 4721.

C M. Rochelau, P A. Romitti, L K. Dennis *2009*Pesticides and hypospadias: A meta analysis. *Journal of Pediatric Urology; 5, 17-24*

Calzolari E, Contiero MR, Roncarati E. 1989 Aetiological factors in hypospadias. J Med Genet;23:222–337.

Carla Arpino et al 2000 Teratogenic effects of antiepileptic drugs: use of an international database on malformations and drug exposure (MADRE) Epilepsia,;41(11):1436-1443,

Carlsen, E., Giwercman, A., Keiding, N., Skakkebaek, N.E., 1992.Evidence for decreasing quality of semen during past 50 years. BMJ305, 609–613

Chen YC, Woolley PV. 1971Genetic studies on hypospadias in males. J Med Genet;8:153–159.

Claude Desjardins 1981Endocrine Signaling and Male Reproduction Biology of Reproduction 24, 1-21

Cohn MJ, Bright PE. 1999 Molecular control of vertebrate limb development, evolution and congenital malformations.Cell Tissue Res;296(1):3–17

Czeizel A, Toth J, Erodi E. 1979 Aetiological studies of hypospadias in Hungary. Hum Hered;38:45–50.

Czeizel A. 1985 Increasing trends in congenital malformations of male external genitalia (Letter). Lancet;1:108.

Dean HJ, Winter JS. 1984 The effect of five synthetic progestational compounds on 5 alpha-reductase activity in genital skin fibrobast monolayers. Steroids;43:13–17.Development 1995;121(5):1373–1385

Dollé P, Izpisúa-Belmonte JC, Brown JM, Tickle C, Duboule D 1991. Hox-4 genes and the morphogenesis of mammalian genitalia. Genes Dev;5(10):1767–1776

Donnenfeld AE, Schrager DS, Corson SL. 1992 Update on a family with hand-foot-genital syndrome: hypospadias and urinary tract abnormalities in two boys from the fourth generation. Am J Med Gen 44(4):482–484 .

Dorland's (1981).*Illustrated Medical History ed. 26.*, W.B. Saunders Co., Philadelphia

Duckett JW, Baskin LS. 1996 Hypospadias. In: Gillenwater JY, Grayhack JT, Howards SS, Duckett JW, editors. Adult and pediatric urology. Vol III, 3rd ed. St. Louis, MO: Mosby Press. p 2549–2589.

Ericson A, Kallen B. 2001 Congenital malformations in infants born after IVF: a population-based study. Hum Reprod;16:504 –509.expression. Development 1996;122(9):2687–2696

Fisch H, Golden RJ, Libearsen GL, et al. 2001 Maternal age as a risk factor for hypospadias. J Urol;165:934 –936.

Forman, D., Moller, H., 1994. Testicular cancer. Cancer Surv. 19– 20,323– 341.

Fredell L, Lichtenstein P, Pedersen NL, et al. 1998 Hypospadias is related to birth weight in discordant mnozygotic twins. J Urol;160:2197–2219.

Fritz G, Czeizel AE. 1996 Abnormal sperm morphology and function in the fathers of hypospadiacs. J Reprod Fertil;106:63– 66.

Fryns JP, Vogels A, Decock P, Van den Berghe H. 1993 The hand-foot-genital syndrome: on the variable expression in affected males. Clin Genet;43(5):232–234 .

Gallentine ML, Morey AF, Thompson IM. 2001 Hypospadias: a contemporary epidemiologic assessment. Urology;57:788 –790.

Garcia AM. 1998 Occupational exposure to pesticides and congenital malformations: a review of mechanisms, methods and results. Am J Ind Med;33:232–240.

Goldman AS, Bongiovanni AM. 1967 nduced genital anomalies. Ann N Y Acad Sci;142:755–767.

Gray LE, Ostby J, Furr J, et al. 2001 Effects of environmental antiandrogens on reproductive development in experimental animals. Hum Reprod Update;7:248 –264.

Gray LE, Ostby J, Furr J, et al. 2000 Perinatal exposure to the phthalates DEHP, BBP, and DINP, but not DEP, DMP, or DOTP, alters sexual differentiation of the male rat. Toxicol Sci;58:350 –365.

Gray LE, Wolf C, Lambright C, et al. 1999 Administration of potentially antiandrogenic pesticides (procymidone, linuron, iprodione, chlozalinate, p,p'-DDE and ketoconazole) and toxic substances (dibutyl- and diethylhexyl phthalate, PCB 169, and ethane dimethane sulphonate) during sexual differentiation produces diverse profiles of reproductive malformations in the male rat. Toxicol Ind Health a; 15:94 –118.

Griffin J, Wilson J. 1989 The androgen resistance syndromes: 5_-reductase deficiency, testicular feminization and related disorders. In: Scriver C,Beaudet A, Sly W, Valle D, editors. The metabolic basis of inherited disease, 6th ed., Vol. II. New York: McGraw-Hill p 1919–1944.

Grumbach, M.M. 1980 The neuroendocrinology of puberty. In: Neuroendocrinology, the interrelationships of the Body's Two major Integrative Systems-in Normal physiology and in clinical Disease (D.T. Krieger and J.C. Hughes, eds.). Sinauer associates, Inc., Sunderland, MA. pp249-258

Gunha GR, Baskin LS. 2004 Development of the penile urethra. Adv Exp Med Biol.;545:87-102

Haraguchi R, Suzuki K, Murakami R, Sakai M, Kamikawa M, Kengaku M, Sekine K, Kawano H, Kato S, Ueno N, et al 2000. Molecular analysis of external genitalia formation: the role of fibroblast growth factor (Fgf) genes during genital tubercle formation. Development;127(11):2471–2479

Hiort O, Willenbring H, Albers N, Hecker W. 1996 Molecular genetic analysis and human chorionic gonadotropin stimulation tests in the diagnosis of prepubertal patients with partial 5_ reductase deficiency. Eur J Pediatr;155:445– 451.

Hussain N, Chaghtai A, Herndon A, et al. 2002 Hypospadias and early gestation growth restriction in infants. Pediatrics;109:473– 478.

Hynes PJ Fraher JP. 2004 The development of the male genitourinary system:III The formation of the spongiose and glandar urethra. Br J Plast Surg.;57:203-214

Imperato-McGinley J, Akgun S, Ertel NH, et al. 1987 The coexistence of male pseudohermaphrodites with 17-ketosteroid reductase deficiency and 5_-reductase deficiencies within a Turkish kindred. Clin Endocrinol (Oxf);27:135–143.

J.E. Skandalakis, S.W.Gray (1994).*Embryology for Surgeons "The Embryological Basis for the treatment of congenital anomalies" 2nd ed*. Williams and Wilkins

K.A.Boisen, et al 2005 Hypospadias in a Cohort of 1072 Danish newborn boys: Prevalence and relationship to placental weight, anthtropometrical measurements at birth and reproductive hormone levels at three months of age J. Clin Endocrinol Metab;90:4041-6

Kallen B, Bertolini R, Castilla A, et al. 1986 A joint international study on the epidemiology of hypospadias. Acta Paediatr Scand Suppl;324:1–52.

Kallen B, Mastroiacovo P, Lancaster PA, et al 1991. Oral contraceptives in the etiology of isolated hypospadias. Contraception;44:173–182.

Kallen B, Winberg J. 1982 An epidemiological study of hypospadias in Sweden. Acta Paediatr Scand Suppl;93:3–21.

Khuri FJ, Hardy BE, Churchill BM. 1981 Urologic anomalies associated with hypospadias. Urol Clin North Am;8:565–571.

Klocker H, Neuschmid-Kaspar F, Culig Z et al. 1995 Androgen receptor alterations in patients with disturbances in male sexual development and in prostatic carcinoma. Urol Int;54:2–5.

Kurzrock EA, Baskin LS, Gunha GR. 1999 Ontogeny of the male urethra: theory of endodermal differentiation. Differentiation.;64:115-122

Latifoglu O, Yavuzer R, Demircliler N, et al. 1998 Extraurogenital congenital anomalies associated with hypospadias: retrospective review of 700 patients Ann Plast Nov;41(5):570-1

Levitt SB, Reda EF. 1988 Hypospadias. Pediatric Ann 17:48 –57.

MacLean HE, Warne GL, Zajac JD. 1995 Defects of androgen receptor function: from sex reversal to motor neuron disease. Molec Cell Endocrinol;112:133–141.

Matlai P, Beral V. 1985 Trends in congenital malformations of external genitalia. Lancet;1:108.

Mau G. 1981 Progestins during pregnancy and hypospadias. Teratology;24:285–287.

McPhaul MJ, Marcelli M, Zoppi S, Griffin JE, Wilson JD. Genetic basis of endocrinedisease. 4. The spectrum of mutations in the androgen receptor gene that causes

Michael P.Porter et al 2005Hypospadias in Washington State: Maternal risk factors and prevalence trends. Pediatrics;115 495-499

Michael Michalakis et al. 2008 Increase in Hypospadias prevalence and the role of endocrine disrupting chemicals. Toxicollogy letters;vol 180 no 1 ISSN:03784274

Monteleone NR, Castilla EE, Paz JE. 1981Hypospadias: an epidemiologic study in Latin America. Am J Med Genet;10:5–19.

Moore KL, Persaud TVN, Charber DB. 2003 The Developing Human: Clinically Oriented Embryology. 7th ed. Philadelphia, Pa: WB Saunders.

Moore KL. The urogenital system. In: The developing human. Clinical

Moore R, Wilson J. Steroid 5_-reductase in cultured human fibroblasts:

Mortlock DP, Innis JW. Mutation of HOXA13 in handfoot-genital syndrome.

Mutations of the androgen receptor coding sequence are infrequent in patients with isolated hypospadias. J Clin Endocrinol Metab 1995;80:2697–2700.

Nat Genet 1997;15(2):179–180

Nurimen T. 1995 Maternal pesticide exposure and pregnancy outcome. J Occup and Environ Med;37:935–940.

O'Neil, J, et al. Pediatric Surgery, 5th ed. Mosby

Paulozzi LJ(1999) "International Trends in Rates of Hypospadias and Cryptorchidism", *Environmental Health Perspectives*; 107, pp297-302.

Paulozzi LJ, Erickson JD, Jackson RJ, "Hypospadias Trends in Two US Surveillance Systems", *Pediatrics* 1997; 100(5), pp831-834.

Peterson R, Imperato-McGinley J, Gautier T, Shackleton C. Urinary steroid metabolites in subjects with male pseudohermaphrodism due to 5 alpha reductase deficiency. 1985 Clin Endocrinol;23:43–53.

Pierik FH, Burdorf A, Nijman JM, et al. 2002 A high hypospadias rate in The Netherlands. Hum Reprod;17:1112–1115.

Podlasek CA, Barnett DH, Clemens JQ, Bak PM, Bushman W. 1999 Prostate development requires Sonic hedgehog expressed by the urogenital sinus epithelium.;Dev Biol 209(1):28–39

Podlasek CA, Duboule D, Bushman W. 1997 Male accessory sex organ morphogenesis is altered by loss of function of Hoxd-13. Dev Dyn;208(4):454–465

Raman-Wilms L, Tseng A, Wighardt S, et al. 1995 Fetal genital effects of first trimester sex hormone exposure: a meta-analysis. Obstet Gynecol;85:141–149.

Roan CC, Matanoski GE, McIlnay C. 1984 Spontaneous abortions, stillbirths and birth defects in families of agricultural pilots. Arch Environ Health;39:56–60.

Rupa DS, Reddy PP, Reddi OS. 1991Reproductive performance in a population exposed to pesticides in cotton fields of India. Environ Res;55:123–128.

Russell DW, Wilson JD. 1994 Steroid 5_ reductase: two genes/two enzymes. Ann Rev Biochem;63:25– 61.

Sharpe, R.M., 2003. The Foestrogen hypothesis_—Where do we stand now?Int. J. Androl. 26, 2– 15.

Siiteri P, Wilson J. 1974 Testosterone formation and metabolism during male sexual differentiation in the human embryo. J Clin EndocrinolMetab;38:113–124.

Silver RI, Rodriguez R, Chang TS, Gearhart JP. 1999 In vitro fertilization is associated with an increased risk of hypospadias. J Urol;161:1954 –1957.

Sinnecker GH, Hiort O, Dibbelt L, Albers N. 1996 Phenotypic classification of male pseudohermaphroditism due to steroid 5 alpha reductase 2 deficiency. Am J Med Genet;63:223–230.

Skakkebaek, N.E., Rajpert-De Meyts, E., Main, K.M., 2001. Testiculardysgenesis syndrome: an increasingly common developmental disorderwith environmental aspects. Hum. Reprod. 16, 972– 978

Smith AH, Fisher DO, Pearce N. 1982 Congenital defects and miscarriages among New Zealand 2,4,5-T sprayers. Arch Environ Health;37:197–120.

Sorenson HR. 1953 Hypospadias with special reference to aetiology. Copenhagen: Munksgaard. p 94.

Susan L. Carmichael Gary M.Shaw et al. 2003 Hypospadias in California trends and descriptive epidemiology.Epidemology;14:701-706

Sutherland RW, Wiener JS, Hicks JP, et al. 1996 Androgen receptor gene mutations are rarely associated with isolated penile hypospadias. J Urol;156:828–831.

Sutherland RW, Wiener JS, Hicks JP, Marcelli M, Gonzales ET Jr, Roth DR, Lamb DJ. 1996 Androgen receptor gene mutations are rarely associated with isolated penile hypospadias. J Urol;156(2 Pt 2):828–831

.Swan, S.H., Elkin, E.P., Fenster, L., 1997. Have sperm densities declined? A reanalysis of global trend data. Environ. Health Perspect.105, 1228– 1232

Sweet RA, Schrott HG, Kurland R. 1974 Study of the incidence of hypospadias in Rochester, Minnesota 1940-1970 and a case control comparison of possible etiologic factors. Mayo Clin Proc;49:52–57.

Tamura H, Maness SC, Reischmann K, et al. 2001 Androgen receptor antagonism by the organophosphate insecticide fenitrothion. Toxicol Sci;60:56–62.

Thigpen A, Davis D, Milatovich A. 1992 Molecular genetics of steroid5α-reductase deficiency. J Clin Invest;90:799–809.

Thigpen A, Silver R, Guileyardo J, et al. 1993 Tissue distribution and ontogeny of steroid 5αreductase isozyme expression. J Clin Invest;92:903–910

Tsatsakis A.M., Tzatzarakis M.N., Tutudaki M. 2008 Pesticide levels in head hair samples of Cretan population as an indicator of present and past exposure. Forensic Science International;vol176, 1, pp67-71

Toppari, J., Larsen, J.C., Christiansen, P., Giwercman, A., Grandjean, P.,Guillette Jr., L.J., Jegou, B., Jensen, T.K., Jouannet, P., Keiding, N.,Leffers, H., McLachlan, J.A., Meyer, O., Muller, J., Rajpert-De Meyts, E., Scheike, T., Sharpe, R., Sumpter, J., Skakkebaek, N.E., 1996. Malereproductive health and environmental xenoestrogens. Environ. HealthPerspect. 104 (Suppl. 4), 741–803.

Virtanen HE., E Rajpert De Meyts, K.M. Main, N.E. Skakkebaek, J.Toppari , 2005, Testicular dysgenesis syndrome and the development and occurrence of male reproductive disorders. Toxicology and applied pharmacology 207 501-505

Wennerholm UB, Bergh C, Hamberger L, et al. 2000I ncidence of congenital malformations in children born after ICSI. Hum Reprod;15:944 –948.

William R. Kelce · Elizabeth M. Wilson1997 Environmental antiandrogens: developmental effects, molecular mechanisms, and clinical implications. J Mol Med; 75:198–207

Wilson JD, George FW, Griffin JE. 1981 The hormonal control of sexual development. Science;211(4488):1278–1284

Winter *et al.* Sex steroid production by the human fetus: its role in morphogenesis and control by gonadotropins Birth defects 1977;13(2):41-58

Zhu Y-S, Ktz MD, Imperato-McGinley J. 1998 Natural potent androgens: lessons from human genetic models. Bailliere's Clin Endocrinol Metab; 12:83–107.

8

Adverse Health Effects of Pesticide Exposure in Agricultural and Industrial Workers of Developing Country

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1. Introduction

Pesticides are used extensively throughout the world. There are several definitions of pesticide; the Food and Agriculture Organization of the United Nations (FAO) defines pesticide as any substance or mixture of substances intended for preventing, destroying or controlling any pest, including vectors of human or animal disease, unwanted species of plants or animals causing harm during or otherwise interfering with the production, processing, storage or marketing of food, agricultural commodities, wood and wood products or animal food stuffs or which may be administered to animals for the control of insects, arachnids or other pests in or on their bodies (FAO, 1986).

Pesticides are playing a pivotal role in meeting the food, cotton fibre and tobacco demand of escalating population and control of vector-borne diseases. However, most of the applied pesticides get dispersed in the environment and affects the health of un-protected agricultural and industrial workers. The three major routes of entry for pesticides include contamination of the skin, lungs and the gut. The skin of a human adult has a superficial surface area of approximately 1.73 m^2, but it is undoubtedly the major focus of accidental acute exposure. Similarly the respiratory tract provides a very efficient surface for the absorption of substances, whether they are in the form of vapors, particles or droplets. Although pesticides furnish some benefits for crop, they entail a number of risks and problems. The public health issue of pesticide exposure is further complicated by the presence of impurities in so-called, inert-ingredients such as solvents, wetting agents and emulsifiers. These chemicals are suspected of producing adverse health effects based on their structural similarity to proven toxicants. The unregulated and excessive use of pesticides has become a major bottleneck in our fight against insect pests.

Exposure to pesticides is one of the most important occupational risks among farmers in developing countries (Wesseling et al., 2001; Konradsen et al., 2003; Coronado et al. 2004). Occupational exposure to pesticides is of great interest in order to identify the hazards of pesticide use and the establishment of safe methods of pesticide handling. This is because

pesticide misuse in various sectors of the agriculture often has been associated with health problems and environmental contamination worldwide (Soares et al., 2003; Mancini et al., 2005; Remor et al., 2009). Misuse of highly toxic pesticides, coupled with a weak or a totally absent legislative framework in the use of pesticides, is one of the major reasons for the high incidence of pesticide poisoning in developing countries (Konradsen et al., 2003). Low education levels of the rural population, lack of information and training on pesticide safety, poor spraying technology, and inadequate personal protection during pesticide use have been reported to play a major role in the intoxication scenario (Hurtig et al., 2003; Atreya, 2008). In general, knowledge of the main determinants of pesticide exposure in developing countries is often poor and also exposure situations may differ among countries.

A major factor of pesticide contamination or poisoning in developing countries is the unsafe use or misuse of pesticides. Elements of unsafe use of pesticides that have been identified by past research include erroneous beliefs of farmers about pesticide toxicity, lack of attention to safety precautions, environmental hazards, and information about first aid and antidotes given by the label, the use of faulty spraying equipment or lack of proper maintenance of spraying equipment, and lack of the use of protective gear and appropriate clothing during handling of pesticides (Hurtig et al., 2003; Damalas et al., 2006a, 2006b; Ajayi and Akinnifesi, 2008; Chalermphol and Shivakoti, 2009; Plianbangchang et al., 2009; Sosan and Akingbohungbe, 2009). Extensive use of domestic utensils and broken equipment for measuring and dispensing pesticides in developing countries often continue unabated because farmers cannot afford equipment that is in good working condition. In view of the adverse health effects from the unsafe pesticide use, the latency of the adverse effects, the reported lack of awareness of the adverse health effects of pesticides by some farmers, and the erroneous belief of invincibility by others, it becomes imperative that the potential hazards of unsafe pesticide use should be clearly communicated to the farmers. Fortunately, many farmers have expressed the need for information and training programs on pesticide safety, and therefore are likely to be responsive to such programs. Research has often emphasized the need to increase the awareness of farmers about the consequences of unsafe pesticide use and the importance of communication and education programs aiming to reduction of risk (Ibitayo, 2006; Hashemi et al., 2008; Oluwole and Cheke, 2009; Sosan and Akingbohungbe, 2009; Damalas and Hashemi, 2010). Incorrect beliefs about pesticides and hazards often associated with pesticide use can reduce the capacity of farmers to protect themselves against these hazards. However, the first step in developing pesticide hazard reduction programs is to identify the extent of the problem by investigating farmers' knowledge, attitudes, and perceptions about pesticide handling and pesticide safety. Agricultural extension is a major channel of communication between farmers and research experts which can improve crop production from many points of view as it provides a good link between farmers and research institutes where several agricultural technologies, including pesticides and the relative technology, are developed, tested, and modified accordingly. Training programs can play a crucial role in pest control decisions, providing farmers with the technical knowledge that is necessary for the selection of appropriate pest management methods and also for safe and effective pesticide use. Despite the appearance of homogeneity, often small farmers have different production practices, needs, and constraints (Carr, 1989). A successful agricultural extension program, therefore, should not consider all individuals in a target group based on several variables such as age, gender, income, and types of crops.

1.1 WHO classification of pesticide

Toxicity of formulated chemical product classified according to WHO hazard classes (table-1). Pesticide belonging to WHO class 1a is extremely hazardous, class 1b is highly hazardous, class11 is moderately hazardous, class111 is slightly hazardous and class 1V is unlikely to present acute serious hazards in normal use (WHO, 2000). Nearly 90 percent of the banned pesticides fall into category 1a/ 1b/11 of the WHO hazard grades. Applications of monocrotophos, cypermethrin, methamidophos and dimethoate have been increased many folds in Pakistan (Tariq et al., 2007).

1.2 Organophosphates

They were developed during the early 19th century, but their effects on insects, which are similar to their effects on humans, were discovered in 1932. Some are very poisonous (they were used in World War II as nerve agents). They are generally highly lipid soluble and may be classified as direct or indirect acetylcholinesterase (AchE) inhibitors. However; they usually do not persist in the environment. The organophosphates (OPs), because of their widespread use and frequently high acute toxicity, are involved in more pesticide poisonings than any other class of pesticides. The organophosphates interfere with the activity of cholinesterase. When the cholinesterase enzyme cannot perform its normal function, the nerves in the body send "messages" to the muscles continuously leading to muscle twitching and weakness. If the poisoning is severe, the victim may have "fits" or convulsions and may even die. Organophosphates are irreversible cholinesterase inhibitors, without medical treatment the level of enzyme activity will return to normal only after several days, weeks or even months. Additive effects of small repeated doses over time, such as in a spraying season, may finally cause poisoning. The effects of mild poisoning include fatigue, headache, and dizziness. Moderate poisoning leads to inability to walk, weakness and chest discomfort. In severe cases there will be unconsciousness, severe constriction of pupils and muscle twitching ultimately resulting in death.

1.3 Methamidophos

It is classified by Environmental Protection Agency (EPA) as a class I compound. Methamidophos is a highly active, systemic, residual organophosphate insecticide/ acaricide/avicide with contact and stomach action. Methamidophos, a potent acetylcholinesterase inhibitor, is highly toxic via oral, dermal and inhalation routes. Early symptoms of acute organophosphate poisoning are dependent on route of exposure, and usually develop during or shortly after exposure (within 12 hours). Weakness, shakiness, blurred vision, tightness in the chest, sweating, confusion, changes in heart rate, convulsions, coma, and cessation of breathing may occur with significant inhalation, ingestion or dermal exposure. The Allowable Daily Intake (ADI) level of methamidophos is 0.0003 mg/kg.

1.4 Carbamates

The effects of carbamates and organophosphates are similar because they both inhibit cholinesterase. Action of carbamates is naturally reversible as compared to methamidophos action of carbamates is naturally reversible (they will be degraded in and/or expelled from the body). Thus, carbamates can cause severe severe poisoning, but they do not normally produce long-term, cumulative poisoning. The symptoms of acute carbamate and organophosphate poisoning are essentially the same.

Pesticide product	Active ingredient	Chemical class	Toxicological class*	Main use
BASUDIN	Diazinon	Organophosphates	II	Insecticide
HERBOXONE	2,4-D	Chlorophenoxy acids	II	Herbicide
TOPIK	Clodinafop-propargyl	Aryloxyphenoxypropionics	III	Herbicide
AATREX	Atrazine q	Triazines	U	Herbicide
MACHETE	Butachlor	Chloroacetanilides	U	Herbicide
CERTAINTY	Sulfosulfuron	Sulfonylureas	U	Herbicide
ERADICANE	EPTC	Carbamides	II	Herbicide
LASSO	Alachlor	Chloroacetanilides	III	Herbicide
DECIS	Deltamethrin	Pyrethroids	II	Insecticide
ALTO	Cyproconazole	Triazoles	III	Fungicide
SENCOR	Metribuzin	Triazines	II	Herbicide
CONFIDOR	Imidacloprid	Neonicotinoids	II	Insecticide
GRANSTAR	Tribenuron-methyl	Sulfonylureas	U	Herbicide

* Ia = Extremely hazardous; Ib = Highly hazardous; II = Moderately hazardous; III = Slightly hazardous; U = Unlikely to present acute hazard in normal use

Table 1. Classification of pesticides used by the farmers surveyed

1.5 Methomyl

Methomyl is a carbamate insecticide. It is broad spectrum fast acting anti-cholinesterase agent. It is a direct contact and stomach poison. It is a plant systemic of high acute toxicity to mammals. It is non-cumulative and rapidly metabolized in both plants and animals to substances of lower toxicity. Methomyl is particularly effective against organophosphorus resistant pests. Methomyl may be absorbed from the gastrointestinal tract, through the intact skin, and, by inhalation of spray mist and dust. The ADI levels of methomyl are 0.01mg/kg.

1.6 Thiodicarb

It is a carbamate pesticide and belongs to class II, moderately hazardous pesticide. Symptoms ymptoms include malaise, muscle weakness, dizziness, sweating headache, salivation, nausea, vomiting, abdominal pain, diarrhea, central nervous system depression and pulmonary edema. The ADI levels of thiodicarb is 0.03 mg/kg.

1.7 Organochlorines

They were commonly used in the past, but many have been removed from the market due to their health and environmental effects and their persistence (e.g. DDT and chlordane).

1.8 Endosulfan

It is an organochlorine pesticide of moderate mammalian toxicity which does not accumulate in the tissues of man or animals to any significant extent. Undiluted endosulfan is slowly and incompletely absorbed in the gastrointestinal tract of warm-blooded animals. Absorption is more rapid in the presence of alcohols, oils and emulsifiers. These substances also accelerate the absorption of endosulfan through skin. It is a central nervous system stimulant, producing convulsions. The ADI level of endosulfan is 0.006 mg/kg.

2. Synthetic organic pesticides

Since the 1940s, pesticide use has expanded because of the development of the synthetic organic compounds. The synthetic organic pesticides (i.e., man-made, carbon-containing chemicals) include the chemical groups; chlorinated hydrocarbons, organophosphates, carbamates, pyrethroids, phenoxy herbicides and a number of other chemical classes. Groups with similar chemical structure tend to be similar in their mode of action, fate in the environment and pest control properties, but not necessarily in their level of toxicity. Though pesticides may have different chemical structures, they can have similar modes of actions. Their activity tends to be highly specific, and they are often harmless to non target species.

2.1 Pyrethroids

They were developed as a synthetic version of the naturally occurring pesticide pyrethrin, which is found in chrysanthemums. They have been modified to increase their stability in the environment. Some synthetic pyrethroids are toxic to the nervous system.

2.2 Cypermethrin

It is a composite pyrethroid; a broad spectrum, non-cumulative insecticide and a fast-acting neurotoxin with good contact and stomach action. It has of moderately high toxicity to mammals and readily metabolized with immediate loss of activity. Cypermethrin is not a plant systemic, it is readily degraded on soil or plants but has good residual activity on inert surfaces. Cypermethrin is primarily absorbed from the gastrointestinal tract. It may also be absorbed by inhalation of spray mist and only minimally through the intact skin. The ADI level of cypermethrin is 0.05 mg/kg.

2.3 Imidacloprid

Acetylcholine esterase enzyme inhibitor when compared to many older synthetic pesticides imidacloprid, is only moderately toxic to mammals, including humans. It is, however, highly toxic to other "non-target" and beneficial insect species. So, as always, care should be taken to avoid misapplication. The ADI level of imidacloprid is 0.06 mg/kg.

3. Pesticide uses

Modern agricultural practices especially the use of pesticides and fertilizers have brought about green revolution in many countries and have infact provided global food security. While pesticide uses have improved world food supply and have been responsible for

better growth and yield, their irresponsible and indiscriminate uses have greatly increased environmental health problems. There are more than 1000 agrochemicals which are being manufactured and used for agriculture as well as public health purposes. Pesticides are an essential tool for increasing the agricultural production. To produce good crop, favorable conditions and suitable chemicals are required. Pakistan is basically an agricultural country, with its economy largely depending upon a good crop yield. Pesticide usage has been undergoing a steady increase in Pakistan, along with the rest of the world. To support a large human population of about 180 million only 254 metric tons of pesticide formulation was used in 1954, however, annual production of pesticides by 18 member companies of the Pakistan Agricultural Pesticide Association (PAPA) was 23,457 and 18,774 metric tons, in the year 1998 and 1999, respectively. The Asia-pacific region is predominantly an insecticide market. National and multinational companies are engaged in the lucrative business of formulating these pesticides in Pakistan. In Pakistan, organophosphorus insecticides dominate the market with 37 percent share, followed by carbamates (23%), synthetic pyrethroids (22%), biopesticides (12%) and organochlorines (6%). In pesticide industry, the principal pollutants are a variety of the active pesticide ingredients, solvents, and other chemicals, wasted during the process, in the form of waste chemicals, solid waste, waste water and air emissions. The pesticides packaging industries in Pakistan are contributing relatively high quantities of toxic pesticides in the environment, as most of them have either no treatment facilities or have grossly inadequate handling arrangements. Moreover, there is no concept of personnel safety in our most of the pesticides industries.

4. Pesticide exposure

Pesticides exposure occurs in different ways in different ways: dermal, oral, respiratory and conjunctival routes.

4.1 Dermal exposure

It occurs by not washing hands after handling pesticides or their containers. Splashing or spilling of pesticide on skin by wearing pesticide-contaminated clothing and applying pesticides in the windy weather. Touching treated plants or soil also leads to dermal exposure. Exposures occur by rubbing eyes or forehead with pesticides contaminated gloves or hands, splashing pesticides in eyes, application in windy weather, drift exposure and mixing/loading of dry formulations without wearing goggles.

4.2 Oral exposure

Hands not washed before eating, smoking or chewing, pesticide splashed into mouth. Accidental application of pesticides to food, storing pesticides in drinking containers and drift on lip or in mouth also leads to oral exposure.

4.3 Inhalational exposure

Exposed to drift during or after spraying, mixing/loading, dusts, powders or other dry formulations. Use of inadequate or poorly fitted respirators.

5. Pesticides poisonings

Agro-chemical industry has offered thousands of compounds. The climatic condition of Pakistan favors pest build up that destroys about 20 percent of potential agricultural crop. The health of the pesticides handlers and farmers in particular are at high risks due to irrational use of pesticides.
Pesticides cause the acute and chronic health effects; organophosphate and carbamate groups are more important. These insecticides inhibit cholinesterase, an enzyme critical for normal functioning of the nervous system

5.1 Prevalence of pesticides poisoning

In USA, more than 18,000 products are licensed for use and each year more than 2 billion pounds of pesticides are applied to crops, gardens, in homes etc. (U.S EPA, 2002). The major economic and environmental losses due to the application of pesticides in public health were 1.1 billion dollars per year in USA (Pimentel, 2005). Such wide spread use results in pervasive human exposure. Evidence continues to accumulate that pesticide exposure is associated with impaired health. Occupational exposure is known to result in an annual incidence of 18 cases of pesticides related illness for every 100,000 workers in U.S (Calvert et al., 2004). Pesticide poisoning is a major public health problem in many developing countries (Xue et al., 1987; Jeyaratnam, 1990). In developing world, pesticide poisoning causes more deaths than infectious diseases. Pesticide poisoning among farmers and occupational workers in developing countries is alarming (McCauley et al., 2006).
WHO estimated approximately 20,000 workers die from exposure every year, the majority in developing countries (Pimentel et al., 1992; Kishi et al., 1995). The number of intoxications with organophosphates is estimated at some 3000,000 per year and the number of deaths and casualties some 300,000 per year (Peter, 2003). Ahmed and co workers have reported 64 percent of fatal cases of acute pesticides poisoning in Multan, Pakistan occurred due to OPs pesticide spraying (Ahmed et al., 2002) However another study revealed 21 percent of occupational pesticides poisoning in hospitalized patient (Afzal et al., 2006).

5.2 Acute toxicity

Organophosphorous compound exert acute systemic toxicity by inhibiting the enzymes AChE through a process of phosphorylation. Pesticides bind to cholinesterase and block the hydrolysis of the acetylcholine and acetic acid at the post synaptic junctions without junctioning acetyl cholinesterase; acetylcholine accumulates (Chan and Critchley, 1998; Mason, 2000). OPs induced neuronal symptoms are a consequence of axonal death. Following OPs exposures inhibition of neuronal enzymes, called neuropathy target esterase, occurs and many of them are irreversible.

6. Health effects of pesticide

6.1 Hepatic dysfunctions

Liver enzymes may be used to detect the effect of pesticides before adverse clinical health effects occur (EI-Demerdash et al., 2001). Prolonged exposure to multiple pesticide, affected liver and kidney (Azmi et al., 2006). There is also an increase in the prevalence of liver

disorders among Ranch Hand workers in high exposure category (Michalek et al., 2001). Statistically significant aspartate aminotransferase (AST) levels were found in agriculture workers continuously exposed to pesticides (Hernandez et al., 2006). Higher levels of gamma glutamyl transferase (GGT), (GOT), were found in the blood of occupational workers chronically exposed to pesticides (Michalek et al., 2001; Misra et al., 1985). An increased rise of liver dysfunction was observed with elevated (AST), alanine aminotransferase (ALT), or lactate dehydrogenase (LDH) (Michalek et al., 2001; El Demerdash et al., 2001). AST and ALT are involved in the breakdown of amino acids into α-keto acid, which are routed for complete metabolism through the Kreb's cycle and electron transport chain. Consequently they are considered as a specific indicator for liver damage (Harper, 1979). The increased activity of serum AST and ALT indicated hepatocellular dysfunctions (Yousaf et al., 2006). A positive correlation of pesticides with the liver enzymes has been reported by many researchers (Carvalho, 1991; Azmi et al., 2006).

Invivo and invitro studies showed that organophosphorous, organochlorine and pyrethroids pesticides caused increase of LDH activity (Bagchi et al., 1995; Yousaf et al., 2006). LDH is used as an indicator for cellular damage and cytotoxicity in pesticides exposure (Bagchi et al., 1995).

Subtle nephrotoxic changes in workers occupationally exposed to pesticides with higher levels of serum creatinine and or blood urea have been reported (Kossomann et al., 2001; Al Qarawri and Adam, 2003). Serum creatinine was also significant in intensive agriculture workers or applicators and pesticides plant workers (Hernandez et al., 2006; Attia, 2006).

Various animal studies also revealed similar findings.

Among other serum markers serum phosphorous was also found to be significantly high in prolong pesticides exposures (Hernandez et al., 2006).Pervious epidemiological studies reported phosphorous levels beyond the normal range in 7 percent of pesticide applicators (Parron et al., 1996).

6.2 Nephrotoxic effects

Cholinesterase inhibitor poisoning has both short and long term neuropsychological sequelae (Tapi et al., 2005). Recent studies have examined a link between pesticides exposure and neurological outcome (Kamel and Hoppin 2004). OPs being inhibitors of esterases lead to the accumulation of acetylcholine at nerve endings leading to cholinergic crises, by initial stimulation and eventually exhaustion of cholinergic synapses (Singh and Sharma, 2000). Clinical features reported anxiety depression, irritability, psychotic symptoms, and erectile disorders were more significant in pesticides applicators than controls (Amr et al., 1997). Chronic low-dose exposure leads to the neuropsychiatric consequences among the tobacco agricultural workers in Brazil (Salvi et al., 2003). Intermediate syndrome is a major cause of morbidity and mortality in patients with acute OP poisoning (Yang and Deng, 2007).

6.3 Chronic toxicity

Delayed effects of pesticide are illnesses or injuries that do not appear immediately (within 24 hours) after exposure to a pesticide. Adverse effects may be delayed for weeks, months or even years after the first exposure to a pesticide. Depending upon the toxicity of the

compound, dosage and exposure time, the adverse effects of pesticides poisoning ranges from headaches, vomiting, skin irritation, respiratory problems to other neurological disorders (Jors et al., 2006). Available evidence suggest that there is a possibility of adverse effects occurring below OP compounds concentrations that are generally considered to be safe based on measurement of AChE inhibition (Singh and Sharma, 2000; Salvi et al., 2003). A study in Srilanka has shown inhibition of AChE enzyme and impairment of sensory and motor nerve conduction due to long term, low level exposure to OPs. (Smit et al., 2003). Farm workers and green house workers exposed to organophosphates reported more symptoms than unexposed workers (Strong et al., 2004). Pesticide poisoning is associated with increased symptom prevalence (Kamel and Hoppin, 2004; Jors et al., 2006). Farmers and farm workers who applied organophosphate had higher symptoms prevalence than did non applicators (London et al., 1998; Beshwari et al., 1999; Ohayo-Mitoko et al., 2000).
Farm workers with little access to information about safety practices or protective equipment may sustain for more exposure (Pimetal et al., 1992). Diverse trends were seen regarding the disposal of pesticide residues. Due to casual attitude towards the handling of pesticide, many farmers threw pesticide containers in field or irrigating water which increases environmental pollution and health hazards in the community. This malpractice causes increased pesticide residues in human blood and water (Ansari et al., 1997; Ahad et al., 2006). The health effects of low dose pesticides exposure are very difficult to evaluate mostly when pesticides mixtures are used (Lewalter and Leng, 1999; Carpy et al., 2000).

6.4 Carcinogenesis

Epidemiological studies have implicated pesticides as causative agent in human cancer (Zahm and Blair, 1992). Cancer and even death is more frequent among farmers rather than general population (Gertrudis et al., 2001). Cytogenetic studies showed an increase in DNA damage and higher chromosomal aberrations (CAs) in exposed farmers compared to the control subjects (Naravaneni, 2007). A study documented significant genotoxic exposure of pesticides on the basis of significant decrease in the level of serum ChE among workers involved in pesticide manufacturing industry (Bhalli et al., 2006). There was an increased risk of lung cancer reported in German agricultural workers, which also increased with the length of exposure (Barthel, 1981).

6.5 Oxidative stress

Recent studies have shown that oxidative stress could be an important component of the mechanism of OP compound toxicity. Several studies explained that oxidative stress is involved in OPs toxicity (Parakasam, 2001; Hsu et al., 2001). Repeated daily oral doses of pesticide in rats altered the biochemical parameters and antioxidant status (Manna, 2004). Several studies reveal that OPs may induce oxidative stress leading to generation of free radicals and alteration of antioxidant status (Bagchi, 1995; Abdhollahi, 2004). Toxic effects of pesticide on human beings especially by omitting radical production can be confirmed by the direct measurement of lipid per oxidation by-product malondialdyhyde (MDA), (Muniz et al., 2007). Our study revealed a significant rise in Plasma malondialdehyde (MDA) levels in exposed farmers than in controls. The results of our study were consistent with other studies suggesting that pesticides increase oxidative stress in humans (Prakasam et al 2001;

Singh et al., 2007). There is increasing evidence that OP and carbamate induced oxidative stress through the generation of free oxygen radicals, leading to lipid peroxidation and DNA damage (Hazarika et al., 2003; Abdollahi et al., 2004; Vidyasagar et al., 2004; Shadnia et al., 2005). Muniz and co workers reported MDA levels 4.9 times and 24 times higher in farm workers and applicators respectively than in controls (Muniz et al., 2007). Pesticides induce a wide array of human health effects through oxidative stress causing cytogenetic damage and carcinogenicity (Mansour, 2004).

7. Monitoring of pesticide exposure

The health effects of pesticides exposure are difficult to monitor in the farmers especially when mixture of pesticides are used over the period of time. The term biomarker is used to include almost any measurement reflecting an interaction between a biological system and an environmental agent, which may be chemical, physical, or biological. Biomarkers can be used to identify causal associations and to make better quantitative estimates of those associations at relevant levels of exposure (WHO, 1993). They may also make it possible to identify susceptible groups or individuals who are at risk of exposure to certain types of environmental and occupational agents. The advances in molecular genetic have led to an upsurge in trust in most susceptibility factors and identification of polymorphism of various enzymes has become possible (Vainio, 2001). Biomarkers include detection of the environmental substance itself or its metabolites in urine or blood, changes in genetic material, and cell death. The biological events detected can represent variation in the number, structure, or function of cellular or biochemical components. Recent advances in molecular and cellular biology allow for measurement of biological events or substances that may provide markers of exposure, effect, or susceptibility in humans. Two kinds of measurement have been used for assessing the exposure to pesticides which includes enzymes activity and pesticides residue in the blood.

Inhibition of plasma cholinesterase serves as a diagnostic tool for the risk assessment of exposure to toxic organophosphates (Amitai et al., 1998). Inhibition of AChE activity has been widely used to assess OPs exposure. A study revealed a high prevalence of pesticides poisoning in agricultural farmers by OPs and carbamates exposure with the reduction of AChE. Green house farm workers also showed the same results (Karabay et al., 2004). Low levels of serum cholinesterase on moderate and prolong pesticide exposure is also reported by many researchers (Gertrudis et al., 2001; Hernandez et al., 2004). Significant decreases in AChE levels were found in pesticides applicators (Kesovachandran et al., 2006). Acetyl cholinesterase activities significantly decrease at the period of maximum exposure to pesticides; pinpoints certain inhibitory effect of pesticides on this esterase (Hernandez et al., 2004; Jors et al., 2006). Decrease in levels of serum cholinesterase found higher in workers with prolong exposure than those with shorter exposures (Karabary et al., 2004; Bhalli et al., 2006). Inhibition of plasma cholinesterase serves as a diagnostic tool for the risk assessment of exposure to toxic OPs (Amitai et al., 1998). As the levels of AChE activity returns to normal within days to weeks after exposure to OPs. It thus serves as a measure for fairly recent exposure (Mason, 2000). Pesticides and their metabolites can be measured in biological samples, serum, fat, urine, blood, or breast milk by the usual analytical techniques or by biological method. A number of reports are available in which insecticides and/or

their metabolites have been measured in body fluids after occupational exposures (Coye, 1986). A positive effect will be indicative of exposure. Pesticides residual analysis is mainly done on High Performance Liquid Chromatography (HPLC) and Gas Chromatography (GC).

8. Factors effecting implementation of waste reduction

Several factors to affect the effectiveness of in-plant modifications and changes upon implementation of the waste reduction proposals can be identified as follows:

i. manufacturing practices,
ii. housekeeping and water conservation practices,
iii. equipment operation and maintenance,
iv. measurement of losses,
v. attitude of workers,
vi. education and training of personnel

Apparently, all factors are largely dependent upon the management stand on environmental issues. Key environmental issues associated with the industry, in order of their significance, are as follows:

8.1 Environmental and public health impacts

All pesticides are potentially toxic and hazardous to human beings. Severity of hazard, posed by a pesticide, depends on its toxicity, route of exposure, whether oral, dermal or inhalation, and the extent of exposure. Xylene and methyl ethyl ketone are the most commonly used solvents for pesticides. Short term exposure to high doses can cause irritation of the skin, eyes, nose and throat, difficulty in breathing, impaired functioning of the lungs, delayed response to a visual stimulus, impaired memory, stomach discomfort and possible changes in the liver and kidneys. Both short and long term exposure can also affect the nervous system.

8.2 Waste chemicals and contaminated solid waste (Metcalf & Eddy, 1985)

The major source of waste chemicals and solid wastes, which are contaminated with pesticides include defective and expired bottles and containers, expired pesticides, corrugated board carton, waste cotton rags, saw dust, spent (charcoal and activated carbon) and emptied drums. Current disposal practices being followed in some units in Pakistan includes, carrying out open burning of the plastic bottles, along with other c contaminated waste either within the factory premises or at some remote areas. Some of the units have installed their own in-house incinerators to dispose waste whereas some units use commercial incineration facilities to dispose their contaminated wastes.

Generally the contaminated solid wastes are collected separately in waste bins and stored, till further disposal. During storage, in most cases, the waste chemical drums and different contaminated solid wastes are not placed on impervious floors. This can lead to contamination of soil and groundwater, consequent to any leakage and spillage.

8.3 Air pollution

Principal emissions, from the liquid pesticides pumping, formulation and filling facilities, are the vapors and fumes of active ingredients and organic solvents. Main emission from the

powder and granule pesticides filling units is toxic particulate matter. In-house waste incinerator (where provided) is another source of air emission. In order to minimize the dispersion of emissions into the general working air, most of the pesticides units have installed spot or local forced ventilation systems. Some of the treatment methods used is charcoal or activated carbon adsorption filters, fabric bag filters and wet scrubbers.

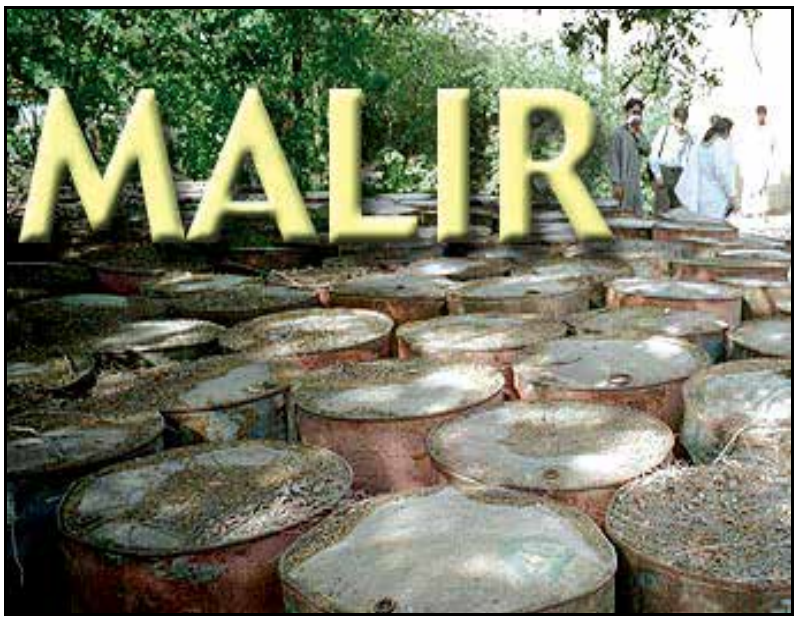

Fig. 1. Improper pesticide storage

Fig. 2. Corroded pesticide container

Fig. 3. Extensive pesticide usage

8.4 Occupational health and safety (OHS)

Most of the units are seriously concerned with workers health issues. Generally, they have their own OHS plans and policies, which they endeavour to, implement and follow. Following OHS issues, which require more attention are identified because without following these practices a proper assessment of the workers exposure cannot be made:

i. Most of the units are not carrying out the required monitoring of the working air quality, with respect to pesticides and solvents.
ii. Records of accidents and disease are not being properly maintained.
iii. Many of the antidotes are not available readily in the market, this situation is not satisfactory to cope with emergency.

Fig. 4. Improper handling of pesticides

Fig. 5. Poor house keeping of pesticides

8.5 Water pollution

Pesticide plant has been operating to a significant degree of water wastage leading to a considerable amount of effluent generated. Possible sources of wastewater are as follows:

i. Wet scrubbers are installed in some plants, the spent scrubbing liquid, discharged from these units, is quite high in pesticides contents.
ii. Laundries practicing washing workers uniform discharge relatively large quantity of polluted wastewater.
iii. The pesticide and organic solvents content in the floor washing wastewater, primarily, depend upon the floor cleaning method, consequently to any chemical spillage.

In some plants, the wastewater which has high pesticides contents is treated prior to its disposal to the drainage system. Plant operation data revealed that most of the colloidal and suspended pesticide contents can be separated from such wastewater, in the form of sludge, by the use of coagulation process at an increased pH value. The treated effluent is reported to have total pesticide contents within the permissible limits of National Environmental Quality Standards (NEQS).

8.6 Common process solid waste

This mainly comprises of the packaging waste, including damaged bottles, containers, cartons, plastic caps, labels and stickers, shrink wrappers, aluminum seals, polythene and paper bags and wooden pallets. Most of these are placed separately from contaminated waste and sold for down stream recycling and reuse. The generation of waste products of pesticides is given in Table 2.

8.7 Treatment

Industry has a modern wastewater treatment facility at their crop protection agricultural division**.** The Hoval pyrolytic non-polluting waste incinerator is especially designed to burn toxic waste materials at high temperatures required for destruction of toxic materials. The effluent treatment unit especially designed for treatment of agrochemical wastewater is able to reduce the concentration of chemicals in the raw effluent to less than 0.001 ppm.

The drum cleaning station is meant for washing empty steel drums, nowadays not in common practice in order to avoid rinsed water. The cleansed are then crushed in the drum crusher and disposed of in steel smelters.

Facilities	Installed Capacity (Quantity per annum per shift)	Capacity utilized
Facility Number One	Liquid 25000 liters (per day)	20,000 liters (per day)
	Granules 8 tons (per day)	6 metric tons (per day)
Facility Number Two	Liquid 4.5 milli liters	Nil
	Powder 1.5 milli kilogram	0.75 milli kilogram
Facility Number Three	Liquid 1.44 milli liters	1 milli liters
	Granules 600 metric tons	Nil
	Powder 1200 metric tons	100 metric tons

Source: Based on data provided by Pakistan Agricultural Pesticides Association (PAPA), 2001

Table 2. Formulating capacities of three major industrial facilities operating in Pakistan

8.7.1 Incinerator

Equipped with the three burners termed as primary and secondary burners, quenching of the water is done automatically whenever required to achieve stable conditions.

Temperature of the primary burner is 500-750°C.

Temperature of the secondary burners 1000-1200°C (holding time is 2-seconds).

Usually solid and liquid wastes are handled by the incinerator: These include all types of plastics, papers, wood products, all expired production stock and active ingredients, solvents and washing water. Total Capacity of the incinerator is approximately 600 kg/day.

8.8 Cleaner production (CP) techniques

In pesticide industry, the principal pollutants are the active pesticide chemicals and solvents, wasted during the process. Some measures for CP techniques are discussed:

8.8.1 Management of waste chemicals and contaminated solid waste

i. All the waste chemicals and contaminated solid waste should be segregated from other wastes and properly stored, till their disposal.
ii. The quantities, generation rates and pertinent characteristics of the wastes produced should be regularly measured and documented.
iii. Incineration is considered to be a suitable technology for the disposal of waste chemicals and contaminated solid waste of pesticide industry.
iv. Suggested contaminated waste incinerator, is rotary kiln type. The air emissions should be monitored for the applicable parameters, to check their conformity to the NEQS.

8.8.2 Air pollution control

There is a need to establish and monitor the occupational air quality, for the protection of the health and safety of the workers. The process of controlling the occupational air quality comprises the following sequential steps:

i. Establishing the occupational air quality criteria for the protection of the health and safety of the workers.
ii. Planning, design, installation and operation of a comprehensive system which ensures maintenance of the occupational air quality, within the desirable limits.
iii. The workers carrying out activities close to the pollutant emissions sources should wear personal protection equipment.
iv. The exhaust air carrying vapors and fumes from liquid pesticides processes should be treated with charcoal or activated carbon adsorption filters.

8.8.3 Occupational health and safety (WHO, 1996-1997)

i. The Material safety data sheets (MSDS) should be readily available on site.
ii. Due to high fire risk, the fire fighting system and emergency planning should be well established.
iii. The management and the workers should be trained to avoid personal exposure to hazards and risks.
iv. The antidotes should be stocked to handle emergency situations.

8.8.4 Reduction of water pollution

It is suggested that waste chemicals including pesticides as well as solids and solvents, should not be allowed to mix into wastewater streams, as far as possible.

i. Unavoidabe liquid pesticide spillage on floors, resulting from leakage and spilling of containers, vessels and process equipment, should not be cleaned by direct water washing, instead these should be dry cleaned, by absorption with sawdust or cotton rags.
ii. The toxic particulate matter, emitted from the powder and granule filling machine should be separated by means of fabric bag filters, instead of wet scrubbers and the collected powder should be reused.
iii. The tested samples from laboratory should be separately stored and treated as waste chemicals, for further proper disposal.

9. KAP information

The industrial workers' knowledge regarding the health hazards of pesticide and safety were assessed on the pre-tested questionnaire by the health team. A model questionnaire used in the study is given in Figure 6.

9.1 Knowledge

Among 184 workers, 62% were not literate and a few had completed their high school level education. Only the workers of FMC had formal training in safe pesticide handling and packing. Most of the workers (90%) had knowledge about the detrimental health effects of pesticide but were not well aware of the safety precautions regarding pesticide handling. However many workers reported to wash hands and bodies after work.

9.2 Attitude

The participants had casual attitude in handling of pesticide and personal protection. We observed that most workers of alpha chemical did not use any personal protective equipment (PPE) during pesticide handling while one industry chemical workers used masks. Farmers did use the gloves and face masks while mixing and filling of tanks. Another industry workers used proper protective head cover, gloves and masks during work.

9.3 Observed practices

Most workers of two industries wore traditional cotton shalwar kameez whereas third industry workers used cover all during work. Majority of the workers were unable to comprehend the safety instructions written in English. FMC workers pesticides exposure was regularly monitored by measuring ChE levels.

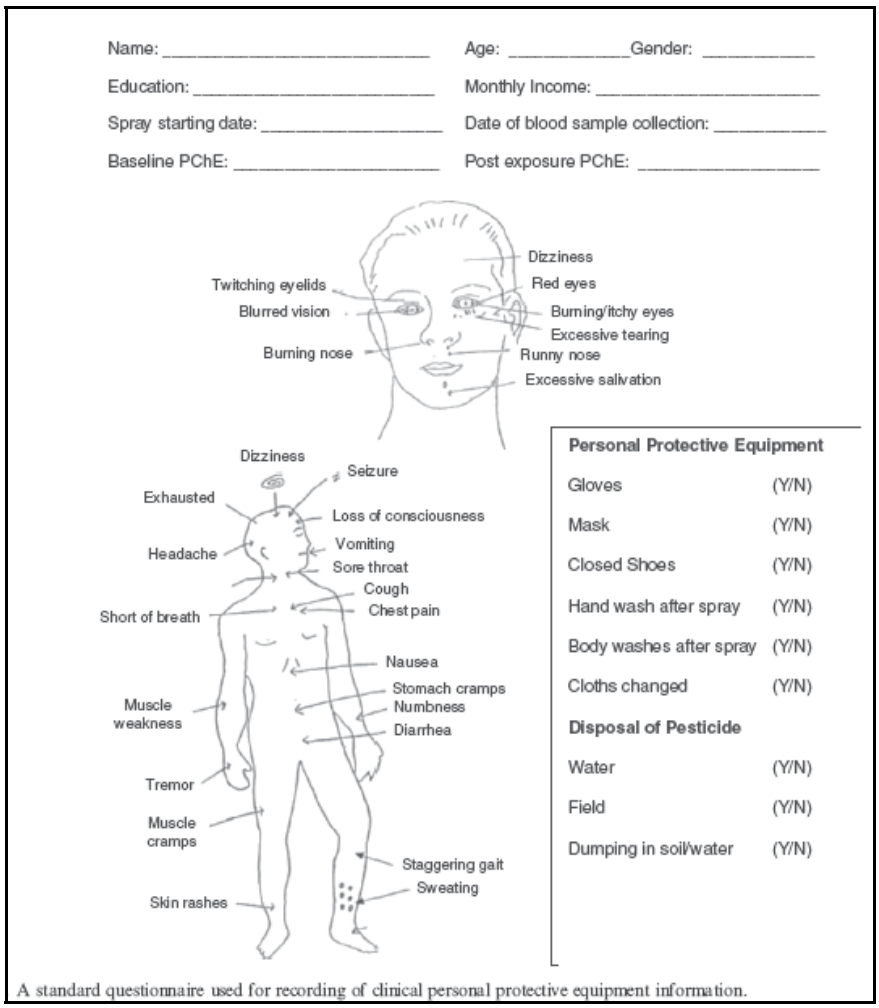

Fig. 6. A standard questionnaire used for recording clinical personal protective equipment information

10. References

Abdollahi, M.; Mostafalou, S.; Pourourmohammadi, S. & Shadnia, S. (2004). Oxidative stress and cholinesterase inhabitation in saliva and plasma of rats following subchronic exposure to malathion Comp. *Biochem. Physiol*. Part C., 137: 29-34.

Australian Government, Department of Health Sciences and Ageing, Office of Chemical Safety (2008). Acceptable daily intakes for agricultural and veterinary chemicals.

Afzal, S.; Ahmad, M.; Mubarik, A.; Saeed, F.; Rafi, Sh.; Saleem, N. & Qureshi, A.H. (2006). Acute organophosphorus poisoning-an experience. *Pak. Armed. Forces. Med. J.*, 56(2):150-56.

Ahad, K.; Mohammad, A.; Mehboob, F.; Sattar, A. & Ahamd, I. (2006). Pesticide residues in Rawal Lake, Islamabad, Pakistan. *Bull. Environ. Contam. Toxicol*., 76(3):463-70.

Ahmad, R.; Ahad, K.; Iqbal, R. & Muhammad, A. (2002). Acute Poisoning due to commercial pesticides in Multan. *Pak. J. Med. Sci*., 18., 3., 227 – 31.

Ahmed, M.; Verma, S.; Kumar, A. & Sidiqui. M. K. J. (2006). Delta-aminolevulinic acid dehydratase inhibition and oxidative stress in relation to blood lead among urban adolescent. *Hum. Experi. Toxicol*., 25., 9., 547–53.

Ajayi, O.C. & Akinnifesi, F.K.(2008). Farmers' understanding of pesticide safety labels and field spraying practices: a case study of cotton farmers in northern Cote d'Ivoire. *Sci. Res. Essays* 2., 204-210.

Al-Qarwai, A. A. & Adam, S.E. (2003). Effects of Malathion plus superphosphate or urea on Najdi Sheep. *Vet. Hum. Toxicol*., 45., 3-6.

Amitai, G.; Moorad, D.; Adani, R. &Doctor, B.P. (1998). Inhibition of acetylcholinesterase and butyl cholinesterase by chlor pyrifos – Oxon. *Biochem. Pharmacol*., 56., 293-9.

Amitai, G.; Moorad, D.; Adani, R. & Doctor, B.P. (1998). Inhibition of acetylcholinesterase and butyl cholinesterase by chlor pyrifos – Oxon. *Biochem. Pharmacol*., 56:293-9.

Amr, M.M.; Halim, Z.S. & Moussa, S.S. (1997). Psychiatric disorders among Egyptian pesticides applicators and formulators. *Environ. Res*., 73., 193-99.

Ansari, MT.; Iqbal, Z. & Ahmad, B. (1997). Organochlorines pesticide residues in human blood in the population of Multan (Pakistan). *Pak .J .Pharm. Sci*., 10.,1., 19-28.

Atreya, K. (2008). Health costs from short-term exposure to pesticides in Nepal. Soc. *Sci. Med*., 67., 511-519.

Attia, M. A. (2006). Risk assessment of occupational exposure to pesticides. Earth. *Environ., Sci*., 3:349-62.

Azmi, M.A.; Naqvi; Azmi, M. A. &Aslam, M. . (2006). Effect of pesticide on health and different enzymes levels in the blood of farm workers from Gadap (rural area) Karachi- Pakistan. *Chemo*., 64., 10., 1739-44.

Azmi, M. A.; Naqvi, S. N.; Azmi, M. A. & Aslam, M. (2006). Effect of pesticide residues on health and different enzyme levels in the blood of farm workers from Gadap (rural area) Karachi-Pakistan. *Chemo*., 64., 10., 1739-44.

Bagchi, D.; Bagchi, M.; Hassoun, E. A. & Stohs, S. J.(1995). In vitro and in vivo generation of reactive oxygen species DNA damage and lactate dehydrogenase leakage by selected pesticides. *Toxicol*., 104., 3129-40.

Barthel, E. (1981). Increased risk of lung cancer in pesticide-exposed male agricultural workers. J. Toxicol. Environ. Health., 8., (5-6)., 1027-40.

Beshwari, M. M. M.; Bener, A.; Amer, A.; Mehdi, A.M.; Onda, H.Z. & Pasha, M. A. H. (1999). Pesticide related health problems and diseases among farmers in the United Arab Emirates. 9., 3., 213-21.

Bhalli, J.A.; Khan, Q. M., Hag, M.A.; Khalid. & Nasim, A. (2006). Cryptogenic analysis of Pakistani individuals occupationally exposed to pesticides in a pesticides production industry. *Mutag*., 21., 143-148.

Calvert, G.M.; Plate D. K.; Das, R.; Rosales, R.; Shafey, O. & Thomsen, C. (2004). Acute occupational pesticide-related illness in the U.S. 1998-1999: Surveillance findings form the SENSOR-pesticides Program. *An. J. Ind. Med*., 45., 14-23.

Carpy, S.A.; Kobe, W. & Doe, J. (2000). Health risk low dose pesticides mixtures: A review of the 1985-1998 literature on combination toxicology and health risk assessment. J. Toxicol. Environ. Health., 3., 1-25.

Carr, S.J. (1989). Technology for small-scale farmers in Sub-Saharan Africa: Experience with food crop production in five major ecological zones. World Bank – Technical Papers, Paper 109, World Bank: Washington, DC.

Carson, R. (1985). The Silent Spring. Harmond Sworth Penguin.

Carvalho, W.A. (1991). Risk factors related with occupational and environmental exposure organochlorine insecticides in the state of Bahia, Brazil. *Bol. Oficina. Sanit. Panam*., 111., 512-24.

Chalermphol, J. & Shivakoti, G.P. (2009). Pesticide use and prevention practices of tangerine growers in northern Thailand. *J. Agr. Educ. Ext*., 15., 21-38.

Chan, T.Y.K. & Critchley, J.A.J.H. (1998). Insecticides poisoning with Organophosphates and carbamates. Hong Kong. *Practioner*., 20., 11., 604-13.

Coronado, G.D.; Thompson, B.; Strong, L.; Griffith, W.C.; Islas, I. (2004). Agricultural task and exposure to organophosphate pesticides among farm workers. *Environ. Health 18 Persp*., 112., 142-147.

Coye, M.J.; Lowe, J.A. & Maddy, K.J. (1986). Biological monitoring of agricultural workers exposed to pesticides. 11: monitoring of intact pesticides and their metabolites. *J. Occup. Med*., 28., 628-36.

Damalas, C.A.; Georgiou, E.B.; Theodorou, M.G. (2006a). Pesticide use and safety practices among Greek tobacco farmers: A survey. *Int. J. Environ. Health Res*. 16., 339-22 348.

Damalas, C.A.; Hashemi, S. M. (2010). Pesticide risk perception and use of personal protective equipment among young and old cotton growers in northern Greece. *Agrociencia* (in press).

Damalas, C.A.; Theodorou, M.G.; Georgiou, E.B. (2006b). Attitudes towards pesticide labelling among Greek tobacco farmers. Int. J. Pest Manag. 52., 269-274.

Economopoulos, A. (1993). Assessment of sources of Air, Water and Land pollution: A guide to rapid source inventory techniques and their use in formulating environmental control strategies. WHO, Geneva.

El-Demerdash, F. M.; Yousaf, M.I. & Elagamy, E.I. (2001). Influence of paraquat, glyphosphate and cadmium on the activity electrophoretic behavior (in vitro) *J.Environ. Sc. Health*. Part B., 36., 29-42.

ETPI (2001). Environmental Technology Programme for Industry. Draft Environmental Report of Pesticides Sector: A joint demonstration project of FPCCI and Government of The Netherlands. Federation House, Karachi, Pakistan.

FAO. (1986). Food and agricultural organization of the United Nation. International code of conduct on the distribution and use of pesticides, Rome.,

Gertrudis, C.; Mario, V.; Arnaldo, V.; Viviana, D.; Isolde, R.; Nicolas, H. & Gloria, C. (2001). A rat mammary tumor model induced by the organophosphorus pesticides parathion and Malathion, possibly through acetyl cholinesterase inhibition. Environ. Health. Perspec. Pect., 109., 5., 211-221

Harper, H.A.; Rodwell, V.W. & Mayers, P.A. (1997). Review of physiological chemistry, Middle East. Edn.

Hashemi, S.M.; Mokhtarnia, M.; Erbaugh, J.M.; Asadi, A. (2008). Potential of extension workshops to change farmers' knowledge and awareness of IPM. Sci. *Total Environ*., 407., 84–88.

Hazarika, A.; Sarkar, S.N.; Hjare, S.; Kataria, M. & Malik, J.K. (2003). Influence of malathion pretreatment and the toxicity of anilofos in male rats: a biochemical interaction study. *Toxicol*., 185., 1-8.

Hernandez, A, F.; Gomez, M.; Penan. A. G.; Fernandez, Gil.; Lourdes, R.,; Villanueva, Enrique. & Antonio, Pla. (2004). Effect of long term exposure to pesticides on plasma esterases from plastic green house workers. *J. Toxicol. Environ. Health*. Part A., 67., 14., 1095-08.

Hernandez, F.A.; Gomez, M.A.; Perez, V.G.; Lario, V.J.; Pena, G. & Gill .F. (2006). Influence of exposure to pesticides on serum components and enzyme activities of cytotoxicity among intensive agricultural farmers. *Environ. Res*., 102., 70-76.

Hsu, D.Z.; Hsu, C.H.; Huang, B.M. & Liu, M.Y. (2001) Abamectin effects on aspartate aminotransferase and nitric oxide in rats. *Toxicol*., 165., 189-193.

Hurtig, A.K.; Sebastian, M.S.; Soto, A.; Shingre, A.; Zambrano, D.; Guerrero, W. (2003). Pesticide use among farmers in the Amazon Basin of Ecuador. *Arch. Environ. Health,* 14., 58., 223-228.

Ibitayo, O.O. (2006). Egyptian farmers' attitudes and behaviors regarding agricultural pesticides: Implications for pesticide risk communication. *Risk Anal*., 26., 989-995.

Javed, A. B.; Khan, Q. M.; Haq, M. A.; Khalid, A. M. & Nasim, A. (2006). Cytogenetic analysis of Pakistani individual's occupationally exposed to pesticides in a pesticide production industry. *Mutagenesis,* 21., 2., 143-148.

Jeyaratnam, J. (1990). Acute pesticide poisoning: A major global health problem. World Health Statistic Quarterly. 43., 3., 139-44.

Jors E,; Morant, R.E.; Aguilar, G. C.; Huici, L. F.; Baelum J & Konradsen, F. (2006). occupational pesticides intoxications among farmers in Bolivia a cross-sectional study. Environ. Health. Global Sci., 5., 10.

Kamal, F. & Hoppin, J.A. (2004). Asocition of pesticide exposure with neurologic dysfunction and disease. Environ. Health. Persp., 112., 9., 950-58

Karabay, N.U.; Cakmak, B.; Saym, F. & Oguz, M.G. (2004). Risk Assessment of Organophosphate Pesticide Exposure on Greenhouse Workers in Menderes Region, Develi Village (Turkey). T. Klin. Tip. Bilimleri., 24., 6-11.

Kesovachandran, C.N.; Rastogi, S.K.; Mathur, N.; Siddiqui, M.K. J.; Singh, V.K.; Biharivipin & Bhasti, R.S. (2006). Health status among pesticide applicators at a Mango plantation in India. *J. Pest. Safety*., 8., 1-8.

Khan, D. A.; Mahwish, M. B.; Farooq, A. K.; Naqvi, T. A. & Karam, A. (2008). Adverse effects of pesticides residues on biochemical markers in Pakistani Tobacco Farmers. *Int J Clin Exp Med*., 1., 274-282.

Khan, D. A.; Hashmi, I. ; Mahjabeen, W. & Naqvi, T. A. [Epub ahead of print] (2009). Monitoring health implications of pesticide exposure in factory workers in Pakistan. *Environ Monit Assess*. DOI: 10.1007/s10661-009-1107-2

Khan, D. A.; Saira, S.; Mahwish, M. B.; Farooq, A. K. & Naqvi, T. A. (2010). Risk assessment of pesticide exposure on health of Pakistani tobacco farmers. Journal of Exposure Science and Environmental Epidemiology. 20., 196–204.

Khan, D. A.; Mahwish, M. B.; Farooq, A. K. & Naqvi, T. A. (2008). Evaluation of pesticides induced toxicity by oxidative stress and inflammatory biomarkers. PAFMJ. 58., 380-386.

Kishi, M.; Hirschhorn, N.; Ojajadisastra, M.; Satterfee, L.N.; Stroman, S. & Dilts, R. (1995). Relationship of pesticide spraying to signs and symptom in Indonesian farmers. Scand. J. *Environ. Health*., 21.,124-33.

Konradsen, F.; Van der Hoek, W.; Cole, D.C.; Hutchinson, G.; 1 Daisley, H.; Singh, S.; Eddleston, M. (2003). Reducing acute poisoning in developing countries-options for restricting the availability of pesticides. *Toxicology*, 192., 249-261.

Konradsen, F.; Van der Hoek, W.; Cole, D.C.; Hutchinson, G.; 1 Daisley, H.; Singh, S., Eddleston, M. (2003). Reducing acute poisoning in developing countries-options for restricting the availability of pesticides. *Toxicology,* 192., 249-261.

Kossmann, S.; Tustanowski, J. & Kołodziej, B. (2001). Renal dysfunction in chemical plant workers producing dust pesticides. *Med. Pr*., 52., 4., 253-6.

Lewwalter, J. and Leng, G. (1999). Consideration of individual susceptibility in adverse pesticide effects. *Toxicol. Lett*., 107., 131-44.

London, L.; Nell, V.; Thompson, M. & Myers, J.E. (1998). Effects of long term organophosphate exposures on neurological symptoms vibration sense and tremor among South African farm workers. *Scand. J. Environ. Health.*, 24., 18-29.

Mancini, F.; Van Bruggen, A.H.C.; Jiggins, J. L.S.; Ambatipudi, A.C.; Murphy, H. (2005). Acute pesticide poisoning among female and male cotton growers in India. *Int. J. Occup. Environ. Health,* 11., 221-232.

Manni, S.; Bhattachryya D.; Mandal, T.K. & Das, S. (2004). Repeated dose toxicity of alfa-cypermethrin in rats'. *l. J .Vet. Sc*., 5., 3., 241-45.

Mansour, S.A. (2004). Pesticide exposure –Egyptian scene .*Toxicol*., 198., 91-15.

Mason, H.J. (2000). The recovery of plasma cholinesterase and erythrocyte acetylcholinesterase activity in workers after over exposure to dichlorovos. *Occup. Med*., 50., 5., 343-37.

Mason, H.J. (2000). The recovery of plasma cholinesterase and erythrocyte acetylcholinesterase activity in workers after over exposure to dichlorovos. *Occup. Med*., 50., 5., 343-37.

McCauley, A.; Linda, Anger, K.W.; Keifer, M.; Langley, R. & Robson, G.M. (2006). Studying health outcomes in farm worker populations exposed to pesticide. *Environ. Health. Perspec.*, 114., 6-8.

Michalek, J.E.; Ketehun, N.S.; and Longneeker, M.P. (2001). Serum dioxin and hepatic abnormalities in veteran of operation Ranch Hand. *Ann. Epidemiol.*, 11., 5., 304-11.

Misra, U.K.; Nag, D.; Dhushan, V. & Ray, P.K. (1985). Clinical and biochemical changes in chronically exposed organophosphate workers. *Toxicol. Let.*, 244., 187-93.

Muniz, J.; Mc Cualey, L.; Scherer, J.; Lsarev, M.; Koshy, M. & Kow, Y. W. (2007). Biomarker of oxidative stress and DNA damage in agricultural wokers: A Pilot study. Toxicol. *Appl. Pharmacol.*, 1-11.

Naravaneni, R. & Jamil, K.(2007). Determination of AChE levels and genotoxic effects in farmers occupationally exposed to pesticides. *Hum. Exp. Toxicol.*, 26., 9., 723-31

Ohayo-Mitoko, G.J.; Kromhout, H.; Simwa, J.M.; Boleij, J.S. & Heederik, D. (2000). Self reported symptoms and inhibition of acetylcholinesterase activity among kenyan agricultural workers. *Occup. Environ. Med.*, 57., 3.,195-00.

Oluwole, O. & Cheke, R.A. (2009). Health and environmental impacts of pesticide use practices: a case study of farmers in Ekiti State, Nigeria. *Int. J. Agr. Sustain.*, 7., 153-17 163.

Pakistan Agricultural Pesticides Association (PAPA), 2001.

Prakasam, A.; Sethupathy, S. & Lalitha, S. (2001). Plasma and RBCs antioxidant status in occupational male pesticide sprayers. *Clinica. Chimica. Acta.*, 310., 107-12.

Parron, T.; Hernandez, A.F.; Pla, A. & Villanueva, E. (1996) Clinical and biochemical changes in greenhouse sprayers chronically exposed to pesticides. *Hum. Exp. Toxicol.*, 15.,12., 957-63.

Peter, E. (2003). The role of oximes in the management of organophosphorus pesticides poisoning. *Oxical. Rev.*, 22., 165-190.

Pimental, D., Acguay, H. and Biltonen, M. (1992). Environmental and economic costs of pesticide use. *Biosci.*, 42. : 750-60

Pimentel. (2005). Environmental and Economic Costs of the Application of Pesticides Primarily in the United States. Environ. *Develop. Sustainability.* 7., 2., 229-52.

Plianbangchang, P.; Jetiyanon, K.; Wittaya-areekul, S. (2009). Pesticide use patterns among small-scale farmers: A case study from Phitsanulok, Thailand. *SE Asian J. Trop. Med. Public Health,* 40., 401-410.

Remor, A.P.; Totti, C.C.; Moreira, D.A.; Dutra, G.P.; Heuser, 1 V.D. & Boeira, J.M. (2009). Occupational exposure of farm workers to pesticides: Biochemical parameters and evaluation of genotoxicity. *Environ. Int.* 35, 273-278.

Salvi, R.M.; Lara, D.R.; Ghisolfi, E.S.; Portela, L.V.; Dias, R.D. & Souza, D.O. (2003). Neuropsychiatric evaluation in subjects chronically exposed to organophosphate pesticides. *Toxicol. Sc.*, 72., 267-71.

Shadnia, S.; Azizi, E.; Hosseni, R.; Khoe,i S.; Fouladdel, S. & Pajoumand, A. (2005). Evaluation of oxidative stress and genotoxicity in organophosphorous insecticides formulators. *Hum. Toxicol.*, 24., 9., 439-45.

Singh, S. & Sharma, N. (2000). Neurological syndromes following organophosphate poisoning. *Neurol. India.*, 48., 308-13.

Singh, V.K.; Jyoti, M.M.; Reddy, K.; Kesavachandran, C.; Rastogi, S.K.; Siddiqui, M. K. J. (2007). Biomonitoring of organochlorines, glutathione, lipid peroxidation and cholinesterase activity among pesticide sprayers in mango orchards. *Clinica. Chimica. Acta.*, 377.,268-72.

Smit, L.A.; Van-Wandel-de-Jodi, B.N. & Heedrik, D. (2003). Neurological symptoms among Srilankan farmers occupationally exposed to acetylcholinesterase- inhibiting insecticides. *Am. J. Ind. Med*., 44., 254-64.

Soares, W.; Almeida, R.M.V.R.; Moro, S. (2003). Rural work and risk factors associated with pesticide use in Minas Gerais, Brazil. *Cad. Saúde Pública.,* 19., 1117-1127.

Sosan, M.B. & Akingbohungbe, A.E. (2009). Occupational insecticide exposure and perception of safety measures among cacao farmers in southwestern Nigeria. *Arch. Environ. Occup. Health,* 64., 185-193.

Strong, L.; Thompson, B.; Gloria, D.; William, C.; Vigoren, E.M. & Islasllda P.T.O. (2004). Health symptoms and exposure to organophosphorus pesticides in farm workers. *An. J. Ind. Med*., 46., 599-06.

Tapi, L.R.; Leyva, A.; Laynez, F. & Santed, F.S. (2005). Chronic neurosycological sequelea of cholinesterase inhibitors in the absensc of structural brain damage: Two cases of acute poisoning. *Environ. Health. Perspec*., 113., 6.,:762-66.

Tariq, I.M.; Afzal, S.; Hussain, I. & Sultana, N. (2007). Pesticides exposure in Pakistan: A review. *Environ. Int.*, 33., 1107-1122.

U.S. EPA office of pesticides programs (2002). F.Y 2002 Annual report Willhington, DC: US. Environmental Protection Agency. Available: http://www.epa.gov/oppfead/ annual/2002/2022 annual report. pdf (accessed 25 March 2004.)

Vianio, H. (2001).Use of biomarkers in risk assessment. I. J. Hyg. Environ. Health., 204., 91-02.

Vidyasagar, J.; Karunakar, N.; Reddy, M.S.; Rajnarayana, K.; Surendar, T. & Krishna, D.R. (2004).Oxidative stress and antioxidant status in acute organophosphorous insecticide poisoning .*Ind. J. Pharmacol*., 36., 76-79.

Wesseling, C.; Aragon, A.; Castillo, L.; Corriols, M.; Chaverri, F.; de la Cruz, E.; Keifer, M.; Monge, P.; Partanen, T.J.; Ruepert, C.; van Wendel de Joode, B. (2001). Hazardous pesticides in Central America. Int. J. Occup. Environ. Health 7., 287-294.

WHO recommended classification of pesticides by hazard and guidelines to classification. 2000-01 Geneva. (2000). World Health Organization (document reference WHO/PCS/01.4)

WHO/IPCS. Environmental health criteria 155. (1993). Biomarkers and risk assessment: Concepts and Principles. Geneva: World Health Organization.

WHO (1996-1997). The WHO Recommended Classification of Pesticides by hazard and guidelines to classification (Eds.), WHO/PCS/96.3, World Health Organization, International Programme on Chemical Safety (IPCS), Geneva, Switzerland.

Xue, S.Z. (1987). Health effects of pesticides: a review of epidemiologic research from the perspective of developing nations. *Am. J. Ind. Med*., 12., 269–70.

Yang, C.C. & Deng, J.F. (2007). Intermediate syndrome following organophosphate insecticides poisoning. *J. Chin. Med. Assoc*., 70.,11., 467-72.

Yousaf, M.I.; Awad, T.I. & Mohammad, E.H. (2006). Deltamethrin- induced oxidative damage and biochemical alterations in rat and its attenuation by Vit. E. *Toxicol*., 227., 240-47.

Zham, S.H. & Blair, A. (1992). Pesticides and non-Hodgkin's lymphoma. *Cancer. Res*., 52., 5485-88.

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Health Risk by Chlorinated Pesticides in Water Bodies Used for Recreational Bathing in Argentina

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1. Introduction

The Buenos Aires province, located in Central Eastern Argentina, has an area of 307,571 km^2, which represents more than 10% of the total surface of the country. It is the province with the largest population (14 million inhabitants, according to Provincial Direction of Statistics, 2010) and accounts for 35 % of the Gross Domestic Product of the country.
The province is one of the main agricultural producers of the country, representing 40 % of the national production (MAA, 2004). The most prominent crops are: soybean, with 3.7x10^6 Ha sowed; wheat, with 2.9x10^6 Ha, sunflower with 1.1x10^6 Ha, and maize, with 0.8x10^6 Ha, according to the 2005/2006 harvest figures (Provincial Direction of Statistics, 2010).
These volumes are the result of a process of agriculturization started in the 1970s and 1980s, in which, the expansion of the agricultural frontier, the conversion of grassland into agricultural lands, and the bigger technification of the activity, brought about an increasing usage of input, mainly herbicides and insecticides (Pengue, 2000; Pengue, 2001). Considering the whole Argentinian territory, soybean is the crop with the greatest surface increase, from barely 37,700 Ha in 1970 (Pengue, 2001), to more than 16x10^6 Ha in 2005 (ISAAA, 2010).
The increase in the use of pesticides (from 73 to 236 million kilograms between 1995 and 2005, according to CASAFE (2007)) triggered arguments on the environmental impacts of this productive process. Stemming from this, many studies have revealed the presence of biocides in the environment in different compartments of Argentina: water environments (Zubillaga et al., 1987, Janiot et al., 1994, Loewy et al., 1999, Menone et al., 2000, 2001, Rovedatti et al., 2001, Miglioranza et al., 2004, Jergentz et al., 2005, Silva et al., 2005, Marino & Ronco, 2005, Peruzzo et al., 2008, Arias et al., 2010), soils (Miglioranza et al., 1999, 2002, 2003a, 2003b, Peruzzo et al., 2008, Gómez et al., 2009), biota, (Miglioranza et al., 1999,

Menone et al., 2000, 2006, Cataldo et al., 2001, Jergentz et al., 2004, Andrade et al., 2005, De la Torre et al., 2005, Martín & Ronco, 2006, Cid et al., 2007, Carriquiriborde et al., 2007, Jofré et al., 2008), humans (García Fernández, 1974, Muñoz de Toro et al., 2006), and agricultural or stockbreeding products (Lenardón et al., 1994, Maitre et al., 1994, Loewy et al., 2003, Ruíz et al., 2008).

The protection of the population from pesticides in Argentina is based on several national regulations (FARN, 2005), both general with implicit references to pesticides, and specific with explicit references to pesticides. One example of a general regulation is the amendment in 1994 of the 41th article of the National Constitution, establishing the right to all inhabitants to enjoy a healthy environment. Another example is Law 25675/2002, or General Law of the Environment, which established the minimal requirements to accomplish sustainable and adequate management of the environment, assuring preservation, conservation, recovery, and improvement of the quality of environmental resources (Congress of Argentina, 2002).

More specific regulations related to the protection from pesticides include Law 18284, or National Food Code (National Goverment of Argentina, 1969) and Regulatory Decree 2126 (National Goverment of Argentina, 1971). This decree established the conditions under which the production and sale of food products are authorized, determining the highest allowed concentrations of pesticide residues in food (FARN, 2005). These regulations, which are under constant revision, stipulate that all elaborated, fractioned, conserved, transported, distributed or displayed food, spices, beverages, their raw materials or food additives must comply with these requirements.

The aforementioned Code, in chapter VII, establishes the requesite characteristics for drinking water (ANMAT, 2010). Pesticides are listed in a group of substances labeled as "organic contaminants":

Aldrin + Dieldrin, max.: 0.03 $\mu g\ L^{-1}$;
Chlordane, max.: 0.30 $\mu g\ L^{-1}$;
DDT (Total + Isomers), max.: 1.00 $\mu g\ L^{-1}$;
Heptachlor + Heptachloroepoxide, max.: 0.10 $\mu g\ L^{-1}$;
Lindane, max.: 3.00 $\mu g\ L^{-1}$;
Metoxichlor, max.: 30.0 $\mu g\ L^{-1}$;
2,4 D, max.: 100 $\mu g\ L^{-1}$;
Metil Parathion, max.: 7 $\mu g\ L^{-1}$;
Parathion, max.: 35 $\mu g\ L^{-1}$;
Malathion, max.: 35 $\mu g\ L^{-1}$;

A bad quality of recreational water is associated with the possibility of contracting pulmonary, sensory organs (eyes, ears), skin and, particularly, gastrointestinal diseases. Among these diseases, the latter (vomits, diarrhea, nauseas) are the most studied as regards water quality and the presence of indicator bacteria which cause such diseases. Prüss (1998) analyzed 22 scientific papers which studied the causal relationship between gastrointestinal symptoms and recreational water quality evaluated from the concentrations of indicator bacteria. In 19 papers, a strong statistical association was verified. WHO (1998), in *Guidelines for Safe Recreational-water Environments: Coastal and Freshwaters* described the impact of water quality on recreational use, placing special stress on fecal contamination with pathogenic microorganisms. Subsequently, general evaluation and monitoring guidelines of the microbiological quality of recreational water were established through the *Annapolis Protocol*

(WHO, 1999). Moreover, 2 chapters were specifically dedicated to the methodology of evaluation of water microbiological quality in *Monitoring Bathing Waters - A Practical Guide to the Design and Implementation of Assessments and Monitoring Programmes* (WHO, 2000). The bacteriological recounts as indicators of recreational water quality have a preferential place when analyzing bathing waters.

Regarding chemical contamination of waters that can be used with recreational purposes, WHO (1998) explains that the occurrence of health risk is much less likely. However, the risk must not be minimized and, hence, bathers' health must be ensured, though how to achieve this is not mentioned. In some cases, the guidelines of quality evaluation of drinking water can be used as evaluation tools, as explained in *WHO Guidelines for drinking-water Quality* (2008).

In Argentina, water quality of superficial aquatic environments with recreational usage and direct contact is evaluated following the *National Guide Levels for Water Quality for Human Recreation*, written by the National Undersecretary of Hydrological Resources (NUHR, 2007). However, only microbiological parameters are established as quality guidelines.

Several projects funded by different Argentinian governmental agencies conducted, among other activities, monitoring of water bodies of the Buenos Aires Province, analyzing for the presence of pesticides among other substances. Given the fact that pesticides have been detected in water, and that the monitored water bodies could potentially be or are currently used with recreational purposes, it is necessary to evaluate the danger of using such water bodies for the formerly mentioned activities since there are no adequate management tools in the environmental legal framework of Argentina. The objective of the hereby work is to evaluate this danger employing Health Risk Analysis (HRA) as a way to replace the lack of other analytical tools.

HRA are management tools that allow establishing, based on the available scientific information, if the chemical substances with particular toxic characteristics present in an environment represent a threat for people's health in accordance with the way in which the exposure to such substances is conducted (NRC, 1983). The risk, according to the USEPA model, is a function of the toxicity of the hazardous substance and the magnitude of the exposure to it, being the latter a measure of the "quality and quantity"of the contact between the substance and the exposed organism (USEPA, 1989, 1992a). The exposure quantifies the relationship between the causal agent of the risk and an organism taking into account contact pathways, scenarios, and time exposure (USEPA, 1992a). The use of HRA for the analysis of bathing waters has few antecedents in Argentina (for example Peluso et al., 2009, 2010), and they have not been recognized in any legal framework as a management tool.

2. Experimental methods and procedures

2.1 Description of the study area

Different investigation projects, whose area of study involves the Buenos Aires province, focus their attention on the quality of water resources and have conducted pesticide monitoring. The following can be mentioned:

Tools for the Sustainable Management of the Water Resources in a Plain Basin (ANPCyT, 2005), which conducted 5 pesticide monitorings at Del Azul creek between January 2005 and December 2007; Monitoring of Organochlorine Pollutants in Buenos Aires Shallow Lakes: Assessment of the Impact on the Ichthyofauna. Implications and Perspectives of

Management (CONICET, 2005), which conducted 4 series of sampling per shallow lake between July 2005 and July 2008 (the shallow lakes were: La Barrancosa, La Salada, El Chifle, San Antonio, Del Estado, Quilla Lauquén, El Paraíso, La Brava, De los Padres, La Peregrina, El Carpincho, Blanca Chica, La Sirena y Monte); Development of Criteria and Guidelines for the Management of Water Resources in Plain Areas (ANPCyT, 2007) which, between January 2008 and July 2010, conducted 5 series of sampling in the 1rst, 2nd and 3rd branches of Tres Arroyos creek, Claromecó, Cristiano Muerto, and Quequén Salado creeks. These projects provided information on the presence of organochlorine pesticides used as basis for the risk analysis applied in this work. Figure 1 presents the geographic location of the analyzed environments.

All these water bodies are located in agricultural areas where pesticides are applied to a greater or lesser extent. Table 1 presents a list of the water bodies, indicating the county of the province where they are located, and the surface and sowed area for the four most important crops (soybean, wheat, maize, and sunflower).

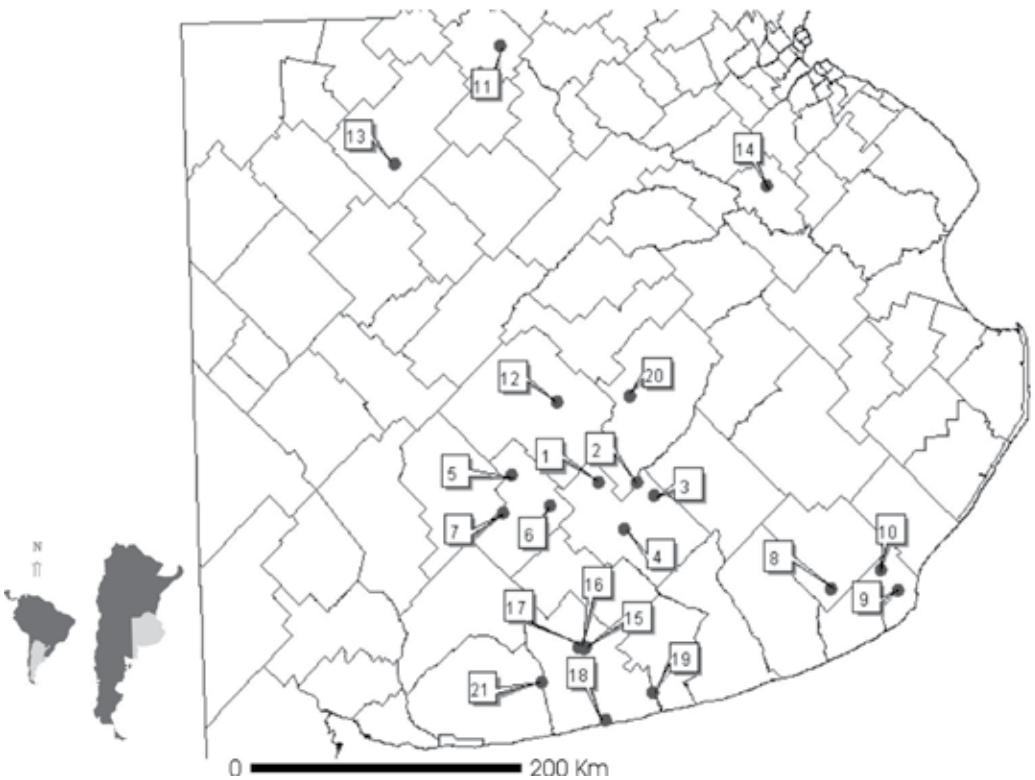

Fig. 1. Geographical location of studied water bodies

Water body	County	County area (in Km²)	Area sowed by county (2006-2007) (in Ha) based in CITAB (2010)			
			Soybean	Wheat	Sunflower	Maize
La Brava SL	Balcarce	4120	40000	70600	25500	11500
El Chifle SL	Benito Juárez	5285	47600	45000	15500	7000
La Barrancosa SL	Benito Juárez					
La Salada SL	Benito Juárez					
San Antonio SL	Benito Juárez					
La Sirena SL	Lincoln	5772	106500	33360	3000	18900
De los Padres SL	General Pueyrredón	1460	16700	22000	5500	3800
La Peregrina SL	General Pueyrredón					
El Carpincho SL	Junín	2260	107900	20500	500	25000
Del Estado SL	Laprida	3440	16000	13000	4000	4000
El Paraiso SL	Laprida					
Quilla Lauquen SL	Laprida					
Monte SL	Monte	1890	18600	6000	700	6000
Blanca Chica SL	Olavarría	7715	69000	15500	5000	10000
Del Azul C	Azul	6615	71000	57000	12000	13000
Quequén Salado C	Coronel Dorrego	5818	56500	191690	58000	6500
Cristiano Muerto C	San Cayetano	3004	41400	112430	70000	7500
1st branch of Tres Arroyos C	Tres Arroyos	5861	97000	228450	150000	12000
2nd branch of Tres Arroyos C	Tres Arroyos					
3rd branch of Tres Arroyos C	Tres Arroyos					
Claromecó C	Tres Arroyos					

Table 1. Studied water bodies indicating the county of location in the province and its area. Moreover, sowed surfaces of soybean, wheat, sunflower and maize are presented. References: SL: shallow lake, C: creek.

2.2 Concentration of hazardous substances in water

The different studies mentioned before were planned so as to obtain spatial and temporal representative water samples according to prefixed objectives in each project. In all cases, sampling consisted of taking one or more representative water samples, obtained by means of mixing a group of subsurface subsamples from different points in the water bodies. The samples were collected according to standard techniques for the analytical determinations to

be carried out (amber glass bottles with internal Teflon tops) and they were refrigerated (4-8 ^{o} C) until analysis, which was carried out in a private laboratory certified by the application authority in environmental issues from the Buenos Aires Province (Reg. 017 Res. 640/02 of the *Provincial Organism for Sustainable Development*. Table 2 presents the substances, their abbreviations for this work, their identification codes according to the Chemical Abstract Service (CAS, 2010) and the applied analytical technique and limit of detection for their determination.

Pesticide	Abbreviation	CAS	Techn. Code	Detect. Lim.
Hexachloro Ciclo Hexane, alpha isomer	α – HCH	319-84-6	EPA SW 846 M 8081	6.00E^{-07}
Hexachloro Ciclo Hexane, gamma isomer	γ – HCH	58-89-9	EPA SW 846 M 8081	5.00E^{-07}
Hexachloro Ciclo Hexane, delta isomer	δ – HCH	319-86-8	EPA SW 846 M 8081	4.00E^{-08}
Chlordane, gamma isomer	γ – Clor	57-74-9	EPA SW 846 M 8081	4.00E^{-07}
Acetochlor	Acet.	34256-82-1	EPA 3510	1.00E^{-04}
Aldrin	Aldr.	309-00-2	EPA SW 846 M 8081	2.00E^{-07}
dichlorodi- phenyldichloroethane (4,4´-DDD)	DDD	72-54-8	EPA SW 846 M 8081	1.00E^{-07}
Dichlorodiphenyltrichloroethane (4,4´-DDT)	DDT	50-29-3	EPA SW 846 M 8081	8.80E^{-06}
Endosulfan, alpha isomer	α – Endo.	959-98-8	EPA SW 846 M 8081	1.00E^{-07}
Endosulfan, beta isomer	β – Endo.	33213-65-9	EPA SW 846 M 8081	9.00E^{-07}
Endosulfan Sulfate	Endo.Sul.	1031-07-8	EPA SW 846 M 8081	2.50E^{-06}
Endrin	Endr.	72-20-8	EPA SW 846 M 8081	5.00E^{-07}
Heptachlor	Hept.	76-44-8	EPA SW 846 M 8081	4.50E^{-06}

Table 2. Pesticides present in the water bodies, their abbreviation, their CAS numbers, and the applied analytical technique and limit of detection for their determination.

Table 3 displays the average concentration, measured in mg L^{-1}. Although USEPA advices utilizing the corrected arithmetic mean as a representative parameter of a reasonably maximum level of exposure (upper confidence limit of the arithmetic mean, whose abbreviation is UCL) (USEPA, 1989, 1992b, 2002a), the arithmetic mean was used due to the extremely limited amount of data, which did not allow appropriate calculation of the UCL.

2.3 Health risk analysis model (HRA)

In this study, the exposure to pesticides through bathing was only based on accidental water intake and skin contact, given the fact that inhalation of substances that may generate vapor was considered irrelevant.

HRA estimation through these two pathways of exposure was carried out using USEPA models. Chronic exposure to a hazardous substance by accidental intake and skin contact was calculated using Eq. 1 and 2, respectively. Each variable, except for substance concentration, was treated probabilistically.

$$ADDI = [C * Ir * ET* EF * ED]/[Bw * AT] \quad (1)$$

$$ADDC = [DA_{event} * SA * ET * EF * ED * FC]/[Bw * AT] \quad (2)$$

Where

ADDI = Average Daily Dose by Accidental Intake (mg kg^{-1} day^{-1})

C = Concentration of the hazardous substance in water (mg L^{-1})

Ir = Daily water intake rate (L day^{-1})

ET = Daily duration of exposure (hour day^{-1})

EF = Annual Exposure frequency (day $year^{-1}$)

ED = Exposure duration (year)

Bw = Weight of the exposed individual (kg)

AT = Correction factors by means of average time (ED * 365 days for non carcinogenic substances; Statistic life expectancy (70) * 365 days for carcinogenic substances)

ADDS = Average Daily Dose by means of Skin Contact (mg kg^{-1} day^{-1})

DA_{event} = Absorbed dose per event (mg cm^{-2} $event^{-1}$)

SA = Skin contact Area with water (cm^2)

FC = Correction factor of surface and volume units (10,000 cm^2 m^{-2} * 0.001 L cm^{-3})

The absorbed dose per event (DA_{event}) is estimated in base to a steady state approach from USEPA (2007), applying Eq. 3.

$$DA_{event} = 2 * FA * Kp * C * (6 * \tau * t_{event})/\pi)^{-0,5} \quad (3)$$

where

DA_{event} = Absorbed dose per event (mg cm^{-2} $event^{-1}$)

FA = Fraction absorbed (dimensionless): is the net fraction available for absorption in the stratum corneum after exposure has ended (USEPA 2007).

Kp = Dermal permeability coefficient of the substance in water (cm hr^{-1}), estimated in base to the molecular weight (Mw, in gr) and the coefficient of octanol-water partition (Kow, dimensionless), as shown in Eq. 4 (USEPA 2007). Table 4 show the Kp used in dermal risk calculation.

$$Log\ Kp = -2.80 + 0.66 \log Kow - 0.0061Mw \quad (4)$$

C = Concentration of the hazardous substance in water (mg L^{-1})

τ = Lag time per event (hr $event^{-1}$)

t_{event} = Event duration (hr $event^{-1}$)

Risk calculation for substances of non carcinogenic toxic effects (NCE) by pathway of exposure was conducted using the quotient of the value of ADD in contrast with a specific

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
α – HCH	2.00 E-08	2.00 E-08	5.00 E-06	2.00 E-08	2.00 E-08	2.00 E-08	2.00 E-08	1.47 E-05	5.30 E-06	9.70 E-06	1.73 E-05	1.80 E-06	2.40 E-05	2.80 E-06	5.50 E-06	1.06 E-05	7.45 E-06	2.45 E-06	4.45 E-06	2.02 E-05	9.45 E-06
γ – HCH	7.30 E-07	2.50 E-07	2.50 E-07	1.10 E-06	2.50 E-07	2.50 E-07	2.50 E-07	2.50 E-07	2.50 E-07	2.80 E-06	2.50 E-07	2.50 E-07	7.00 E-07	8.00 E-07	7.50 E-07	7.50 E-07	1.08 E-06	2.50 E-07	2.50 E-07	2.50 E-07	4.17 E-07
δ – HCH	1.50 E-09	1.50 E-09	1.50 E-06	1.50 E-09	1.50 E-09	1.50 E-09	1.50 E-09	6.00 E-07	3.00 E-07	1.50 E-09	1.25 E-05	5.20 E-06	1.50 E-09	1.50 E-09	2.00 E-08	2.00 E-08	3.60 E-07	2.00 E-08	2.00 E-08	5.50 E-07	6.67 E-08
γ – Clor.	5.00 E-08	5.00 E-08	5.00 E-08	5.00 E-08	5.00 E-08	5.00 E-08	5.00 E-08	5.00 E-08	5.00 E-08	5.00 E-08	5.00 E-08	5.00 E-08	5.00 E-08	5.00 E-08	2.00 E-07	2.00 E-07	2.00 E-07	3.00 E-07	2.00 E-07	1.95 E-06	2.00 E-07
Acet	1.50 E-08	1.50 E-08	1.50 E-08	1.50 E-08	1.50 E-08	1.50 E-08	1.50 E-08	1.50 E-08	1.50 E-08	1.50 E-08	1.50 E-08	1.50 E-08	1.50 E-08	1.50 E-08	1.50 E-08	1.50 E-08	1.50 E-08	1.50 E-08	1.50 E-08	1.42 E-02	1.50 E-08
Aldr.	5.00 E-09	5.00 E-09	5.00 E-09	5.00 E-09	5.00 E-09	5.00 E-09	5.00 E-09	5.00 E-09	5.00 E-09	5.90 E-06	5.00 E-09	5.00 E-09	5.00 E-09	5.00 E-09	1.00 E-07	1.00 E-07	1.00 E-07	1.00 E-07	1.00 E-07	2.80 E-07	1.33 E-07
DDD	5.00 E-09	3.50 E-06	1.50 E-06	1.60 E-06	5.00 E-09	5.00 E-09	5.00 E-09	5.00 E-09	5.00 E-09	5.00 E-09	5.00 E-09	5.00 E-09	5.00 E-09	5.00 E-09	5.00 E-08	5.00 E-08	5.00 E-08	5.00 E-08	5.00 E-08	5.00 E-09	1.25 E-07
DDT	2.59 E-07	2.59 E-07	2.59 E-07	2.59 E-07	2.59 E-07	2.59 E-07	2.59 E-07	2.59 E-07	2.59 E-07	2.59 E-07	2.59 E-07	1.66 E-05	2.59 E-07	2.59 E-07	2.59 E-07	2.59 E-07	2.59 E-07	2.59 E-07	2.59 E-07	2.59 E-07	2.59 E-07
α – Endo	5.00 E-09	5.00 E-09	3.00 E-07	5.00 E-09	5.00 E-09	5.00 E-09	5.00 E-09	5.00 E-09	5.00 E-09	4.60 E-06	5.00 E-09	5.00 E-09	1.04 E-05	5.00 E-09	5.00 E-09	5.00 E-09	5.00 E-09	5.00 E-09	5.00 E-09	1.24 E-06	5.00 E-09
β – Endo	2.30 E-06	4.50 E-07	4.50 E-07	4.50 E-07	4.50 E-07	4.50 E-07	4.50 E-07	4.50 E-07	4.50 E-07	8.80 E-06	2.30 E-06	4.50 E-07	4.50 E-07	4.50 E-07	4.50 E-07	4.50 E-07	4.50 E-07	4.50 E-07	4.50 E-07	4.50 E-07	4.50 E-07
Endo Sul.	5.70 E-08	5.70 E-08	5.70 E-08	5.70 E-08	5.70 E-08	5.70 E-08	5.70 E-08	8.10 E-06	3.20 E-06	5.70 E-08	9.20 E-06	3.30 E-06	5.70 E-08	6.30 E-06	1.25 E-06	1.25 E-06	1.25 E-06	1.25 E-06	8.04 E-06	6.20 E-06	5.61 E-06
Endr	2.00 E-08	2.00 E-08	7.00 E-07	2.00 E-08	2.00 E-08	2.00 E-08	2.00 E-08	2.00 E-08	2.00 E-08	2.00 E-08	2.00 E-08	2.00 E-08	2.00 E-08	2.20 E-06	2.00 E-08	2.00 E-08	2.00 E-08	2.00 E-08	2.00 E-08	2.00 E-08	2.00 E-08
Hept	2.25 E-06	2.25 E-06	2.25 E-06	2.25 E-06	2.25 E-06	2.25 E-06	2.25 E-06	2.25 E-06	2.25 E-06	2.25 E-06	2.25 E-06	2.25 E-06	2.25 E-06	2.25 E-06	2.25 E-06	2.25 E-06	2.25 E-06	2.25 E-06	2.25 E-06	8.15 E-06	2.25 E-06

Table 3. Concentrations (in mg L^{-1}) of pesticides in the studied water bodies. References for the pesticides: see Table 2. References for water bodies: 1- La Barrancosa SL, 2- La Salada SL, 3- El Chifle SL, 4- San Antonio SL, 5- Del Estado SL, 6- Quilla Lauquén SL, 7- El Paraíso SL, 8- La Brava SL , 9- De los Padres SL, 10- La Peregrina SL, 11- El Carpincho SL, 12- Blanca Chica SL, 13- La Sirena SL, 14- Monte SL, 15- 1rst branch Tres Arroyos creek, 16- 2nd branch Tres Arroyos creek, 17- 3rd branch Tres Arroyos creek, 18- Claromecó creek, 19- Cristiano Muerto creek, 20- Del Azul creek, 21- Quequén Salado creek

reference dose for such route, being the value under which there are no toxicological effects on the exposed individual: risk quotient R (USEPA, 1992 a). The Reference Dose (RfD) (USEPA, 1992a) was used as threshold dose. If R exceeds the unit value, it is considered to be a potential adverse health effect over the exposed population.
For risk calculation for substances with carcinogenic toxic effects (CE), the exposure was also estimated in the light of the ADDI or ADDS, though the duration of the exposure in the AT correction factor was 70 years. Risk calculation was conducted from the product of each ADD multiplied by a referential toxicological value, utilizing for that purpose the Slope Factor SF (USEPA, 1996), also particular for each route of exposure. In fact, this methodology calculates the excess of individual risk due to cancer assuming a linear relationship between the exposure concentrations and the carcinogenic effects. Such method was applied by USEPA (1996, 2005).

Pesticides	Kp
α – HCH	$2.97E^{-02}$
γ – HCH	$2.97E^{-02}$
δ – HCH	$2.97E^{-02}$
γ – Clor.	$1.57E^{-01}$
Acet	$6.10E^{-03}$
Aldr.	$4.67E^{-01}$
DDD	$4.00E^{-01}$
DDT	$1.06E^{+00}$
α – Endo	$3.29E^{-03}$
β – Endo	$3.29E^{-03}$
Endo Sul.	$3.29E^{-03}$
Endr	$4.45E^{-02}$
Hept	$2.16E^{-01}$

Table 4. Coefficients of Skin Permeability (Kp) for the different pesticides.

References for the pesticides: see Table 2.

In Argentina, the maximum accepted individual risk value due to exposure to CE substances in drinking water is $10E^{-5}$. This limit is established in the local guideline levels of water quality for human consumption (Goransky and Natale, 1996; SRHN, 2010). No criterion is available on the accepted limits for NCE, for which it is assumed the unit as reference value.
The aggregated and cumulative risks (for simultaneous exposure to the same hazardous substance through different pathways of contact and for simultaneous exposure to different substances, respectively) were calculated using an additive model into a Risk Index, which was used by USEPA for screening HRA (USEPA, 1992a; 2001a; 2003).

2.4 Model parameters

From the group of analyzed water bodies, two subgroups can be distinguished: water bodies visited throughout the year given their proximity to populated centres (the creeks),

and the less visited ones, whose main attraction is sport fishing are not normally near populated centres (the shallow lakes). Following this division, it could be distinguished:

a. An exposure scenario of a *recreational residential* type, which is defined by a larger quantity of annual contact episodes, and by a longer daily duration, given the proximity to the residence location of the exposed population. The annual usage frequency is mainly defined by temperature, and it is usually carried out in bathing resorts. As a representative user for this environment, a 10-year-old child was chosen. The parameters used to define the exposure (morphometric characteristics, frequency and duration of contact) are displayed in Table 5, along with the information source.
   The estimation of the body surface was conducted applying the DuBois & DuBois (1916) formula, as shown in Eq. 5, using weight and height for the age of the selected individual.

$$SC = H^{0.725} * P^{0.425} * 0.007184 \quad (5)$$

   Where:
   SC: body surface (m^2)
   H: height (cm)
   P: weight (kg)

Parameter	Det-Prob	Type of P. curve	Min	Max	AM	SD	Source
Ir ($L\ h^{-1}$)	Det		0,05				USEPA, 1989
ET (h)	Prob	Triangular	0.5	2	1		self judgment
EF ($d\ a^{-1}$)		Beta	0.82	45.71	20.7	11.07	Peluso et al., 2006
ED (a)		Triangular	1	30	15		self judgment
BW (kg)		Normal	24	44	32	3.33	Lejarraga & Orfila, 1987
Height (m)		Normal	1.25	1.48	1.36	0.04	Lejarraga & Orfila, 1987
SA (m^2)		Normal	0.93	1.28	1.10	0.05	Estimated in the light of DuBois & DuBois, 1916

Table 5. Parameters of exposure for the *recreational residential* scenario. References: Det-Prob. Deterministic or Probabilistic; Min: minimum; Max: maximum; AM: Arithmetic Mean; SD: Standard Deviation. Source: source of information.

Given the fact that it is assumed that the bather would have full water contact, the SC value was the one that was utilized as a replacement of SA in equation 2.

b. A *rural fishing* scenario, with a visit pattern of more sporadic annual visits, of lesser daily duration, and which requires some means of transportation to the location. In this case, although temperature is important, it is not usually a determinant of attendance to the site. Conversely, the attendance will be conditioned by the fishing opportunities that the location may offer. Sport fishing in Argentinian Pampean lakes is an activity that attracts fishermen throughout the year. In winter, the focus is set on silverside fishing (*Odontesthes bonariensis*), and in winter, on wolf fish (*Hoplias malabaricus*), silver catfish (*Rhamdia quelen*), and carps (*Cyprinus carpio*) (Grosman, 2006). The amount of sport fishermen is estimated at 1,125,000 in the Buenos Aires province alone (Lopez et al., 2001). As representative of the exposed individual in this scenario, a 60-year-old adult was chosen. In Table 6, the parameters of this exposure scenario are shown.

The frequency of exposure derives from sociological studies of a fishery in El Carpincho, where the annual attendance rates were determined (Grosman & Benito, 2004). Based on these, the frequencies of use, only for the summer (three months), were calculated, being the probabilities those presented in Table 7.
The duration of the exposure was obtained based on the amount of years in sport fishing in the location (data obtained from Grosman and Benito (2004)). Based on the group of values of this work, the best fitted curve of distribution of frequencies with Crystal Ball (Decisionnering, 2007) was tested and the descriptive parameters were obtained.
Body weight values were derived from the study of De Girolami et al., (2003) based on body mass index (BMI), extracting weight and height corresponding to the 60-year-old stratum of BMI. Subsequently, using weight and height for such age range, the body surface based on the DuBois & DuBois formula was estimated, applying Eq. 5. Following the criteria used for the other exposure scenario, SC replaces SA in Eq. 2.

Parameter	Det-Prob	Type of P. curve	Min	Max	AM	SD	Source
Ir (L h^{-1})	Det		0.05				USEPA, 1989
ET (h)	Prob	Triangular	0.5	1	0.75		self judgment
EF (d a^{-1})		Uniform	1.00	12.00	5.51	4.07	Grosman & Benito, 2004
ED (a)		Gamma	5.46	54.99	29.33	11.46	Estimated in the light of Grosman & Benito, 2004
BW (kg)		Normal	58.96	141.1	83.27	17.76	Estimated in the light of De Girolami et al., 2003
Height (m)		Normal	1.56	1.85	1.72	0.05	Estimated in the light of De Girolami et al., 2003
SA (m^2)		Normal	1.59	2.51	1.98	0.15	Estimated in the light of DuBois & DuBois, 1916

Table 6. Parameters of exposure for the rural fishing scenario. References: Det-Prob. Deterministic or Probabilistic; Min: minimum; Max: maximum; AM: Arithmetic Mean; SD: Standard Deviation. Source: information source.

FAP (events a^{-1})	P
1	0.09
2	0.04
3	0.49
6	0.11
12	0.26

Table 7. Probability (P) of annual frequencies of visits with sport fishing purposes for the summer in El Carpincho shallow lake (based on Grosman & Benito, 2004).

2.5 Calculation of risk level and usage of the toxicological reference value

Risk was calculated first by substance and by pathway of exposure. Secondly, risk was calculated for all substances and both routes of exposure simultaneously, applying an additive

model. The calculation, both for NCE and CE, was conducted with Crystal Ball 7.1 (Decisioneering, 2007), applying Monte Carlo for 5,000 repetitions (USEPA, 2001b) on the basis of the types of the distribution of probabilities of each variable. Table 8 shows the toxicological referentials for both NCE (RfDs) and CE (SFs) by oral intake and by skin contact, coming from the Integrated Risk Information System (IRIS) database of USEPA (2010).
From the probabilistic distributions of risk values in each case, the arithmetic mean, the standard deviation, the maximum value, and the 95 percentile were calculated.

Pesticides	RfD int	RfDskin	SFint	SFskin
α – HCH	$3.00E^{-04}$	$2.91E^{-04}$	$6.30E^{+00}$	$6.49E^{+00}$
γ – HCH	$3.00E^{-04}$	$2.91E^{-04}$	$6.30E^{+00}$	$1.98E^{+00}$
δ – HCH	$3.00E^{-04}$	$2.91E^{-04}$	$1.30E^{+00}$	$1.34E^{+00}$
γ – Clor.	$5.00E^{-04}$	$2.50E^{-04}$	$3.50E^{-01}$	$7.00E^{-01}$
Acet	$2.00E^{-02}$	$1.00E^{-02}$	N.A.	N.A.
Aldr.	$3.00E^{-05}$	$1.50E^{-05}$	$1.70E^{+01}$	$3.40E^{+01}$
DDD	N.A.	N.A.	$2.40E^{-01}$	$3.43E^{-01}$
DDT	$5.00E^{-04}$	$3.50E^{-04}$	$3.40E^{-01}$	$4.86E^{-01}$
α – Endo	$6.00E^{-03}$	$3.00E^{-03}$	N.A.	N.A.
β – Endo	$6.00E^{-03}$	$3.00E^{-03}$	N.A.	N.A.
Endo Sul.	$6.00E^{-03}$	$3.00E^{-03}$	N.A.	N.A.
Endr	$3.00E^{-04}$	$6.00E^{-06}$	N.A.	N.A.
Hept	$5.00E^{-04}$	$3.60E^{-04}$	$4.50E^{+00}$	$6.25E^{+00}$

Table 8. Toxicological referential for non carcinogenic effects (RfDs) and carcinogenic ones (SFs), by oral intake (int) and by skin contact (skin). References: N.A. Not applicable. References for pesticides: see Table 2

3. Results

The results indicate there is no health risk in the two considered exposure scenarios, neither due to the pesticides present in the water bodies, nor to the non carcinogenic effects or the carcinogenic ones (see Tables 9a and 9b, 10a and 10b).
The aggregated cumulative risk values of NCE for both scenarios, which is the worst condition given that the exposure is simultaneous to all substances and through both pathways of exposure at the same time, differs greatly from the value of significance (R = 1). As shown in Tables 9a and 9b, the highest risk for the *recreational residential* scenario is $9.06E^{-03}$, while for the *rural fishing* scenario is around $1.83E^{-03}$ data taken from La Peregrina shallow lake (water body 10) in both cases. The first scenario is around 5 times riskier than the second one. The second riskiest environment is the Blanca Chica shallow lake (water body 12). The aggregated cumulative risk derived from CE is also much lower than reference values ($R = 10^{-5}$), reaching values of $8.66E^{-07}$ and $9.66E^{-07}$ for the *recreational residential* and the

rural fishing scenarios respectively, for La Peregrina shallow lake (see Tables 10a and 10b). In this case, the rural fishing scenario generates a similar risk to the recreational residential one. The second water body with highest risk values is Del Azul creek (water body 20).

The highest cumulative risk values for both pathways of exposure for NCE and CE, can be seen in Tables 11a and 11b. In nearly all cases, the riskiest environment is La Peregrina shallow lake. However, for accidental intake exposure and NCE, for both scenarios, the riskiest compared scenario is Del Azul creek (water body 20), followed by the former mentioned shallow lake. As shown in Table 10a, for skin contact exposure and NCE, for both scenarios, the second riskiest environment is Blanca Chica shallow lake (water body 12). In the case of CE, for both pathways of exposure and both scenarios, the second riskiest environment is Del Azul creek.

Table 11c shows the percentual risk of skin contact with regard to aggregated risk, both for NCE and CE. It can clearly be seen that skin contact risk is extremely important for both scenarios and types of effects, with average values around 90%.

It is always assumed that the highest risk occurs in children, which is proven in this study by comparing the accidental intake for the recreational residential and the fishing rural scenarios. For NCE, the arithmetic mean of the maximum risks by accidental intake in the recreational residential scenario for the 21 water bodies is 14.18 times higher (SD=0.03) than in the fishing rural one; through skin contact, the mean is 4.95 times higher (SD=0.01). For CE, the relationship varies. The risks for both pathways of exposure continue being higher for the child's scenario, but the value ranges are lower. The arithmetic mean of the maximum risk values for the recreational scenario is 3.22 times (SD=0.001) higher than in the fishing rural one, while the skin contact is 0.86 times (SD=0.06) greater.

The reason why in all cases the highest risks are found in the scenarios where the child is used as representative of exposed individuals is due to the fact that in children the dose becomes higher as it is distributed in a smaller body weight. However, for both NCE and CE, the difference between accidental intake and skin contact is reduced for both children and adults.

In addition to a lower body weight, the daily volume of water intake that emerges from the multiplication of the intake rate (the same for child and adult) by the duration of the event and the frequency of exposure is higher in the child, contributing to an increase in dose. The combination of highest ET and EF, and lowest BW results in a 14 times higher average risk of accidental intake in the child compared to the adult, not compensated by the higher ED of the adult. For skin contact, the intake is negligible. Although BW continues being lower and EF higher in the child, the fisherman's body surface increases, which added to the higher ED, reduced the differences up to the point where they are minimal for EC. With a small change in the scenario, as a rise of EF that would be completely logical for some fishermen that conduct more than 12 annual fishing excursions, the risk value could match or even exceed that of the child's.

The analysis of which substances generate the highest risk values in each case is displayed in Table 12. For both NCE and CE, Aldrin is of the most importance. For accidental intake, Acetochlor (in Del Azul Creek) and the α isomer of HCH (in La Peregrina shallow lake) appear as important for NCE and CE, in both water bodies. For skin exposure, after Aldrin, Heptachlor stands out as one of the main pesticides present in all water bodies, for NCE and CE.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
AM	4.87 E^{-05}	4.75 E^{-05}	1.57 E^{-04}	4.94 E^{-05}	4.75 E^{-05}	4.75 E^{-05}	4.75 E^{-05}	8.22 E^{-05}	6.02 E^{-05}	3.54 E^{-03}	1.15 E^{-04}	9.95 E^{-04}	1.03 E^{-04}	3.59 E^{-04}	1.19 E^{-04}	1.30 E^{-04}	1.25 E^{-04}	1.12 E^{-04}	1.15 E^{-04}	5.81 E^{-04}	1.47 E^{-04}
SD	2.74 E^{-05}	2.67 E^{-05}	8.83 E^{-05}	2.78 E^{-05}	2.67 E^{-05}	2.67 E^{-05}	2.67 E^{-05}	4.63 E^{-05}	3.39 E^{-05}	1.99 E^{-03}	6.48 E^{-05}	5.60 E^{-04}	5.80 E^{-05}	2.02 E^{-04}	6.68 E^{-05}	7.32 E^{-05}	7.01 E^{-05}	6.29 E^{-05}	6.49 E^{-05}	3.28 E^{-04}	8.25 E^{-05}
Max	1.24 E^{-04}	1.21 E^{-04}	4.00 E^{-04}	1.26 E^{-04}	1.21 E^{-04}	1.21 E^{-04}	1.21 E^{-04}	2.08 E^{-04}	1.52 E^{-04}	9.06 E^{-03}	2.94 E^{-04}	2.55 E^{-03}	2.62 E^{-04}	9.20 E^{-04}	3.02 E^{-04}	3.29 E^{-04}	3.16 E^{-04}	2.85 E^{-04}	2.94 E^{-04}	1.52 E^{-03}	3.72 E^{-04}
P95	9.62 E^{-05}	9.40 E^{-05}	3.10 E^{-04}	9.76 E^{-05}	9.40 E^{-05}	9.40 E^{-05}	9.40 E^{-05}	1.63 E^{-04}	1.19 E^{-04}	6.99 E^{-03}	2.27 E^{-04}	1.97 E^{-03}	2.04 E^{-04}	7.10 E^{-04}	2.35 E^{-04}	2.57 E^{-04}	2.46 E^{-04}	2.21 E^{-04}	2.28 E^{-04}	1.15 E^{-03}	2.90 E^{-04}

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
AM	8.92 E^{-06}	8.73 E^{-06}	2.87 E^{-05}	9.05 E^{-06}	8.73 E^{-06}	8.73 E^{-06}	8.73 E^{-06}	1.45 E^{-05}	1.08 E^{-05}	6.53 E^{-04}	2.00 E^{-05}	1.84 E^{-04}	1.80 E^{-05}	6.63 E^{-05}	2.16 E^{-05}	2.35 E^{-05}	2.26 E^{-05}	2.05 E^{-05}	2.11 E^{-05}	9.79 E^{-05}	2.66 E^{-05}
SD	6.74 E^{-06}	6.60 E^{-06}	2.17 E^{-05}	6.84 E^{-06}	6.60 E^{-06}	6.60 E^{-06}	6.60 E^{-06}	1.10 E^{-05}	8.20 E^{-06}	4.93 E^{-04}	1.51 E^{-05}	1.39 E^{-04}	1.36 E^{-05}	5.01 E^{-05}	1.63 E^{-05}	1.78 E^{-05}	1.71 E^{-05}	1.55 E^{-05}	1.59 E^{-05}	7.41 E^{-05}	2.01 E^{-05}
Max	2.50 E^{-05}	2.45 E^{-05}	8.06 E^{-05}	2.54 E^{-05}	2.45 E^{-05}	2.45 E^{-05}	2.45 E^{-05}	4.10 E^{-05}	3.05 E^{-05}	1.83 E^{-03}	5.67 E^{-05}	5.15 E^{-04}	5.09 E^{-05}	1.86 E^{-04}	6.08 E^{-05}	6.62 E^{-05}	6.36 E^{-05}	5.76 E^{-05}	5.92 E^{-05}	2.79 E^{-04}	7.49 E^{-05}
P95	2.15 E^{-05}	2.10 E^{-05}	6.92 E^{-05}	2.18 E^{-05}	2.10 E^{-05}	2.10 E^{-05}	2.10 E^{-05}	3.51 E^{-05}	2.62 E^{-05}	1.57 E^{-03}	4.83 E^{-05}	4.42 E^{-04}	4.34 E^{-05}	1.60 E^{-04}	5.21 E^{-05}	5.67 E^{-05}	5.45 E^{-05}	4.94 E^{-05}	5.08 E^{-05}	2.37 E^{-04}	6.42 E^{-05}

Table 9a and 9b. NCE aggregated cumulative risk for *recreational residential* and *rural fishing* scenarios. References for water bodies: see Table 3. AM: Arithmetic mean; SD: Standard deviation. Max: Maximum value; P 95: 95 percentile

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
AM	6.66 E^{-09}	7.52 E^{-09}	9.34 E^{-09}	7.16 E^{-09}	6.57 E^{-09}	6.57 E^{-09}	6.57 E^{-09}	1.32 E^{-08}	8.95 E^{-09}	1.97 E^{-07}	1.54 E^{-08}	2.45 E^{-08}	1.73 E^{-08}	7.91 E^{-09}	1.21 E^{-08}	1.44 E^{-08}	1.31 E^{-08}	1.07 E^{-08}	1.16 E^{-08}	4.06 E^{-08}	1.49 E^{-08}
SD	4.71 E^{-09}	5.32 E^{-09}	6.60 E^{-09}	5.06 E^{-09}	4.64 E^{-09}	4.64 E^{-09}	4.64 E^{-09}	9.32 E^{-09}	6.33 E^{-09}	1.39 E^{-07}	1.09 E^{-08}	1.73 E^{-08}	1.23 E^{-08}	5.59 E^{-09}	8.58 E^{-09}	1.02 E^{-08}	9.26 E^{-09}	7.57 E^{-09}	8.18 E^{-09}	2.87 E^{-08}	1.05 E^{-08}
Max	2.95 E^{-08}	3.33 E^{-08}	4.19 E^{-08}	3.18 E^{-08}	2.91 E^{-08}	2.91 E^{-08}	2.91 E^{-08}	5.99 E^{-08}	4.02 E^{-08}	8.66 E^{-07}	7.05 E^{-08}	1.08 E^{-07}	7.95 E^{-08}	3.54 E^{-08}	5.43 E^{-08}	6.48 E^{-08}	5.88 E^{-08}	4.76 E^{-08}	5.16 E^{-08}	1.82 E^{-07}	6.69 E^{-08}
P95	1.61 E^{-08}	1.82 E^{-08}	2.26 E^{-08}	1.73 E^{-08}	1.59 E^{-08}	1.59 E^{-08}	1.59 E^{-08}	3.19 E^{-08}	2.17 E^{-08}	4.76 E^{-07}	3.74 E^{-08}	5.92 E^{-08}	4.20 E^{-08}	1.91 E^{-08}	2.94 E^{-08}	3.49 E^{-08}	3.18 E^{-08}	2.59 E^{-08}	2.80 E^{-08}	9.83 E^{-08}	3.61 E^{-08}

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
AM	4.91 E^{-09}	1.33 E^{-08}	1.61 E^{-08}	1.26 E^{-08}	1.16 E^{-08}	1.16 E^{-08}	1.16 E^{-08}	2.20 E^{-08}	1.54 E^{-08}	3.50 E^{-07}	2.55 E^{-08}	4.34 E^{-08}	2.85 E^{-08}	1.37 E^{-08}	2.10 E^{-08}	2.45 E^{-08}	2.25 E^{-08}	1.88 E^{-08}	2.01 E^{-08}	7.03 E^{-08}	2.56 E^{-08}
SD	4.36 E^{-09}	9.93 E^{-09}	1.20 E^{-08}	9.38 E^{-09}	8.66 E^{-09}	8.66 E^{-09}	8.66 E^{-09}	1.64 E^{-08}	1.14 E^{-08}	2.61 E^{-07}	1.90 E^{-08}	3.24 E^{-08}	2.12 E^{-08}	1.02 E^{-08}	1.56 E^{-08}	1.83 E^{-08}	1.68 E^{-08}	1.40 E^{-08}	1.50 E^{-08}	5.24 E^{-08}	1.91 E^{-08}
Max	2.44 E^{-08}	3.68 E^{-08}	4.44 E^{-08}	3.47 E^{-08}	3.21 E^{-08}	3.21 E^{-08}	3.21 E^{-08}	6.09 E^{-08}	4.25 E^{-08}	9.66 E^{-07}	7.07 E^{-08}	1.20 E^{-07}	7.90 E^{-08}	3.78 E^{-08}	5.80 E^{-08}	6.78 E^{-08}	6.21 E^{-08}	5.19 E^{-08}	5.56 E^{-08}	1.94 E^{-07}	7.08 E^{-08}
P95	1.47 E^{-08}	3.16 E^{-08}	3.81 E^{-08}	2.99 E^{-08}	2.76 E^{-08}	2.76 E^{-08}	2.76 E^{-08}	5.23 E^{-08}	3.65 E^{-08}	8.32 E^{-07}	6.07 E^{-08}	1.03 E^{-07}	6.78 E^{-08}	3.25 E^{-08}	4.99 E^{-08}	5.83 E^{-08}	5.34 E^{-08}	4.46 E^{-08}	4.79 E^{-08}	1.67 E^{-07}	6.08 E^{-08}

Table 10a and 10b. CE aggregated cumulative risk for *recreational residential* and *rural fishing* scenarios. References for water bodies see Table 3. AM: Arithmetic mean; SD: Standard deviation. Max: Maximum value; P 95: 95 percentile

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Rec. Res.	Int	3.21 E^{-06}	2.47 E^{-06}	1.18 E^{-05}	3.57 E^{-06}	2.47 E^{-06}	2.47 E^{-06}	2.47 E^{-06}	2.28 E^{-05}	9.91 E^{-06}	9.57 E^{-05}	4.18 E^{-05}	2.45 E^{-05}	3.48 E^{-05}	1.00 E^{-05}	1.17 E^{-05}	1.82 E^{-05}	1.51 E^{-05}	7.15 E^{-06}	1.01 E^{-05}	3.17 E^{-04}	1.71 E^{-05}
	Skin	1.23 E^{-04}	1.20 E^{-04}	3.95 E^{-04}	1.25 E^{-04}	1.20 E^{-04}	1.20 E^{-04}	1.20 E^{-04}	1.96 E^{-04}	1.48 E^{-04}	9.02 E^{-03}	2.67 E^{-04}	2.54 E^{-03}	2.41 E^{-04}	9.16 E^{-04}	2.97 E^{-04}	3.22 E^{-04}	3.10 E^{-04}	2.82 E^{-04}	2.90 E^{-04}	1.28 E^{-03}	3.65 E^{-04}
Rur. Fish.	Int	2.26 E^{-07}	1.74 E^{-07}	8.30 E^{-07}	2.52 E^{-07}	1.74 E^{-07}	1.74 E^{-07}	1.74 E^{-07}	1.61 E^{-06}	6.99 E^{-07}	6.75 E^{-06}	2.95 E^{-06}	1.72 E^{-06}	2.46 E^{-06}	7.07 E^{-07}	8.24 E^{-07}	1.29 E^{-06}	1.06 E^{-06}	5.04 E^{-07}	7.13 E^{-07}	2.23 E^{-05}	1.21 E^{-06}
	Skin	2.48 E^{-05}	2.43 E^{-05}	7.99 E^{-05}	2.52 E^{-05}	2.43 E^{-05}	2.43 E^{-05}	2.43 E^{-05}	3.96 E^{-05}	2.99 E^{-05}	1.82 E^{-03}	5.41 E^{-05}	5.13 E^{-04}	4.87 E^{-05}	1.85 E^{-04}	6.00 E^{-05}	6.51 E^{-05}	6.26 E^{-05}	5.71 E^{-05}	5.86 E^{-05}	2.59 E^{-04}	7.38 E^{-05}

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Rec. Res.	Int	9.24 E^{-10}	7.90 E^{-10}	2.81 E^{-09}	1.09 E^{-09}	7.39 E^{-10}	7.39 E^{-10}	7.39 E^{-10}	6.47 E^{-09}	2.81 E^{-09}	1.16 E^{-08}	8.43 E^{-09}	2.18 E^{-09}	1.02 E^{-08}	2.03 E^{-09}	3.16 E^{-09}	5.11 E^{-09}	4.07 E^{-09}	1.79 E^{-09}	2.56 E^{-09}	1.06 E^{-08}	4.60 E^{-09}
	Skin	2.72 E^{-08}	3.09 E^{-08}	3.70 E^{-08}	2.91 E^{-08}	2.69 E^{-08}	2.69 E^{-08}	2.69 E^{-08}	5.04 E^{-08}	3.54 E^{-08}	8.13 E^{-07}	5.84 E^{-08}	1.01 E^{-07}	6.52 E^{-08}	3.16 E^{-08}	4.85 E^{-08}	5.65 E^{-08}	5.18 E^{-08}	4.35 E^{-08}	4.66 E^{-08}	1.62 E^{-07}	5.90 E^{-08}
Rur. Fish.	Int	2.87 E^{-10}	2.45 E^{-10}	8.72 E^{-10}	3.39 E^{-10}	2.29 E^{-10}	2.29 E^{-10}	2.29 E^{-10}	2.01 E^{-09}	8.71 E^{-10}	3.61 E^{-09}	2.62 E^{-09}	6.78 E^{-10}	3.16 E^{-09}	6.29 E^{-10}	9.80 E^{-10}	1.59 E^{-09}	1.26 E^{-09}	5.54 E^{-10}	7.94 E^{-10}	3.28 E^{-09}	1.43 E^{-09}
	Skin	2.42 E^{-08}	3.66 E^{-08}	4.39 E^{-08}	3.46 E^{-08}	3.19 E^{-08}	3.19 E^{-08}	3.19 E^{-08}	5.98 E^{-08}	4.20 E^{-08}	9.64 E^{-07}	6.93 E^{-08}	1.19 E^{-07}	7.73 E^{-08}	3.75 E^{-08}	5.75 E^{-08}	6.70 E^{-08}	6.15 E^{-08}	5.16 E^{-08}	5.52 E^{-08}	1.92 E^{-07}	7.00 E^{-08}

Table 11a and 11b. Highest NCE and CE aggregated cumulative risk for both *recreational residential* (Rec. Res.) and *rural fishing* (Rur.Fish.) scenarios, discriminating in each case, the two pathways of exposure: accidental intake (Int) and skin contact (Skin). References for water bodies: see Table 3.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
NCE	Rec.Res.	96.24	96.81	95.39	96.01	96.81	96.81	96.81	81.17	89.08	98.86	75.02	99.11	76.88	98.84	93.78	90.86	92.21	96.06	94.65	62.67	92.41
	Rur. Fish.	98.08	98.43	97.97	98.13	98.43	98.43	98.43	92.59	95.60	98.96	90.31	99.18	90.87	99.06	97.31	96.34	96.73	98.22	97.70	84.85	96.84
CE	Rec.Res.	94.17	95.49	87.13	93.46	95.17	95.17	95.17	79.06	86.59	97.46	76.73	96.16	74.98	89.02	88.86	84.81	86.72	92.84	90.53	88.87	86.81
	Rur. Fish.	98.00	98.97	96.96	98.50	98.90	98.90	98.90	94.89	96.83	99.43	94.27	99.13	93.80	97.43	97.39	96.38	96.86	98.35	97.79	97.39	96.88

Table 11c. Percentage of the risk value through skin exposure with regard to the aggregated risk, for the *recreational residential* and the *rural fishing* scenarios differentiating NCE and CE. References for water bodies: see Table 3.

Pest.	ENC								EC							
	Int.				Skin				Int.				Skin			
	Rec. Res.		Rur. Fish.		Rec. Res.		Rur. Fish.		Rec. Res.		Rur. Fish.		Rec. Res.		Rur. Fish.	
	10	21	10	21	10	12	10	12	10	21	10	21	10	21	10	21
α – HCH	1.26 E-05	2.62 E-05	8.87 E-07	1.85 E-06	4.77 E-05	3.67 E-05	9.64 E-06	8.29 E-06	4.10 E-09	8.55 E-09	9.81 E-10	2.04 E-09	1.61 E-08	8.90 E-09	1.82 E-08	1.57 E-08
γ – HCH	3.63 E-06	3.24 E-07	2.56 E-07	2.29 E-08	1.38 E-05	1.06 E-05	2.78 E-06	2.39 E-06	1.18 E-09	1.06 E-10	2.83 E-10	2.53 E-11	1.42 E-09	7.84 E-10	1.60 E-09	1.38 E-09
δ – HCH	1.95 E-09	7.14 E-07	1.37 E-10	5.03 E-08	7.37 E-09	5.68 E-09	1.49 E-09	1.28 E-09	1.31 E-13	4.80 E-11	3.13 E-14	1.15 E-11	5.15 E-13	2.84 E-13	5.82 E-13	5.00 E-13
γ – Clor.	3.89 E-08	1.52 E-06	2.74 E-09	1.07 E-07	1.51 E-06	1.16 E-06	3.06 E-07	2.63 E-07	1.18 E-12	4.58 E-11	2.81 E-13	1.10 E-11	4.74 E-11	2.62 E-11	5.35 E-11	4.60 E-11
Acet	2.92 E-10	2.77 E-04	2.06 E-11	1.95 E-05	4.41 E-10	3.39 E-10	8.91 E-11	7.66 E-11								
Aldr.	7.66 E-05	3.63 E-06	5.40 E-06	2.56 E-07	8.85 E-03	6.81 E-03	1.79 E-03	1.54 E-03	6.74 E-09	3.20 E-10	1.61 E-09	7.64 E-11	8.08 E-07	4.46 E-07	9.13 E-07	7.84 E-07
DDD									8.06 E-14	8.06 E-14	1.93 E-14	1.93 E-14	5.92 E-12	3.27 E-12	6.68 E-12	5.74 E-12
DDT	2.02 E-07	2.02 E-07	1.42 E-08	1.42 E-08	3.78 E-05	2.91 E-05	7.64 E-06	6.57 E-06	5.91 E-12	5.91 E-12	1.41 E-12	1.41 E-12	1.15 E-09	6.35 E-10	1.30 E-09	1.12 E-09
α – Endo	2.98 E-07	8.04 E-08	2.10 E-08	5.67 E-09	2.43 E-07	1.87 E-07	4.91 E-08	4.22 E-08								
β – Endo	5.71 E-07	2.92 E-08	4.03 E-08	2.06 E-09	4.65 E-07	3.58 E-07	9.40 E-08	8.08 E-08								
Endo Sul.	3.70 E-09	4.02 E-07	2.61 E-10	2.84 E-08	3.01 E-09	2.32 E-09	6.09 E-10	5.23 E-10								
Endr	2.60 E-08	2.60 E-08	1.83 E-09	1.83 E-09	7.15 E-06	5.50 E-06	1.44 E-06	1.24 E-06								
Hept	1.75 E-06	6.35 E-06	1.24 E-07	4.47 E-07	6.50 E-05	5.01 E-05	1.32 E-05	1.13 E-05	6.80 E-10	2.46 E-09	1.62 E-10	5.88 E-10	2.62 E-08	1.45 E-08	2.96 E-08	2.54 E-08

Table 12. Highest risk values by pesticide for both pathways of exposure (accidental *intake* and *skin* contact) of each of the scenarios for the two water bodies with highest risk according to Tables 11a and 11b. References for water bodies: see Table 3. References for pesticides: see Table 2.

4. Discussion

When hazardous chemical substances are detected in waters that can be used with recreational purposes, the management procedure to evaluate whether there is risk for human populations in Argentina is to compare the concentrations found in water with Guide Levels or Highest Permitted Levels for human consumption water. Such procedure coincides with the guidelines written by WHO (1998). For such cases, the *National guidance levels for environment water quality for sources for human consumption* (SRHN, 2007b) or the Argentinian Food Code (PEN, 1969) with its Regulatory Decree 2126 (PEN, 1971), and its updates (ANMAT, 2010) are used. In the case of the analyzed water bodies in this work, because of being located in the Buenos Aires province, it is usual to resort to quality regulations for drinking water in Law 11820 (Legislature of Buenos Aires province, 1996) for comparison. This procedure, although it shares some similarities with the guidelines indicated by WHO (1998), it has some drawbacks.

The first and most evident drawback is that there are substances present in the water bodies analyzed in this work for which the regulatory framework does not have limit values (for example both Endosulfan isomers, Endosulfan Sulfate, and Endrin).

On the other hand, the comparison with regulatory levels is a management procedure that, though simple, is rigid and unrealistic in terms of the exposure. Firstly, the analysis is conducted by single substances for only one pathway of exposure. In this specific case, there are only normative regulations for water intake, not for skin contact. With regard to intake, the limit values are considering consumption intake, not recreational intake. Consumption water intake assumes intake rates much higher than the ones correspondent to accidental intake during recreational use, which causes that these values prove to be excessively conservative. The regulatory framework assumes an intake water rate of 2 L day^{-1} (SRNH, 2007b), which is much higher than 0.1 L day^{-1}, the highest value of intake utilized in this work for the recreational residential scenario.

Conversely, this work has demonstrated that, at least for this type of chemicals, evaluating substances toxicity by ingestion alone leads to underestimation of the risk. This study proved that in the aggregated cumulative risk value by organochlorine pesticides, the risk due to skin exposure is much higher than the one produced by intake.

HRA have operational advantages over the regulatory values as management tools. These methodologies allow conducting a more exhaustive and realistic study of all exposure processes, being able to classify between routes of exposure (digestive, respiratory, skin), scenarios (recreational, residential, working), exposed individuals (children, adults), and even to consider simultaneous pathways of exposure (aggregated HRA) and substances (cumulative HRA). The regulatory framework, on the contrary, does not allow for any particular analysis with regard to the two analyzed scenarios in accordance with the technical decisions that define them.

Another advantage that the HRA offer is the possibility to operate them probabilistically. The regulatory values assume deterministic values. Therefore, a child drinks 1 liter of water every day while an adult drinks 2 liters; a child weights 30 kg and an adult 70 kg (USEPA, 1997a; USEPA, 2002b). It is obvious that this simplification, although it makes the operational aspects easier, masks the existance of variability in human populations. It has to be admitted that within the "child" category, for example, there is an extremely important

dispersion of values if different age ranges are included. Moreover, population variability for one age, between sexes and for each sex also exists. It could even be argued that similar age ranges could differ, even within a population, due to the dependency on the intake rate, or weight rate, of some socioeconomic factors (USEPA, 1997a; USEPA, 2002b). The regulatory value is a unique representative value of these distributions, which leads to estimating the danger level from a unique exposure scenario, accurate and invariant towards the hazardous substance. The probabilistic studies note that the different participating variables have, intrinsically, uncertainty and variability, which influence the risk study and, consequently, the management based on them (Thompson and Graham, 1996).

The probabilistic techniques in the HRA operate with value distributions for each variable, resulting in the estimated risk being a value distribution also, with a different level of probability. This allows the inclusion of the uncertainty and/or variability resulting from the model, which a deterministic procedure cannot. Although in this work the analysis was carried out based on the highest values obtained for each application of Monte Carlo in order to have a more simplistic model, the result of each estimation was actually a distribution from which any statistic parameter could be obtained.

Therefore, if the regulatory levels were used as the only bathing waters management tool, the question whether the presence of these pesticides could generate health conditions because of the use of these water bodies for recreational bathing, would have remained unanswered. The hereby work shows that, given the lack of another tool to control the physical and chemical quality of water for recreational use with direct skin contact, the HRA could be a possible substitute management strategy providing information which is unattainable today with the currently available management tools.

5. Conclusions

This study indicates that health risk for non carcinogenic and carcinogenic effects due to accidental intake and skin contact during bathing activity in superficial water in the Buenos Aires province would not be relevant.

The application of health risk analysis allowed identifying La Peregrina shallow lake as the riskiest environment, when considering the exposure to accidental intake and skin contact simultaneously (aggregated risk) with all substances at the same time (cumulative risk). For the recreational residential scenario (which has a child as representative exposed individual) the aggregated cumulative risk is $9.06E^{-03}$ for non carcinogenic effects (NCE) and $8.23E^{-07}$ for carcinogenic effects (CE), whereas in the fishing rural scenario (whose representative exposed individual is an adult), the risks are $1.83E^{-03}$ for NCE and $9.66E^{-07}$ for CE.

For each scenario, skin contact risks are higher than those of accidental intake (in average, skin risk reaches 90 % of the aggregated risk of the scenario, for both NCE and CE).

If the differences between scenarios are evaluated, the child's scenario always has higher risk values than the adult's, but these differences are variable. The biggest difference occurs in NCE, between accidental intakes of both scenarios, where the cumulative risk for the child is 14 times higher than the risk for the adult. When CE are analyzed, the differences between both scenarios become narrower: the recreational scenario is 3 times and less than

one time higher than the fishing rural scenario for accidental intake and skin contact, respectively.

The substance that generates the highest risk values, for both NCE and CE, is Aldrin. For accidental intake, in addition to Aldrin, Acetochlor and the α isomer of HCH appear as important for NCE and Heptachlor for CE. For skin exposure, in addition to Aldrin, Heptachlor is relevant for both NCE and CE.

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7. References

Andrade M.L., Reyzábal, M.L., Covelo E.F., Vega, F.A. (2005). Organochlorine pesticides in soils of the horticultural belt of Bahía Blanca (Argentina). *Canadian Journal of Soil Science* 85, 2, May 2005, pp 273-282.

ANMAT National Administration of Drugs, Food and Medical Technology http://www.anmat.gov.ar/normativas_alimentos_cuerpo.asp (last accessed in July 2010) (in Spanish).

ANPCyT, National Agency for Promotion of Science and Technology (2005). *Tools for the Sustainable Management of Water Resources in a Plain Basin*. Project BID 1728/OC-AR-PID 452 (2005-2008). Dir. Dr. E. Usunoff. (in Spanish).

ANPCyT, National Agency for Promotion of Science and Technology (2007). *Development of Criteria and Guidelines for the Management of Water Resources in Plain Areas.* Project AR PID 35765 –UNCPBA PMT III BID 1728/OC (2007-2010) Dir. Dr. A. Villanueva. (in Spanish).

Arias A.H., Pereyra M.T., Marcovecchio J.T. (2010). Multi-year monitoring of estuarine sediments as ultimate sink for DDT, HCH, and other organochlorinated pesticides in Argentina. *Environmental Monitoring and Assessment* D.O.I. 10.1007/s10661-010-1315-9. Published online: 06 February 2010.

Carriquiriborde P., Díaz J., Mugni H., Bonetto C., Ronco A. (2007). Impact of cypermethrin on stream fish populations under field-use in biotech-soybean production. *Chemosphere* 68, 4, June 2007, pp 613–621.

CAS Chemical Abstract Service (2010). CAS database. http://www.cas.org. (last accessed in July 2010).

CASAFE Chamber of Agricultural Health and Fertilizer of Argentina (2007). http://www.casafe.org/comptox.htm. (last accessed in July 2010) (in Spanish)

Cataldo D., Colombo J.C., Boltovskoy D., Bilos C. & Landoni P. (2001). Environmental toxicity assessment in the Paraná River delta (Argentina): simultaneous evaluation of selected pollutants and mortality rates of Corbicula fluminea (Bivalvia) early juveniles. *Environmental Pollution* 112, 3, May 2001, pages 379-389.

Cid F., Antón R.I. & Caviedes-Vidal E. (2007). Organochlorine pesticide contamination in three bird species of the Embalse La Florida water reservoir in the semiarid midwest of Argentina. *Science of the Total Environment* 385, 1-3, October 2007, pp 86–96.

CITAB Statistical Information of Buenos Aires province. Center of Territorial and Environmental Investigations of Buenos Aires province. CITAB site (2010). http://www.bancoprovincia.com.ar/citab/estadisticas.htm (last accessed in July 2010). (in Spanish).

CONICET, National Council of Scientific and Technical Investigations (2004). *Monitoring of Organochlorine Pollutants in Buenos Aires Shallow Lakes: Impact Assessment on Icthyofauna. Implications and Perspectives of Application.* Project Pip 5877-CONICET 2004. (Dir. Dr. C. Maggese). (in Spanish).

De Girolami D., Freylejer C., González C., Mactas M., Slobodianik N. & Jáuregui Leyes P. (2003). Description and analysis of the Body Mass Index and estatural category by ages, in a registry of 10.338 individuals of Argentina. *Actualización en nutrición* 4, 2, April-June 2003, pp 12-17. (in Spanish).

De la Torre F., Ferrari L. & Salibian A. (2005). Biomarkers of a native fish species (*Cnesterodon decemmaculatus*) application to the water toxicity assessment of a peri-urban polluted river of Argentina. *Chemosphere* 59, 4, April 2005, pp 577 – 583.

Decisioneering, Inc. (2007). Crystal Ball software Version 7.1.

Directorate of Provincial Statistics (2010). http://www.ec.gba.gov.ar/Estadistica/FTP/index.html. (last accessed in July 2010) (in Spanish).

DuBois D. & DuBois D.F. (1916). A formula to estimate the approximate surface area if height and weight be known. *Archives of Internal Medicine* 17, (6_2), June 1916, pp 863-71.

FARN Environmental and Natural Resources Foundation (2005). Legal framework applicable to the integrated management of pesticides in Argentina. Final Report. http://www.farn.org.ar/arch/informe_pesticidas.pdf (last accessed in July 2010) (in Spanish).

García Fernández J.C. (1974). Studies and comments on human impregnation with organochlorine pesticides in Argentina. *Medicina* 34, 4, April 1974, pp 393-410. (in Spanish).

Gómez E., Ferreras L., Lovotti L. & Fernández E. (2009). Impact of glyphosate application on microbial biomass and metabolic activity in a Vertic Argiudoll from Argentina. *European Journal of Soil Biology* 45, 2, March-April 2009, pp 163–167.

Goransky R & Natale O. (1996). *Methodological bases for the establishment of local guidelines of water quality for human consumption*. Final Report. Undersecretary of Water Resources of Argentina, National Institute of Science and Technology of Water, Autonomous city of Buenos Aires, Argentina. (in Spanish).

Grosman F. & Benito A. (2004). *Socio-economic assessment of sport fishing in Junín.* Editorial Universidad Nacional del Noroeste de la Provincia de Buenos Aires. ISBN 987-21506-3-X. Junín, Argentina. (in Spanish)

Grosman F. (2006). *Typology of different actors linked to the pampean shallow lakes of Argentina*. In *Gestión y cultura del agua* Soares Moraes D., Vázquez García V., Serrano Sánchez

A., de la Rosa Regalado A., Coord. Pp. 134-150. Mexican Institute of Water Technology and Postgraduates' College in Agricultural Sciences. Mexico DF, Mexico. (in Spanish)

ISAAA, International Service for the Acquisition of Agri-biotech Applications (2010). *Executive Summary of Global Status of Commercialized Biotech/GM Crops: 2007*. ISAAA Briefs No. 37. http://www.isaaa.org/resources/publications/briefs/37/executivesummary/default.html (last accessed in July 2010).

Janiot L., Sericano J.L. & Roses O.E. (1994). Chlorinated pesticide occurrence in the Uruguay River (Argentina-Uruguay). *Water, Air, & Soil Pollution* 76, 3-4, August 1994, pp 323-331.

Jergentz S., Mugni H., Bonetto C. & Schulz R. (2004). Runoff-related endosulfan contamination and aquatic macroinvertebrate response in rural basins near Buenos Aires, Argentina. *Archives of Environmental Contamination and Toxicology* 46, 3, April 2004, pp 345-352.

Jergentz S., Mugni H., Bonetto C. & Schulz R. (2005) Assessment of insecticide contamination in runoff and stream water of small agricultural streams in the main soybean area of Argentina. *Chemosphere* 61, 6, November 2005, pp 817-826.

Jofré M.B., Antón R.I. & Caviedes-Vidal E. (2008). Organochlorine contamination in anuran amphibians of an artificial lake in the semiarid midwest of Argentina. *Archives of Environmental Contamination and Toxicology* 55, 3, October 2008, pp 471-480.

Legislature of Buenos Aires province (1996). Law 11820. Regulatory Framework for the Supply of Public Drinking Water and Sewage in the Province of Buenos Aires. Official Bulletin of 11/09/96. http://www.gob.gba.gov.ar/legislacion/legislacion/l-11820.html. (last accessed in July 2010). (in Spanish).

Lejarraga H. & Orfila G. (1987). Height and weight standards for Argentinian children from birth to maturity. *Archivos Argentinos de Pediatría* 85, March 1987, pp 209-222. (in Spanish).

Lenardón A., Maitre M.I. & Enrique S. (1994). Organochlorine pesticides in Argentinian butter. *Science of The Total Environment* 144, 1-3, April 1994, pp 273-277.

Loewy M., Kira V., Carvajal G., Venturino A. & Pechen de D'Angelo A. (1999). Groundwater contamination by azinphos methyl in the Northern Patagonic Region (Argentina). *The Science of the Total Environment* 225, 3, January 1999, pp 211-218.

Loewy M., Carvajal G., Novelli M. & Pechen de D'Angelo A. (2003). Effect of pesticide use in fruit production orchards on shallow ground water. *Journal of Environmental Science and Health - Part B Pesticides, Food Contaminants, and Agricultural Wastes* 38, 3, April 2003, pp 317-325

López H.L., Baigún C.R., Iwaszkiw J.M., Delfino R.L., Padín O. (2001) *The Salado basin: use and possibilities of its fishing resources*. Editorial de la Universidad de La Plata, Serie Ambiente y Desarrollo 1, ISBN 950-34-0221-2. La Plata, Argentina. (in Spanish)

MAA Ministerio de Asuntos Agrarios (2004). *Report of the Institutional Strengthening Project for Rural Development of the Buenos Aires Province*. Ministry of Land Affairs. http://www.maa.gba.gov.ar/dir_des_rural/proinder.php (last accessed in July 2010) (in Spanish).

Maitre M.I., de la Sierra P., Lenardon A., Enrique S. & Marino F. (1994). Pesticide residue levels in Argentinian pasteurised milk. *The Science of The Total Environment* 155, Issue 2, October 1994, pp 105-108.

Marino D. & Ronco A. (2005). Cypermethrin and Chlorpyrifos concentration levels in surface water bodies of the Pampa Ondulada, Argentina. *Bulletin of Environmental Contamination and Toxicology* 75, 4, October 2005, pp 820–826.

Martin M.L. & Ronco A. (2006). Effect of Mixtures of Pesticides Used in the Direct Seeding Technique on Nontarget Plant Seeds. *Bulletin of Environmental Contamination and Toxicology* 77, 2, August 2006, pp 228-236.

Menone M.L., Aizpún de Moreno J.E., Moreno V.J., Lanfranchi A.L., Metcalfe T.L. & Metcalfe C.D. (2000a). PCBs and organochlorines in tissues of silverside (*Odontesthes bonariensis*) from a coastal lagoon in Argentina. *Archives of Environmental Contamination and Toxicology* 38, 2, February 2000, pp 202-208.

Menone M.L., Bortolus A., Botto F., Aizpún de Moreno J.E., Moreno V,J, Iribarne O., Metcalfe T.L. & Metcalfe C.D. (2000b). Organochlorine contaminants in a coastal lagoon in Argentina: Analysis of sediment, crabs, and cordgrass from two different habitats. *Estuaries and Coasts* 23, 4, August 2000, pp 583-592.

Menone M.L., Aizpún de Moreno J.E., Moreno V.J., Lanfranchi A.L., Metcalfe T.L. & Metcalfe C.D. (2001). Organochlorine pesticides and PCBs in a Southern Atlantic Coastal Lagoon Watershed, Argentina. *Archives of Environmental Contamination and Toxicology* 40, 3, March 2001, pp 355-362.

Menone M.L., Miglioranza K.S.B., Botto, F., Iribarne, O., Aizpún de Moreno, J.E. & Moreno, V.J. (2006). Field accumulative behavior of organochlorine pesticides. The role of crabs and sediment characteristics in coastal environments. *Marine Pollution Bulletin* 52, 12, December 2006, pp 1717-1724.

Miglioranza K.S.B., Aizpún de Moreno J., Moreno V.J. (2004). Land-based sources of marine pollution: organochlorine pesticides in stream systems. *Environmental Science and Pollution Research* 11, 4, July 2004, pp 227-232.

Miglioranza K.S.B., Aizpún de Moreno J.E., Moreno V.J., Osterrieth M.L. & Escalante A.H. (1999). Fate of organochlorine pesticides in soils and terrestrial biota of Los Padres pond watershed, Argentina. *Environmental Pollution* 105, 1, April 1999, pp 91-99.

Miglioranza K.S.B., González Sagrario M., Aizpún de Moreno J.E., Moreno V.J., Escalante A.H.& Osterrieth M.L. (2002). Agricultural soil as a potential source of input of organochlorine pesticides into a nearby pond. *Environmental Science and Pollution Research*, 9, 4, July 2002, pp 250-256.

Miglioranza, K.S.B., Aizpún De Moreno, J.E. & Moreno, V.J. (2003a). Dynamics of organochlorine pesticides in soils from a southeastern region of Argentina. *Environmental Toxicology and Chemistry* 22, 4, April 2003, pp 712-717.

Miglioranza K.S.B., Aizpún de Moreno J.E. & Moreno V.J. (2003b). Trends in soil science: organochlorine pesticides in argentinean soils. *Journal of Soils and Sediments* 3, 4, December 2003, pp 264-265.

Muñoz de Toro M., Beldoménico H.R., García S.R., Stoker C., De Jesús J.J., Beldoménico P.M., Ramos J.G. Luque E.H. (2006). Organochlorine levels in adipose tissue of women from a litoral region of Argentina. *Environmental Research* 102, 1, September 2006, pp 107–112.

NRC, National Research Council (1983). *Risk assessment in the federal government: managing the process. 206* p. ISBN 0–309–03349–7. National Academic Press, Washington, USA.

Peluso F., González Castelain J., Cazenave G. & Usunoff E. (2006). Estimation of water intake rate and frequency of exposure in natural recreational waters for its use in probabilistic health risk assessment. *Cuadernos del CURIHAM*, 12, 1, December 2006, pp 1 – 7. (in spanish).

Peluso F., González Castelain J., Rodríguez L. & Jaime S. (2010). Compared bathing of two places of the Del Azul creek (Azul county, Buenos Aires province) for health risk analysis. In press. *REGA (Revista de Gestión del Agua en América Latina)* 7, 1, January-July 2010. (in Spanish).

Peluso F., Grosman F. & González Castelain J. (2009). Health risk from organochlorine pesticides in water of an Argentinian pampean shallow lake. *Acta Bioquímica Clínica Latinoamericana* 43, 2, April-June 2009, pp 233-240. (in Spanish).

Peluso F.; González Castelain J., Othax N. & Rodríguez L. (2010). Health risk from toxic substances in surface waters of Tres Arroyos, Argentina. Inédito. (In Spanish).

National Goverment of Argentina Republic (1969). National Law 18284, National Food Code. *Anales de la Legislación Argentina* XXIX-B, 1456, 18/VII/69. http://www.anmat.gov.ar/codigoa/Ley_18284_1969.pdf (last accessed in July 2010) (in Spanish).

National Goverment of Argentina Republic (1971). Decree 2126/71 of the Law 18284. *Anales de la Legislación Argentina* XXXI-C, 3086, 30/VI/71 (in Spanish).

Congress of Argentina Republic (2002). Law 25675: General Law of Environment. Official Bulletin of 28/11/2002. http://www.ambiente.gov.ar/?aplicacion=normativa&IdNorma=85 (last accessed in July 2010) (in Spanish).

Pengue W. (2000*). Transgenic Crops. Where are we going?* Lugar Editorial S.A., Buenos Aires, Argentina, pp 1–190. (in Spanish).

Pengue W. (2001). Soybean expansion in Argentina. Globalization, Development and Agricultural Genetic Engineering: A model for assembly. Grain site. http://www.grain.org/seedling/?id=328. (in spanish).

Peruzzo P.J., Porta A.A. & Ronco A.E. (2008). Levels of glyphosate in surface waters, sediments and soils associated with direct sowing soybean cultivation in north pampasic region of Argentina. *Environmental Pollution* 156, 1, November 2008, pp 61-66.

Prüss A. (1998). Review of epidemiological studies on health effects from exposure to recreational water. *International Journal of Epidemiology* 27, 1, February 1998, pp 1–9.

Rovedatti M.G., Castañe P.M., Topalián M.N. & Salibián A. (2001). Monitoring of organochlorine and organophosphorus pesticides in the water of the Reconquista River (Buenos Aires, Argentina). *Water Research* 35, 14, October 2001, pp 3457–3461.

Ruíz A.E., Wierna, N. & Bovi Mitre G. (2008). Organochlorine pesticides in raw milk sold in Jujuy (Argentina). *Revista de Toxicología* 25, 1-3, May 2008, pp 61-66. (in Spanish).

Sericano J.L. & Pucci A. (1984). Chlorinated hydrocarbons in the seawater and surface sediments of Blanca Bay, Argentina. *Estuarine, Coastal and Shelf Science* 19, 1, July 1984, pp 27-51.

Silva C., Boia C., Valente J. & Borrego C. (2005). Pesticides in Esteros del Iberá (AR): evaluation of impacts and proposal of guidelines for water quality protection. *Ecological Modelling* 186, 1, July 2005, pp 85–97.

SRHN, Undersecretariat of Water Resources of the Argentina Nation site (2007a). *National guidance water quality levels for human recreation*. http://www.hidricosargentina.gov.ar/metodologia5.pdf (last accessed in July 2010) (in Spanish).

SRHN, Undersecretariat of Water Resources of the Argentina Nation site (2007b) *National guidance water quality levels for human recreation*. http://www.hidricosargentina.gov.ar/metodologia11.pdf (last accessed in July 2010) (in Spanish).

SRHN, Undersecretariat of Water Resources of the Argentina Nation site (2010). National guidance levels for water quality. http://www.hidricosargentina.gov.ar/NivelCalidad.html (last accessed in July 2010) (in Spanish).

Thompson K. & Graham J. (1996). Going beyond the single number: using probabilistic risk assessment to improve risk management. *Human and Ecological Risk Assessment* 2, 4, September 1996, pp 1008 – 1034.

USEPA, US Environmental Protection Agency (1989). *Risk assessment guidance for superfund*. Volume 1: human health evaluation manual. Part A. EPA/540/1-89/002. 287 p. US Environmental Protection Agency, Office of Emergency and Remedial Response. Washington DC, USA. http://www.epa.gov/oswer/riskassessment/ragsa/ (last accessed in July 2010)

USEPA, US Environmental Protection Agency (1992a). *Guidelines for exposure assessment*. EPA/600/Z-92/001. 139 p. US Environmental Protection Agency, Risk Assessment Forum. Washington DC, USA. http://cfpub.epa.gov/ncea/cfm/recordisplay.cfm?deid=15263 (last accessed in July 2010)

USEPA, US Environmental Protection Agency (1992b). *Supplemental guidance to RAGS: calculating the concentration term*. Intermittent Bulletin. Volume 1 Number 1. 8 p. Publication 9285.7-081. US Environmental Protection Agency, Office of Emergency and Remedial Response, Washington DC, USA. http://www.deq.state.or.us/lq/pubs/forms/tanks/UCLsEPASupGuidance.pdf (last accessed in July 2010).

USEPA, US Environmental Protection Agency (1996). *Proposed guidelines for carcinogen risk assessment*. EPA/600/P-92/003C. US Environmental Protection Agency, Office of Research and Development. Washington DC, USA.

USEPA, US Environmental Protection Agency (1997). *Exposure Factor Handbook.* (Final Report) USEPA/600/P-95/002. 1193 p. National Center for Environmental Assessment, Office of Research and Development US Environmental Protection

Agency, Washington D.C., USA. http://cfpub.epa.gov/ncea/cfm/recordisplay.cfm?deid=12464 (last accessed in July 2010).

USEPA, US Environmental Protection Agency (2001a). *General principles for performing aggregate exposure and risk assessments*. 79 p. US Environmental Protection Agency, Office of Pesticide Programs. Washington DC, USA. http://www.epa.gov/pesticides/trac/science/aggregate.pdf (last accessed in July 2010).

USEPA, US Environmental Protection Agency (2001b). *Risk assessment guidance for superfund*. Volume III. Part A: Process for conducting probabilistic risk assessment. EPA 540-R-02-002. 385 p. US Environmental Protection Agency, Office of Emergency and Remedial Response, Washington DC, USA.

USEPA, US Environmental Protection Agency (2002a). *Calculating upper confidence limits for exposure point concentrations at hazardous waste sites*. OSWER 9285.6 -10. US Environmental Protection Agency, Office of Emergency and Remedial Response Washington DC, USA. http://www.epa.gov/swerrims/riskassessment/pdf/ucl.pdf (last accessed in July 2010).

USEPA, US Environmental Protection Agency (2002b). *Child-Specific Exposure Factors Handbook*. EPA-600-P-00-002B. 448 p. National Center for Environmental Assessment, Office of Research and Development, U.S. Environmental Protection Agency, Washington D.C., USA. http://cfpub.epa.gov/ncea/cfm/recordisplay.cfm?deid=199243 (last accessed in July 2010).

USEPA, US Environmental Protection Agency (2003). *Framework for cumulative risk assessment*. EPA/630/P-02/001F. 129 p. Environmental Protection Agency; National Center for Environmental Assessment; Office of Research and Development; Washington (DC). http://oaspub.epa.gov/eims/eimscomm.getfile?p_download_id=36941 (last accessed in July 2010)

USEPA, US Environmental Protection Agency (2004). *Risk assessment guidance for superfund*. Volume I: Human Health Evaluation Manual. Part E. EPA/540/R/99/005. 156 pp. US Environmental Protection Agency, Office of Superfund Remediation and Technology Innovation, Washington DC, USA.

USEPA, US Environmental Protection Agency (2005). *Guidelines for carcinogen risk assessment*. EPA/630/P-03/001F. 166 p. US Environmental Protection Agency, Risk Assessment Forum, Washington DC, USA. http://www.epa.gov/raf/publications/pdfs/CANCER_GUIDELINES_FINAL_3-25-05.PDF (last accessed in July 2010).

USEPA, US Environmental Protection Agency (2010). IRIS (Integrated Risk Information System) database. http://www.epa.gov/IRIS.

WHO, World Health Organization (1998). *Guidelines for Safe Recreational-water Environments: Coastal and Freshwaters*. Consultation Draft. World Health Organization, Geneva. http://www.who.int/water_sanitation_health/bathing/srwe1/en/ (last accessed in July 2010).

WHO, World Health Organization (1999). *Health-Based Monitoring of Recreational Waters: the Feasibility of a New Approach (The 'Annapolis Protocol')*. World Health Organization, Geneva. http://www.who.int/water_sanitation_health/bathing/wsh9901/en/index.html (last accessed in July 2010).

WHO, World Health Organization (2000). *Monitoring Bathing Waters - A Practical Guide to the Design and Implementation of Assessments and Monitoring Programmes.* Published on behalf of WHO by F & FN Spon. 311 p. ISBN 0-419-24390-1. London, UK.

WHO, World Health Organization (2008). *Guidelines for drinking-water quality incorporating 1st and 2nd addenda, Vol.1, Recommendations.* 3rd ed. 668 p. ISBN 978-92-4-154761-1, World Health Organization, Geneva, Switzerland.

Zubillaga H., Sericano J.L. & Pucci A.E. (1987). Organochlorine pesticide contents of tributaries into Blanca Bay, Argentina. *Water, Air, & Soil Pollution* 32, 1-2, January 1987, pp 43-53.

10

Trace Organic Contaminants (PAHS, PCBs, and Pesticides) in Oysters *Crassostrea virginica*, from the Caloosahatchee Estuary and Estero Bay, SW Florida.

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1. Introduction

Estuaries and coastal marine environments are some of the most anthropogenically-stressed environments in the world and are known to receive large amounts of hazardous organic chemicals in traces but deleterious quantities. Some of these chemicals include carcinogenic and toxic polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), and pesticides and herbicides. These chemicals can not only adversely benthic (Thompson et al. 1996, Capuzzo 1996, McDowell et al. 1999) and pelagic organisms but also accumulate to higher trophic levels, ultimately affecting humans.

The Caloosahatchee River and Estuary is located on the southwest coast of Florida (Fig. 1) and runs from Lake Okeechobee to the Franklin Lock and Dam (S-79) where it empties into the estuary at Shell Point. The estuary between the Franklin Lock and Dan and Shell Point is ~ 42 kilometers long. The Caloosahatchee River is the major source of freshwater to the estuary.

Historically, the Caloosahatchee River was a meandering riverine system with numerous oxbows, flowing from its headwaters at the marshlands of Lake Flirt and Lake Hicpochee, west of Lake Okeechobee, to the Gulf of Mexico. Alterations to this system have begun in the late 1800's, with Hamilton Disston's dredging and channelization project, which included a connection to Lake Okeechobee and construction of an extensive canal network associated with agricultural development in the watershed (Barnes 1995). The river has been permanently connected to Lake Okeechobee and about 20% of the water entering the estuary now comes from the Lake, mainly as regulatory releases to maintain the Lake at a prescribed water level (SFWMD 2009). The river has also been straightened, deepened and three water control structures (S-77, S-78, and S-79) have been added. The last structure S-79, was completed in 1966 to act, in part, as a salinity barrier. The channelization and canal building process (C-43) has changed the timing, quantity, quality and direction of runoff within the watershed and led to abnormal salinity fluctuations. The operation of three water

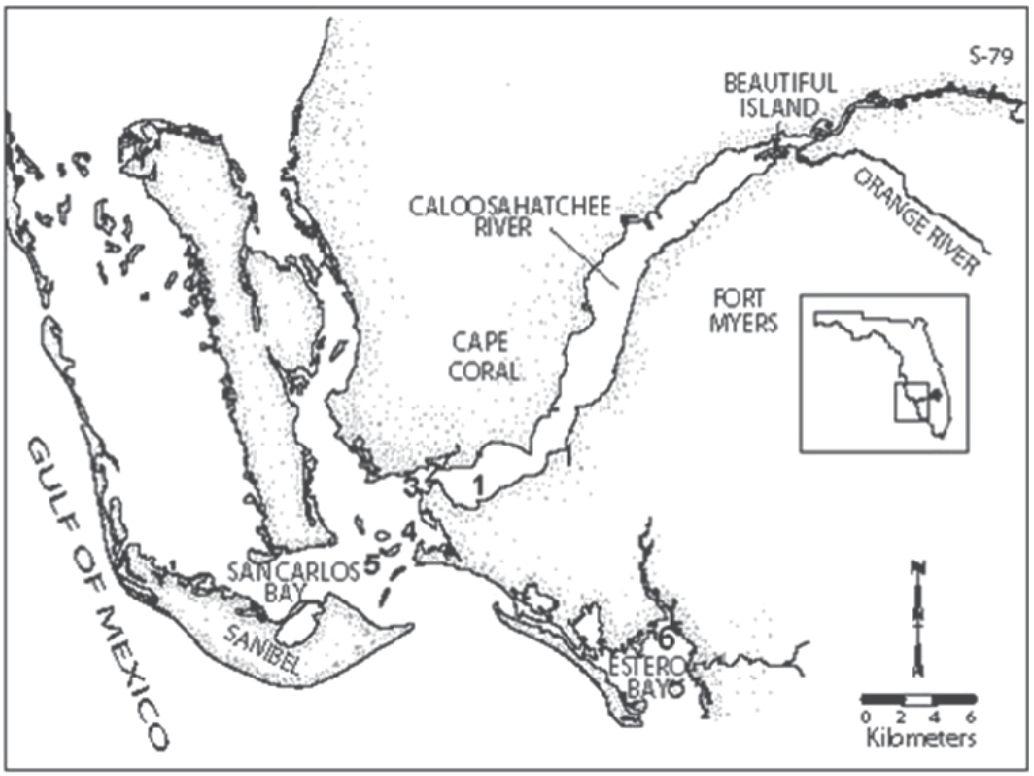

Fig. 1. Sampling Locations in the Caloosahatchee Estuary (1-5) and Estero Bay (6)), Southwest Florida.

control structures allowing large periodic regulatory releases from Lake Okeechobee has reduced the tidally influenced portion of the estuary. The combination of over-draining and the addition of S-79 as a salinity barrier results in a truncated and highly variable salinity gradient in the estuary. During periods of low freshwater discharge typically during the dry season, salt water regularly intrudes all the way to the structure, often exceeding 10 ppt. By contrast, high freshwater discharge can cause salinity to drop below 5 ppt at the mouth. The transition between the two states can be rapid, sometimes requiring less than a week. The fluctuations observed at the head and mouth of the estuary exceeds the salinity tolerances of oligohaline and marine species. The result is that freshwater delivery to the estuary has been altered and shows characteristics consistent with a watershed that has lost storage capacity. Stormwater is not retained but runs off quickly and at higher peak flows. Because there is no retention base flows are low or non-existent during the dry season (Volety 2008, Volety et al. 2009).

In SW Florida, in addition to lake releases from Okeechobee, runoff from agricultural lands, golf courses, marinas, and housing subdivisions is directed into sensitive estuarine environments such the Caloosahatchee estuary. These water flow alterations have resulted in the input of organic and inorganic pollutants and altered salinity regimes, stressing the organisms inhabiting these ecosystems.

Oysters *Crassostrea virginica* are ubiquitous features in the estuaries along the eastern seaboard of the United States and the Gulf of Mexico including SW Florida and the

Caloosahatchee estuary (Volety et al. 2009). Given the enormous filtration activity (Newell 1988), and low detoxification capability of oysters, oysters are used for contaminant monitoring (O'Connor and Lauenstein 2006). The purpose of this study was to determine temporal and spatial variation in baseline concentrations of PAHs, PCBs, and selected pesticides in oyster tissue in Caloosahatchee Estuary and a reference site, Estero Bay.

2. Methods

2.1 Field collections

Oysters were collected by hand from the Caloosahatchee Estuary and Estero Bay in Southwest Florida (Fig. 1). Most of the sampling locations are intertidal to shallow-subtidal in nature and are subject to tidal flushing. Samples were scrubbed clean of fouling organisms and were kept frozen (-20 ^{o}C) until further processing as noted below.

2.2 Analytical protocol

Just prior to contaminant analyses, oysters were thawed at room temperature for ~ 2-4 h after which time they were shucked and all soft tissue removed for determination of water content and trace organic contaminants. Approximately 15 oysters were pooled and homogenized per composite sample. Composite wet oyster tissue from each station was placed in 100 mL pre-cleaned (450 ^{o}C for 4h) and tared glass jars and then dried at 60 ^{o}C. The drying process was continued for at least one week or longer until dry weights stabilized. Sample water content was determined using the difference in weight before and after drying.

Desiccated oyster tissue was homogenized in a stainless steel blender. In between samples, the blades were wiped down pre-cleaned glass wool soaked with GC-grade methanol. Dried homogenized oyster tissue samples were then placed in pre-weighed Pyrex test tubes. A surrogate standard consisting of deuterated PAHs and PCBs was added, followed by 30 mL of a dichloromethane:methanol (2:1, v:v) cocktail. Samples were extracted twice using ultrasonication for 30 minutes each time. Each extract was centrifuged and transferred to a rotary evaporator flask. Extracts were concentrated to 0.25 mL, exchanged to 100% hexane, and then purified using silica gel. The purification process consisted of passing the extract through a column containing 10 g of precleaned (Soxhlet extraction with DCM for 24 h) deactivated silica (mesh size 100-200), topped with 0.5 cm precombusted (4 h at 450 ^{o}C) Na_2SO_4 for further purification. As an additional precautionary step against tissue lipids confounding gas chromatographic analysis, Florisil chromatography was also conducted. The purified extract was then reduced by rotoevaporation followed by nitrogen blow down to 1 mL. An internal standard containing deuterated select PAHs and pesticides was added, and the extract further reduced to 100 µL under N_2 for analysis and quantification using gas chromatography-mass spectrometer.

Sample extracts were analyzed on a Shimadzu QP5050A gas chromatograph-mass selective detector (GC/MS). A J&W DB-35MS capillary column (30 m length, 0.25 mm diameter, 0.25 m film thickness) was used and compounds were quantified using selective ion monitoring. Method parameters for PAH and pesticide analysis were as follows: 70 ^{o}C initial hold time of 1 min; 70-150 ^{o}C at 20 ^{o}C min^{-1}; 150-280 ^{o}C at 4 ^{o}C min^{-1}, hold for 15 min; 280-295 ^{o}C at 5 ^{o}C min^{-1}, hold for 2 min; source temperature 150 ^{o}C. The carrier gas was helium set to flow at 1.0 mL min^{-1} with a velocity of 39 cm s^{-1}. The PAHs analyzed in this manner included

naphthalene, azulene, 2-methylnaphthalene, 1-methylnaphthalene, acenaphthylene, biphenyl, acenaphthene, fluorene, phenanthrene, anthracene, 1-methylfluorene,2-methylphananthrene, 2-methylanthracene, 1-methylanthracene,1-methylphenanthrene, 9-methylanthracene, fluoranthene, pyrene, 3,6-dimethylphenanthrene, 9,10-dimethylanthracene, benz[a]anthracene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[e]pyrene, benzo[a]pyrene, perylene, indeno[1,2,3-cd]pyrene, benzo[ghi]perylene, dibenz[a,h]anthracene, coronene. The sum of these compounds are referred to as total PAHs. The organochloride compounds analyzed in this manner include DDT and metabolites, chlordane and isomers, hexachlorocyclohexanes, and heptachlor. Organophosphates targeted in this study include: methylparathion, fenthion, parathion, and azinophos (methyl,ethyl). Triazine herbicides, carbamates, and pyrethroids were also quantified. Minimum detectable quantities were established via quantification of relative response factor dilutions of known concentrations, with minimum acceptable signal to noise ratio of 10. Instrument detection limits for the GC/MS ranged from 0.002 ng to 0.056 ng per 1 μL injection.

3. Results and discussion

3.1 Trace organic contaminant concentrations in oyster tissue – compound class totals

In general, the concentrations of PAHs, PCBs, organochlorides, and organophosphates found in oyster tissue in this study are similar in magnitude to concentrations found in oyster tissue globally (KUMAR et al., 2008; LAUENSTEIN et al., 2002a). Detectable concentrations of total PAHs (tPAHs) in oysters ranged from 2 to 800 ng grams dry weight^{-1} throughout both Caloosahatchee Estuary as well as in Estero Bay (Table 1), with generally higher levels in Caloosahatchee Estuary. There did not seem to be any obvious seasonal trend in tPAH concentrations at both sites (Figure 2). Samples which were designated as "not quantifiable" (NQ) contained excessive lipid interferences in the chromatography which precluded confirmation of PAH compound identity. In general, the range of tPAH concentrations in oyster tissue from both sites in this study are lower than that found in Gulf of Mexico oysters (WADE et al., 1988) but similar in concentration to that found in coastal waters of North and South Carolina (LAUENSTEIN et al., 2002b). Many of the PAHs detected at both sites are known to be carcinogens (e.g. benzo[a]pyrene). In general, polychlorinated biphenyl (PCBs) concentrations were only detected in a oyster tissue from a few months during the sampling period and moreover, they were detected slightly more frequently at the reference site, Estero Bay (Table 1). Their limited detection precludes us from making specific statements about their spatial or temporal trends at each location.

The organochlorides analyzed in the oysters in this study are listed in Table 2. Interestingly, organochloride compound concentrations which were detected at mg L^{-1} concentrations in some cases, were higher in oysters from the reference site, Estero Bay, in December of 2007 and in March, June, September and November of 2008. These levels of organochlorides are higher than those found in oysters isolated from a marsh/estuarine environment in coastal Georgia, USA (Kumar et al., 2008). Concentration ranges of organophosphate pesticides varied broadly between ~5 to 15,000 ng gdw^{-1} (Table 2). While there were no discernable trends across both sites (Figure 5), organophosphate levels were higher in Estero Bay, the reference site than in Caloosahatchee Estuary. Both organochlorides and organophosphate compounds have relatively high vapor pressures (SCHWARZENBACH et al., 2003). Their

	polycyclic aromatic hydrocarbons (ng g^{-1} dry wt)			
	Caloosahatchee		Estero	
Month	average	Std. Dev.	average	Std. Dev.
Dec-07	213.04	20.52	100.04	22.68
Jan-08	144.57	1.44	43.48	10.68
Febuary - 08	143.92	0.00	5.58	2.51
March-08	8.04	4.96	78.79	59.03
April-08	556.66	47.56	11.37	1.55
May-08	0.00	0.00	61.49	20.26
June-08	57.11	46.22	181.21	0.00
July-08	2.79	0.00	0.00	0.00
August-08	293.67	94.41	1.76	0.00
September-08	497.58	313.36	154.34	101.08
October-08	760.91	746.92	82.33	10.76
November-08	151.86	146.75	33.75	0.00

	polychlorinated bihenyls (ng g^{-1} dry wt)			
	Caloosahatchee		Estero	
Month	average	Std. Dev.	average	Std. Dev.
Dec-07	0.00	0.00	1.50	0.61
Jan-08	0.69	0.00	0.00	0.00
Febuary - 08	1.03	0.00	0.00	0.00
March-08	0.83	0.00	1.34	0.00
April-08	0.00	0.00	0.47	0.00
May-08	0.00	0.00	0.81	0.00
June-08	0.00	0.00	0.79	0.00
July-08	0.00	0.00	0.00	0.00
August-08	0.73	0.12	0.00	0.00
September-08	0.00	0.00	21.41	0.00
October-08	0.00	0.00	0.81	0.00
November-08	3.40	0.00	0.00	0.00

Table 1. Polycyclic aromatic hydrocarbons and polychlorinated biphenyls in oyster tissue.

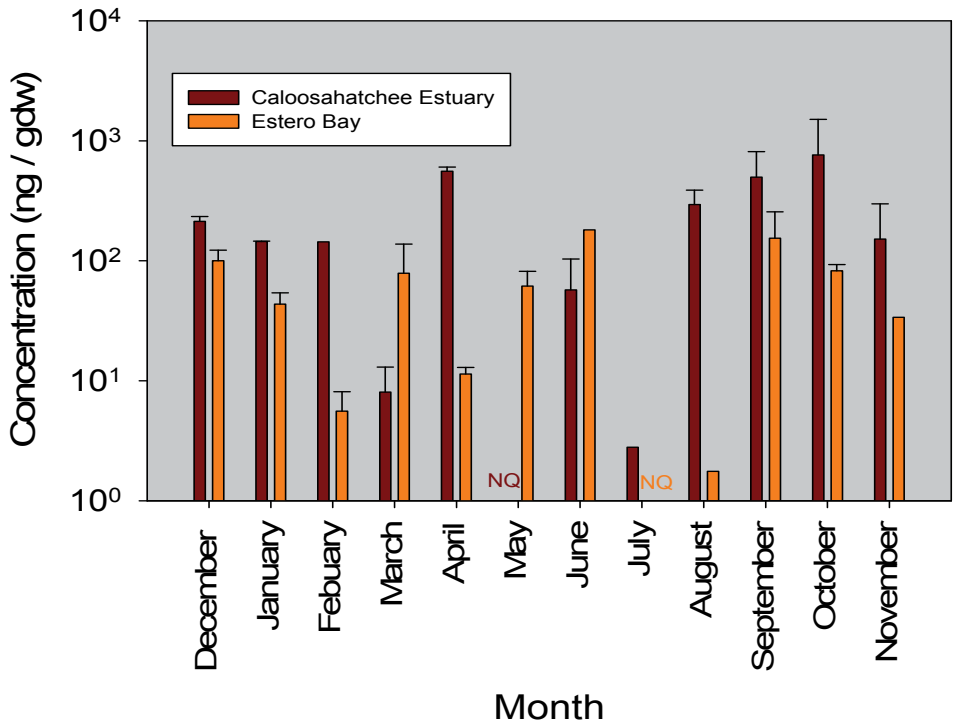

Fig. 2. Total polycyclic aromatic hydrocarbons (PAH) concentrations in Caloosahatchee Estuary and Estero Bay oyster tissue.

presence at detectable levels in both the Caloosahatchee Estuary and Estero Bay may ultimately have resulted from volatilization from agricultural soils from proximal or distal regions (THOMANN and CONNOLLY, 1984). This would account for similar levels of occurrence at both sites. Typically, oysters accumulate trace organic contaminants from sediments in the seabed in areas where they reside. In that context, sedimentary resuspension may have resulted in periodic exposure of the trace organic contaminants in this study. Sediment resuspension events have been demonstrated to result in greater bioaccumulation of metals by oysters in the Gulf of Mexico (JOHNSON et al., 2009). Given that all the sampling collection areas are interidal and shallow-subtidal, oysters from these areas are exposed to sediment re-suspension through wind- and tidal-driven forces as well as boat wakes.

Triazine herbicides and carbamates were detected periodically in oyster tissue throughout the sampling period at both sites. Concentration ranges varied broadly from the low ng g^{-1} levels to mg g^{-1} levels per dry weight of oyster tissue. Both systems are located proximal to upland areas and/or receive water from agricultural and urban areas that lie within the watershed, where triazine and carbamate compounds may be applied as herbicides and pesticides. Triazines and carbamates remain some of the mostly widely used classes of trace organic pesticides in the continental US. Thus, their detection in these samples is not surprising, given some of the regional applications noted above.

	organochlorides (ng g^{-1} dry wt)			
	Caloosahatchee		Estero	
Month	average	Std. Dev.	average	Std. Dev.
Dec-07	32.71	8.18	136.75	36.93
Jan-08	593.84	12.89	37.12	27.07
Febuary - 08	3.73	0.28	2.54	0.21
March-08	54.20	13.16	95.38	5.53
April-08	28.56	6.97	3.76	0.12
May-08	0.00	0.00	29.30	5.60
June-08	88.23	47.87	9346.45	15.65
July-08	10.29	0.00	0.00	0.00
August-08	74.49	59.77	40.88	0.00
September-08	26.44	7.60	1033.27	55.13
October-08	41.27	27.70	24.10	8.89
November-08	5.43	0.46	486.25	0.00

	organophosphates (ng g^{-1} dry wt)			
	Caloosahatchee		Estero	
Month	average	Std. Dev.	average	Std. Dev.
Dec-07	25.03	22.74	179.54	122.26
Jan-08	0.00	0.00	5.86	4.21
Febuary - 08	0.51	0.00	1.14	0.00
March-08	303.49	294.65	1344.75	8.29
April-08	38.84	26.34	36.80	0.00
May-08	0.00	0.00	5.88	6.12
June-08	318.45	2.38	10873.40	0.00
July-08	0.00	0.00	0.00	0.00
August-08	2994.87	4697.27	215.17	0.00
September-08	15.52	8.92	15507.29	0.00
October-08	9.98	6.12	15.61	11.07
November-08	25.24	24.48	0.00	0.00

Table 2. Organochlorides and organophosphates in oyster tissue.

	triazines (ng g^{-1} dry wt)			
	Caloosahatchee		Estero	
Month	average	Std. Dev.	average	Std. Dev.
Dec-07	2.44	0.00	0.56	0.00
Jan-08	0.00	0.00	2.73	0.29
Febuary - 08	2.37	0.00	24.70	15.64
March-08	37.55	2.19	2.30	1.22
April-08	291.42	264.53	1.36	0.00
May-08	0.00	0.00	1.33	0.00
June-08	11.65	2.61	421.15	0.00
July-08	0.00	0.00	0.00	0.00
August-08	5.76	0.00	0.00	0.00
September-08	77.87	75.58	0.00	0.00
October-08	5.49	0.00	0.54	0.00
November-08	0.00	0.00	0.00	0.00

	carbamates (ng g^{-1} dry wt)			
	Caloosahatchee		Estero	
Month	average	Std. Dev.	average	Std. Dev.
Dec-07	11.63	10.52	63.06	0.00
Jan-08	749.80	0.00	0.82	0.00
Febuary - 08	0.00	0.00	2.53	0.83
March-08	0.00	0.00	15.24	0.00
April-08	0.23	0.00	0.00	0.00
May-08	0.00	0.00	0.98	0.00
June-08	3.17	0.00	35.18	0.00
July-08	0.00	0.00	0.00	0.00
August-08	13.93	0.00	0.00	0.00
September-08	0.00	0.00	0.00	0.00
October-08	3.24	0.00	6.03	0.00
November-08	13.21	0.00	2586.04	0.00

Table 3. Triazine herbicides and carbamates in oyster tissue.

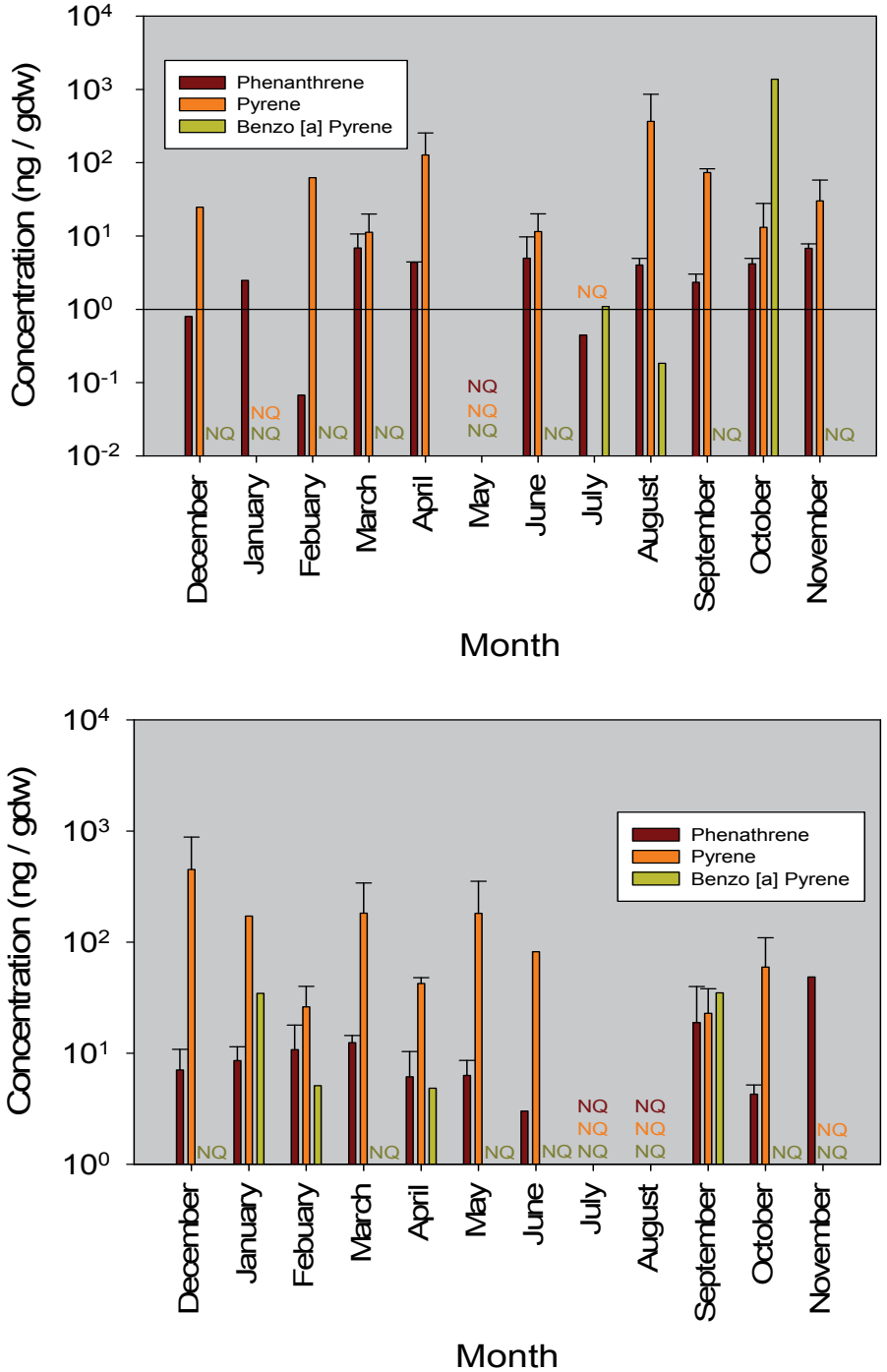

Fig. 3. Selected PAHs in oyster tissue isolated from Caloosahatchee Estuary (top panel) and Estero bay (bottom panel).

	pyrethroids (ng g^{-1} dry wt)			
	Caloosahatchee		Estero	
Month	average	Std. Dev.	average	Std. Dev.
Dec-07	0.00	0.00	0.00	0.00
Jan-08	0.00	0.00	0.00	0.00
Febuary - 08	0.00	0.00	0.00	0.00
March-08	2.21	0.00	2.21	0.00
April-08	0.00	0.00	0.41	0.00
May-08	0.00	0.00	0.21	0.00
June-08	0.00	0.00	0.00	0.00
July-08	0.00	0.00	0.00	0.00
August-08	0.00	0.00	0.00	0.00
September-08	0.00	0.00	0.00	0.00
October-08	1.51	0.00	0.33	0.00
November-08	0.00	0.00	174.17	0.00

Table 4. Pyrethroids in oyster tissue.

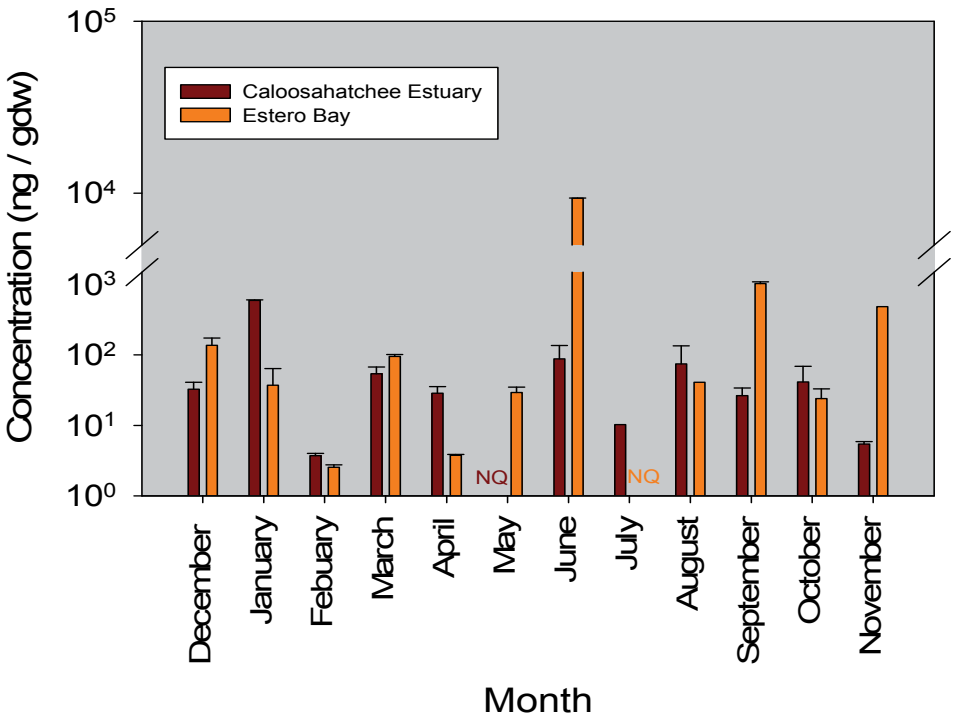

Fig. 4. Organochlorides in Caloosahatchee Estuary and Estero Bay oyster tissue.

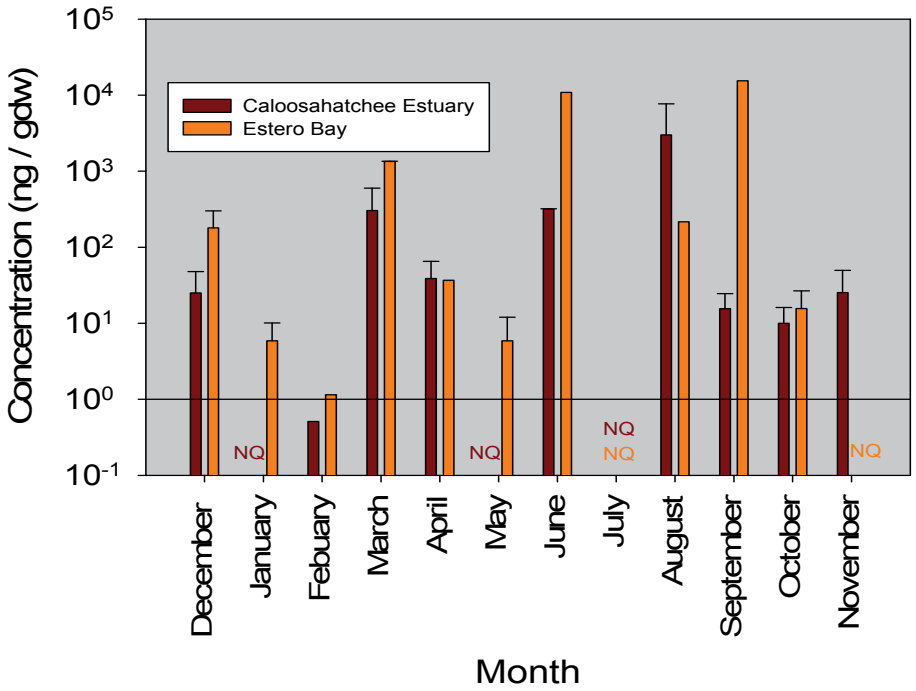

Fig. 5. Organophosphates in Caloosahatchee Estuary and Estero Bay oyster tissue.

3.2 Trace organic contaminant concentrations in oyster tissue – individual PAHs

In general, PAHs enter modern aquatic environments as a byproduct of combustion processes or are petroleum derived. While some PAHs are low enough in molecular weight that they remain in solution, in general, mid to high molecular weight PAHs (e.g. phenanthrene to indeno[1,2,3-cd]pyrene) associate with particulate or dissolved organic matter in the environment. Such particulate or dissolved organic matter upon association with sediments in aquatic systems can enter the benthic food webs. Although we did not quantify organic contaminants in sediment, we can use the size/molecular weight of various PAHs to help understand PAH fate and transport, and ultimately their sources in the environment. Low molecular weight PAHs are more water soluble relative to their high molecular weight counterparts (MACKAY et al., 1992). Thus, observations of low molecular weight PAH bioaccumulation may imply that the aqueous phase served as a relatively more efficient route of entry into an organism. In contrast, high molecular weight PAHs tend to be more particle reactive so their abundance in oyster tissue could be indicative of more-particle rich sources of trace organic contaminants (MACKAY et al., 1992). Alternatively, an absence of high molecular weight PAH accumulation may suggest that PAH availability to oysters was limited by desorption from particles. Indeed, PAHs tightly absorbed to combustion-derived particles such as soot can be minimally bioavailable (ACCARDI-DEY and GSCHWEND, 2003; LOHMANN et al., 2005).

Concentrations of phenanthrene, pyrene, and benzo[a]pyrene were examined closely in oyster tissue from both sites. In most cases, oyster tissue was higher in pyrene relative to

phenanthrene in both Caloosahatchee Estuary and in Estero Bay. This observation of a mid-molecular weight PAH being accumulated to a greater extent than a low molecular weight PAHs, has been observed in other contaminant accumulation studies with benthic organisms (Landrum, 1989). One explanation for this observation is that lower molecular weight PAHs such as phenanthrene may be accumulated and then metabolized and subsequently excreted by benthic organisms in contrast to larger PAHs which are more difficult to metabolize (Landrum, 1989). Alternatively, low molecular weight PAHs as well as other low molecular weight trace organic contaminants may simply not be in abundance as they have higher vapor pressures, volatilization rates, and microbial mineralization rates, resulting in faster disappearance from the aquatic ecosystem in which they reside (MACKAY et al., 1992), In contrast, high molecular weight and more particle reactive PAHs may have a higher residence time in the environment within which the oysters reside. Benzo[a]pyrene was only detectable in oyster tissue in 3 months out of the year 1 samples at Caloosahatchee Estuary and in four months out of year in samples from Estero Bay. We propose that the infrequence periods of detectable levels of benzo[a]pyrene compared to other PAHs can be explained by benzo[a]pyrene may be irreversibly be bound to soot-like residues obtained from combustion as noted above (LOHMAN and GSCHWEND, 2003).

In conclusion, we observe that middle molecular weight PAH's were present in the highest concentrations. This shows oysters are accumulating their contaminants from both dissolved and particulate-associated compounds. Highest concentrations of PAH's were observed in the Caloosahatchee Estuary. Varying levels of organochlorides were observed within the Caloosahatchee Estuary and Estero Bay. Slightly higher levels of organophosphates were found within the Estero Bay Aquatic Buffer Preserve. Continued monitoring is needed in both of these systems along with an approach designed to target the possible routes of entry of these trace organic contaminants into oysters.

4. Acknowledgements

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5. References

Accardi-Dey, A. and Gschwend, P. M., 2003. Reinterpreting literature sorption data considering both adsorption into organic carbon and adsorption onto black carbon. *Environmental Science and Technology* 37, 99-106.

Barnes, T. K. 2005. Caloosahatchee estuary conceptual ecological model. *Wetlands* 25, 884-897.

Capuzzo, J. M. 1996. The bioaccumulation and biological effects of lipophilic organic contaminants. In. *The Eastern Oyster Crassostrea virginica*. V. S. Kennedy, R. I. E. Newell, and A. F. Eble (eds). Maryland Sea Grant College Publication, College Park, MD. pp. 539-557.

Johnson, W. E., Kimbrough, K. L., Lauenstein, G. G., and Christensen, J., 2009. Chemical contamination assessment of Gulf of Mexico oysters in response to hurricanes Katrina and Rita. *Environmental Monitoring and Assessment* 150.

Kumar, K. S., Sajwan, K. S., Richardson, J. P., and Kannan, K., 2008. Contamination profiles of heavy metals, organochlorine pesticides, polycyclic aromatic hydrocarbons and alkylphenols in sediment and oyster collected from marsh/estuarine Savannah GA, USA. *Marine Pollution Bulletin* 56, 136-149.

Landrum, P. F., 1989. Bioavailability and toxicokinetics of polycyclic aromatic hydrocarbons sorbed to sediment for the amphipod *Pontoporeia hoyi*. *Environmental Science and Technology* 23, 588-595.

Lauenstein, G. G., Cantillo, A. Y., and O'Connor, T. P., 2002a. The status and trends of trace element and organic contaminants in oysters, Crassostrea virginica, in the waters of the Carolinas, USA. *The Science of the Total Environment* 285, 79-87.

Lauenstein, G. G., Cantillo, A. Y., and O'Connor, T. P., 2002b. The status and trends of trace element and organic contaminants in oysters, *Crassostrea virginica*, in the waters of the Carolinas, USA. *The Science of the Total Environment* 285, 79-87.

Lohman, R. and Gschwend, P. M., 2003. Experimentally-derived values of K_{BC} for selected PAHs in Boston and NY-NJ Harbor.

Lohmann, R., MacFarlane, J. K., and Gschwend, P. M., 2005. On the Importance of Black Carbon to Sorption of PAHs, PCBs and PCDDs in Boston and New York Harbor Sediments. *Environmental Science and Technology* 39, 141-148.

Mackay, D., Shiu, W. Y., and Ma, K. C., 1992. Illustrated handbook of physical-chemical properties and environmental fate of organic chemicals. Lewis Publishers, Chelsea.

McDowell, J. E., B. A. Lancaster, D. F. Leavitt, P. Rantamaki, and B. Ripley. 1999. The effects of lipophilic organic contaminants on reproductive physiology and disease processes in marine bivalves. Limnology and Oceanography. 44: 903-909.

Newell, R. I. E. 1988. Filtration capacities of oysters based on historic evidence. Proceedings of the Chesapeake Research Consortium 1988 meeting, Baltimore, MD. Chesapeake Research Consortium Publication. 129: 536-546.

O'Connor, T., and Lauenstein, G.G. 2006. Trends in chemical concentrations in mussels and oysters collected along the US coast: Update to 2003. *Marine Environmental Research*, vol 26, pp. 261-285.

Schwarzenbach, R. P., Gschwend, P. M., and Imboden, D. M., 2003. *Environmental Organic Chemistry*. Wiley, Hoboken.

SFWMD. 2009. Caloosahatchee River Watershed Protection Plan, South Florida Water Management District. http://www.sfwmd.gov/portal/page/portal/pg_grp_sfwmd_koe/portlet_northerneverglades/tab2302089/ne_crwpp_main_123108.pdf

Thomann, R. V. and Connolly, J. P., 1984. Model of PCB in the Lake Michigan lake trout food chain. *Environmental Science and Technology* 18, 65-71.

Thompson, R. J., R. I. E. Newell, V. S. Kennedy, and R. Mann. 1996. Reproductive processes and early development. In. *The Eastern Oyster Crassostrea virginica*. V. S. Kennedy, R. I. E. Newell, and A. F. Eble (eds). Maryland Sea Grant College Publication, College Park, MD. pp.335-370.

Volety, A. K. 2008. Effects of salinity, heavy metals and pesticides on health and physiology of oysters in the Caloosahatchee Estuary. Ecotoxicology. 17: 579-590. DOI 10.1007/s10646-008-0242-9.

Volety, A. K., M. Savarese, G. Tolley, P. Sime, P. Goodman, and P. Doering. 2009. Eastern oysters (*Crassostrea virginica*) as an indicator for restoration of Everglades Ecosystems. Ecological Indicators. 9: S120-S136. DOI:10.1016/j.ecolind.2008.06.005

Wade, T. L., Atlas, E. L., Brooks, J. M., Kennicutt II, M. C., Fox, R. G., Sericano, J., Garcia-Romero, B., and DeFreitas, D., 1988. NOAA Gulf of Mexico Status and Trends Program: Trace Organic Contaminant Distribution in Sediments and Oysters. *Estuaries* 11, 171-179.

11

Cholinergic Pesticides

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1. Introduction

The use of protection plant products for the control of pests in agriculture is very ancient: thousands of years ago, Greek and Chinese people knew the insecticide properties of sulfur and arsenic compounds, respectively. The roman Plinio suggested the use of organic insecticides, such as the sedum and marrobium extracts for fighting insects, while Virgil suggested treating the seeds with olive oil to avoid fungine infestation.
After that period, up to the XIX century, the agricultural economy allowed the use of natural remedies, without need of chemical products. Actually, the agricultural sites were relatively small, and with differentiated cultures, so that the effects of infestation of the single cultures were not relevant on the general economy. With the XX century, a new way of regarding to agriculture was diffused, thanks to the availability of large agricultural areas to be used for monocultures. This made necessary the prevention and fight against pests. Paul Herman Muller (1899-1965, Nobel prize for medicine in 1948) first understood the properties of DDT to be effective not only against the common housefly, but also against a wide variety of pests, including the louse, Colorado beetle, and mosquito. This compound was extensively used also in agriculture, but recently it was banned in several Countries cause of its long persistence in environment. High persistence pollutants have been called POPs (Persistent organic pollutants). Persistence is a dangerous feature, because it causes accumulation in the environment and possible bioaccumulation in the organisms.
The studies on toxicity of DDT and other organochlorine insecticides (dieldrin and heptachlor) and the ascertainment of their interference in the endocrine system caused their ban in the US in 1972 (Mellanby, 1992) and in Europe (Council Directive 79/117/EEC* and Regulation EC No 850/2004 of the European Parliament and of the Council).
Banning organochlorine agents caused an increase in the use of organophosphate and carbamate (CB) pesticides. Organophosphate (OP) is the general name for the esters of phopsphoric acid. Organophosphates are the most diffused organophosphorus compounds, and are also the basis of a number of pesticides and insecticides worldwide used and

poured into the environment in the amount of hundred tons every season. These compounds are easily synthesized, and their hemi life lasts from some days to some months in the Laboratory, at room temperature. The discovery of their effects on living organisms was made by the German chemist Willy Lange and his graduate student, Gerde von Krueger (cited by Khurana & Prabhakar, 2000), who first described the effects on cholinergic nervous system. This discovery inspired German chemist Gerhard Schrader (Nobel prize in 1948) in the 1930s to experiment with these compounds as insecticides at company IG Farben. Along these studies, he discovered Tabun, an enormously toxic organophosphate compound towards a number of organisms, including man. Thus, the potential use of OPs as chemical warfare agents induced the Nazi government to develop organophosphate nervine agents (Buckley et al., 2004). In that period the G series of weapons, which included Sarin, Tabun and Soman, was produced. These weapons were not used during World War II. British scientists also synthesized diisopropyl fluorfosfate (DFP), during the war. After World War II, American companies gained access to information from Schrader's laboratory, and began synthesizing organophosphate pesticides in large quantities. Parathion was among the first marketed, followed by Malathion and Azinphosmethyl. These compounds and their formulate derivatives are used for a wide range of aims: from chemical weapon, to pest control and also medical compounds (anxiolytic, antispasmoic, regulators of eye pressure, etc.) (*part of information obtained from Wikipedia)

Carbamate pesticides (Aldicarb, Carbaryl, Carbofuran, and their formulate derivatives are also largely employed for agricultural, garden and even domestic pest control, and are proposed for substitution of pyrethroid and organophosphorus compounds against *Anopheles* in Third Countries (Akogbéto et al., 2010). The first synthesized and used carbamate is Carbaryil, which was commercialized in 1956. Their persistence in the environment is short, but the persistence is higher in aquatic medium. Thus, environmental effects are exerted mainly on fish and aquatic organisms.

In addition, a new generation of neurotoxic compounds, with effect on a part of the cholinergic system, is represented by neo-nicotinoids, of which the most known is Imidachloprid

2. Mode of action of cholinergic pesticides

Organophosphorus and carbamate compounds exert their neurotoxic activity by inhibition of cholinesterase activities (acetylcholinesterase, AChE, E.C. 3.1.1.7 and pseudo cholinesterase, BChE: E.C. 3.1.1.8) and, consequently, the status of the cholinergic neurotransmitter system. These enzymes are modulators of the cholinergic signaling, as their function is exerted by removing the signal molecule acetylcholine (ACh) from its receptors (see the review of Hayes, 1991 for organophosphates and of Fischel, 2008, for carbamates). Consequently their inhibition causes an overflow of ACh at receptor sites, that in turn affects intracellular responses driven by both nicotinic and muscarinic receptors. In this way, neurotoxic compounds may cause alteration of all functions of the cholinergic neurotransmission system, and of other neurotransmitters, whose release is regulated by the pre-synaptic ACh receptors. These insecticides are strongly suspected to cause damage to the human health, and clearly they do in case of acute intoxication, when people gets in contact with high doses, generally for accidents, or occupational causes. But up to date a few data are present about the possibility of subtle chronic (low-dose, long-term) damage due to aerosol diffusion, or to residuals in crops and vegetables, possibly reinforced by the co-

formulated compounds and /or traces of other pollutants, such as other neurotoxic substances, heavy metals, hydrocarbons, or else. On the other hand, the no-effect concentration for man (NOEC), indicated by the pharmaceutical firms and databases, is not surely ascertained, because it is obtained by experimental exposure of animals, generally rats or mice, and then by estimating it as several fold lower. Moreover, very few is known about the possible bioaccumulation in the body, which is different between models and also is subject to individual variability. In addition, the doses that do not affect adults may strike heavily embryonic differentiation, which represents a very sensitive stage of the organism life. The signs and symptoms of carbamate poisonings are similar to those caused by the organophosphate pesticides. The carbamate's principal route of entry is either by inhalation or ingestion or secondarily by the dermal route. Dermal exposure tends to be the less toxic route than inhalation or ingestion. For example, carbofuran has a rat oral LD_{50} of 8 mg/kg, compared to a rat dermal LD_{50} of greater than 3,000 mg/kg, making it much more toxic when ingested. The carbamates are hydrolyzed enzymatically by the liver; degradation products are excreted by the kidneys and the liver. Respiratory depression combined with pulmonary edema is the usual cause of death from poisoning by carbamate compounds. As with organophosphates, the signs and symptoms are based on excessive cholinergic stimulation. Unlike organophosphate poisoning, carbamate poisonings tend to be of shorter duration because the inhibition of nervous tissue acetylcholinesterase is reversible, and carbamates are more rapidly metabolized (Fischel, 2008).

The pharmacology of OPs and carbamates has been extensively studied, and the differences are resumed as follows: 1) OPs irreversibly link the AChE molecule by the phosphate group (Guo et al., 2003) thus preventing the ingress of ACh in the active site of the gorge, while carbamates compete for the substrate acetylcholine (ACh), allowing reversibility of the effects (Minneau, 1991); 2) OPs can leave residuals in the environment, while carbamates only leave small inorganic molecules (Fishel, 2008).

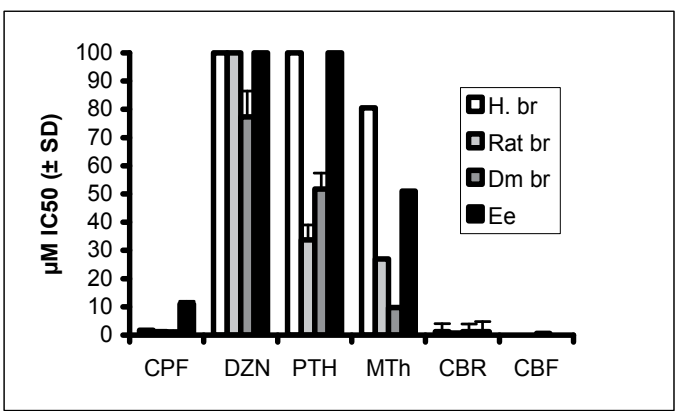

Fig. 1. (Made by Rakonczay, in the frame of the EC project SENS-PESTI, QLK4, and reported in the paper by Aluigi et al., 2005). Effect of organophosphates and carbamates on acetylcholinesterase activity from different sources. IC50 values are reported as means ± SD, of 3-6 independent experiments with triplicate samples. Preincubation with inhibitor was 30 min, the inhibitors were solved in MeOH, final concentration of the MeOH in the incubation mixture was 0,5%.Purified electric eel AChE was purchased from Sigma, by use of three different lot numbers of enzyme preparations.

3) Most of OPs are soluble in lipids, and this allows passage through the cell membranes and accumulation in fat tissues.

Metabolites toxicity

The metabolites of both carbamates and Ops are more than tenfold active in ChEs inhibition than their parent molecules (Sultatos, 1994, Aluigi et al., 2005) Actually, the link between the oxon derivatives and the serine residuals present in the gorge of AChE molecule is much more persistent, and it is said that oxonized OP compounds definitely "kill" the AChE molecule (Sultatos, 1994).

The IC50 for CPF was between 1 and 10 μM, depending on the different organisms sensitivity; the IC50 of DZN and PTH was between 30 and more than 100 μM (over the diagram scale). The carbamates showed IC50 around 1 μM for all the organisms, including the purified Electric eel AChE, used as a control.

Student's t-tailed with 2-tailed significance values showed:

Drug	**H Br vs**	**P value**	**H Br vs**	**P value**	**H Br vs**	**P value**
CPF	rat br	< 0,002:	*Dm* br	< 0,05	Ee	< 0,0001
DZN	rat br	NS	*Dm* br	< 0,01	Ee	NS
PTH	rat br	< 0,0001	*Dm* br	< 0,0002	Ee	NS
MTh	rat br	< 0,0001	*Dm* br	< 0,0001	Ee	< 0,0004
CBR	rat br	< 0,0004	*Dm* br	NS	Ee	< 0,04
CBF	rat br	< 0,0008	*Dm* br	< 0,0004	Ee	< 0,0001

Table 1. Significance of the different effect of the drugs on human AChE molecules vs neural tissue of different organisms. CBF = carbofuran; CBR= carbaryl; CPF = Chlorpyrifos; CBR; DZN=diazinon; MTH = malathion; PTH = fenthion; H br = human brain; *Dm* br= *Drosophila melanogaster* brain: rat br = rat brain; Ee= electric eel purified cholinesterase (Sigma)

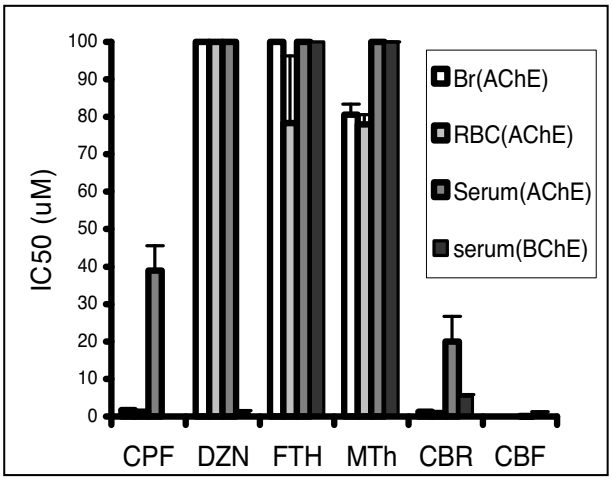

Fig. 2. IC50 of OP and CB compounds on AChE and BChE of different human tissues

2.1 Primary and secondary targets of toxicity

AChE activity is the primary, but not the only one target of cholinergic compounds toxicity: actually, according to Casida & Quistad (2004), secondary non-AChE targets are represented by inhibition of pseudocholinesterases, and ACh receptors, such as the muscarinic ones, that can be affected directly, besides the effect mediated by AChE inhibition. This causes sometimes contradictory effects, such as increase of AChE activity in the affected organs (Aluigi et al, 2005; Aluigi et al., 2010a, 2010b) showing a sort of paradox effect.

The effect of some cholinergic inhibitors is different not only among different organisms, but also among different brain parts. Actually, Rakonczay and Papp (2001) also found that after an acute (4 h) treatment with an irreversible cholinesterase inhibitor organophosphate, metrifonate (100 mg:kg i.p.), the activities of both acetyl- and butyrylcholinesterase were inhibited (66.0–70.7% of the control level) in the rat brain cortex and hippocampus. There were no significant changes in the acetyl- and butyryl-cholinesterase activities in the olfactory bulb, or in the choline acetyltransferase activity in all three brain areas.

The third class of cholinergic substances (neonicotinoids) are insecticides which act on the neuromuscular system of insects with lower toxicity to mammals. Neonicotinoids are among the most widely used insecticides worldwide, because they affect a molecular form of nicotinic ACh receptor, which is typical of insects. The mode of action of neonicotinoids is similar to the natural insecticide nicotine, that (like ACh) activates the response of nicotinic ACh receptors, but is not cleaved by ChEs. In insects, neonicotinoids cause paralysis which leads to death, often within a few hours. The main concern for the use of these insecticides is due to a possible connection to honey bee Colony Collapse Disorders and generally for the disappearance of pollinator insects. According to what is reported in the literature, no damage may be exerted on man, cause of the specific binding to insect receptors, but recent data may suggest a certain caution. Preliminary experiments show competition between Imidachloprid and α-BuTx, a snake venom from *Bungarus multicinctus*, selectively binding to the α-7 subunit of the mammalian nicotinic receptor.

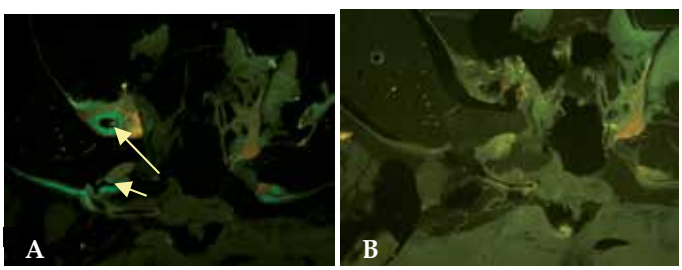

Fig. 3. Cross section of bee heads, embedded in Kulzer 7100 resin, sectioned 3 μm thick. Both the sections were incubated 1 h in the dark with 10^{-8}M FITCH-conjugated α-BuTx, in PBS pH 7.4. A: untreated bee; B: bee pre-exposed for 10 min to 10^{-5}M Imidachloprid. (Thesis of Dr. Guglielmo Castagnoli, 2009).

2.2 Persistence in the environment and crops

The persistence of these compounds in the environment is generally considered fairly short, but evidences have been found that in sediments they may remain for long times, as occurred in the river Rhine (Dauberschmidt et al, 1996) where lethality of fish, mollusks, and aquatic birds lasted for months and kilometers downstream. According to Ragnarsdottir

(2000) an OP pesticide presenting short half-life in the laboratory increases to one year in conditions of low pH and temperature. The same author reported that OPs are detected in soils years after application, probably due to sorption of the OPs to soil particles, making them unavailable for microbial metabolism (Ragnarsdottir, 2000). As far as living organisms are concerned, the effects of such compounds may last much more, because the AChE of blood may be affected up to several months (see the case report Romero et al. 1989).

2.3 Studies in USA, South America, Australia, Eastern Countries

These two classes of pesticides are directed towards both insects and other small pest organisms, and act similarly. They interfere with cholinergic transmission in the nervous system of their target, and affect human health because AChE is a common enzyme, active in the nervous system of all the living organisms, and involved in cell-to-cell communications, including those leading embryonic development and differentiation. For this reason, their effects on human health have been studied more intensively in countries such as USA, South America and Australia, and even eastern Countries, such as India, where the agricultural sites cover big areas, and agriculturers represent an important part of the population. In Europe only a few researcher groups work on this argument.

Some commonly used organophosphates in these countries include malathion, methyl parathion, chlorpyrifos, azinphosmethyl, and diazinon. Common N-methyl carbamates include aldicarb and carbaryl (*List of chemicals evaluated for carcinogenic potential.* U.S. EPA Office of Pesticide programs, 26 August 1999).

Anyway, in these countries also, the study of the effects of low-dose exposure to contaminants is rather neglected, because OPs and CBs, introduced to replace organo-chlorines, are generally shorter-lived in the environment, and more acutely toxic. This way of regarding the problem causes an underestimate of the possible contact with consumers, due to residuals in the crops, and also to the fact that after collection, during transport, the merchandises are packed and again treated with pesticides (e.g. bananas from Costa Rica are packed in plastics with chlorpyrifos and shipped to European markets: personal communication from distributors).

2.4 Europe

The main bulk of studies in Europe are represented by environmental diagnostics: i.e. identification of the presence of contaminants in the environment by use of AChE biochemical detection as a bio marker. Two of these projects were recently supported by the European Community (Project Reference: ACHEB QLK3-2000-00650; SENS_PESTI, QLK4-CT2002-02264). The last one involved our group, and most of the reported results were obtained along development of this project (2003-2006), and after, as a proceeding of work. Here we report some of the results obtained in the frame of SENS-PESTI, together with other outstanding reports available in the literature. The studies worldwide are an enormous number, cause of the socio-economical relevance of the topic, thus our report cannot be as complete as I would like.

3. Human diseases possibly related to occupational exposure

3.1 Acute intoxication

This term refers to the immediate sensible effects (generally within 24 hours) of a particular dose of cholinergic pesticide on human health.

The exposure to such high doses of the contaminants is generally caused by accidents such as the one occurred in the river Rhine in November, 1986 (Dauberschmidt et al., 1996), which caused an ecological disaster, including lethality of fish, mollusks, and aquatic birds for months and kilometers downstream. The effects of acute intoxication are mainly exerted on the nervous system, through the hyper activation of receptors, causing peripheral nervous symptoms, also called *cholinergic crisis,* up to death (Jamal, 1997). The symptoms are due to muscarinic receptors (cardiac arrhythmia, salivation, lacrimation, hypotension, respiratory problems, headache, dizziness), and to nicotinic receptors, causing paralysis, muscular cramps, and titanic contraction of muscles (Aardema et al., 2008). This crisis is sometimes followed by a more dangerous late onset of symptoms, such as asystole, which may appear after weeks, when the patient is released from the Hospital (Chacko and Elangovan, 2010). The effects of the acute intoxication are well known and classified as well as the first aid practice and antidotes, such as oxime and atropine (see Sultatos, 1994, Aardema et al., 2008, for extensive reviews).

3.2 Chronic intoxication from low continuous or repeated doses

At long term, nervous system disorders may occur: for instance, it was discussed for many years and now definitely established, from epidemiological studies in California, that in areas where pesticides are spread, the incidence of certain neurodegenerative diseases is increased (Davis et al., 1978; Betarbet et al., 2000). Respiratory effects may lead to aggravation of pre-existing conditions such as asthma (Underner et al., 1987). Actually, it is known that one of the effects of OPs is exerted on bronco constriction (Reeves et al., 1999). Carbamates such as Carbaryl, may also cause morphologically deformed sperms etc.

Between 1991 and 1996, California EPA reported 3, 991 cases of occupational poisoning by agricultural pesticides (O'Malley, 1997). Domestic use of pesticides may cause symptoms that are similar or identical to those caused by other illnesses, so that chronic pesticide poisoning is often misdiagnosed.

In particular, neurotoxic pesticides effects are directed towards embryonic development as shown by experiments on invertebrates and vertebrates differentiation (Sherman, 1966; Morale et al., 1998, Pesando et al., 2002, Aluigi et al., 2005, 2010a). Numerous case reports and case series present various combined severe congenital anomalies following occupational or accidental exposure of pregnant women to OP pesticides (Romero et al., 1989, Soreq and Zakut, 1990).

Chronic intoxication is due to prolonged or repeated exposure to low doses of pesticides. This is slow and may cause subtle health effects, and every body may be exposed, for the diffusion of aerosols, or by consuming agricultural products (in some agricultural sites, a survey of 1997 revealed that the large-leaf vegetables on the market were found to contain from 0.3 to 0.007 mg/Kg organophosphate residues (Ligurian EPA, personal communication).

Chronic health effects from pesticides are problematic to study in humans, because most people are exposed to low doses of pesticide mixtures, symptoms appear late in time, and delayed health effects are difficult to link to past exposures.

3.2.1 Cancer facts

Among the effects on human health, several are known or suspected: cancer facts, such as inheritable gene amplification suspected to cause tumors in families of agriculturers (Soreq

and Zakut, 1990; Shapira et al., 2000); multiple recent reports link hairy cell leukemia (HCL) with pesticide exposure, in particular with organophosphate exposure (Clavel et al. 1996). More recent studies (Cabello et al, 2001) have demonstrated a relationship between malathion and parathion and the induction of mammary tumors (possibly related to the function of these two compounds as endocrine disrupters, as all the liposoluble organic compounds are potentially able to interfere in steroid hormones reception). In human adults, Gorell et al. (1998) reported about the possibility that neurotoxic pesticides may induce neurodegenerative diseases in the population of agricultural areas.

In addition, the increased permanence of ACh at the receptors, caused by the impairing of AChE by ChE inhibitors, may act as a coadjuvant of tumor progression. Actually, in some tumour types, following activation of nicotinic and/or muscarinic receptors (Dodds et al., 2001; Minna, 2003); the MAP Kinase cascade is activated, driving cell proliferation (Ukegawa et al., 2003; Trombino et al., 2004). MAPK are important signal molecules, leading to cell growth and proliferation (Davis et al., 2009). At the same way, in the lung cancers following hyperactivation of nicotinic receptors, cell death regulation is compromised, thus causing the enhancement of cell proliferation. This can explain why tumour progression is enhanced by tobacco smoking (Cooke & Bitterman, 2004).

The researchers group lead by H. Soreq recently provided epidemiological and molecular evidence that the "readthrough" AChE, (AChE-R), a variant form of AChE induced by stress, and in particular by stress induced by pesticides, can cause inheritable diseases, including some cancers, in agriculturers' families (Soreq & Zakut, 1990; Shapira et al., 2000). During the last years this Researchers group provided evidence of the involvement of such a stressed form of AChE in anxiety (Ofek et al., 2007; Adamec et al., 2008), inflammation (Dori et al., 2007) and also in the modulation of beta-amyloids (Berson et al., 2008; Buznikov et al., 2008).

3.2.2 Neurological facts

Due to the fact that AChE is directly involved in the modulation of signals during the primary neural induction from the notochord to the neurogenic ectoderm (Aluigi et al., 2005) toxicological implication of AChE inhibitors on this process appears evident (Brimijoin and Koenigsberger, 1999). All the anticholinesterase drugs, by increasing the cholinergic tone of receptors, can cause neuropsychological defects (Colosio et al., 2009); organophosphates cause impairment of neural development (Aluigi et al., 2005), as well as of memory and psychomotor speed, and affective symptoms such as anxiety, irritability and depression (Frost, 2000), visual-spatial deficits, and from recent experiments OPs are suspected to be involved in new variant transmissible spongiform encephalopathy (Purdey, 1998).

Neurological development in children is particularly at risk of disruption. Animal studies demonstrate periods of vulnerability, particularly to anticholinesterase, during early life (Karczmar et al., 1970). Recent evidence that AChE may play a direct role in neuronal differentiation supports these findings (Biagioni et al., 2001).

3.2.3 Reproductive and developmental anomalies

Reproductive (Nelson, 1990) and developmental facts (Chanda and Pope, 1996; Aluigi et al. 2005, 2008, 2010a, 2010b) were also demonstrated to be caused by maternal, embryonic or differentiating cells exposure to neurotoxic pesticides.

Moreover, in animal experiments, AChE activity was shown to be involved in limb bud chondrogenesis (Falugi & Raineri, 1985), and the amount of AChE and ChE present in blood

was shown to be depleted for several months in persons exposed to organophosphate pesticides in USA (Romero et al., 1989). In one case, the consequence of the depletion of AChE activity (due to professional OP exposure) in the maternal blood, lasting throughout the pregnancy, caused the birth of a baby with only one eye, brain and heart anomalies, who died after some days (Romero et al., 1989).
During the last decades, neurotransmitter systems (Buznikov, 1990; Buznikov and Shmukler, 1996) and in particular the cholinergic systems (Drews, 1975; Minganti et al., 1981;Fluck et al., 1980 and Falugi, 1993) have been found responsible for cell interactions leading early development. In particular, molecules belonging to the cholinergic system, whose presence and amount is regulated by AChE and BChE activity, were found to exert a neurotrophic effect (Filogamo & Marchisio, 1971), and a strong input to neurogenesis and axon differentiation (Biagioni et al., 2001). In this light, a danger to early neural development of human fetus is strongly suspected as well as to the later establishment of neural function in children and in adults.
From recent studies OPs are suspected to be involved in adolescent behavioural disturbance (Bouchard et al., 2010) attention-deficit/hyperactivity disorder (ADHD) in children 8 to 15 years of age. Cross-sectional data from the National Health and Nutrition Examination Survey (2000-2004) were available for 1139 children, who were representative of the general US population. From a structured interview with parents, one hundred nineteen children met the diagnostic criteria for ADHD. These children also presented high levels of DMAP, a metabolite of thionophosphates, supporting the hypothesis of a relationship between exposure to OP drugs and ADHD.

3.2.4 Developmental anomalies

The developmental anomalies occurring after exposure to cholinergic drugs generally regard tissues and organs where in normal conditions AChE activity is mainly localized. At early stages, which Buznikov called "pre-nervous" and Drews called "embryonic", the effects are linked to the role of AChE and the molecules to it related, in cell-to-cell communication, generally due to intercellular messages, mediated by ionic fluxes and intracellular ionic changes. AChE activity has been found in vertebrate embryos (e.g. chick embryos, Aluigi et al., 2005) since the first stages, localized in the Hensen's node, and successively in the wall of the primitive streak, in the somites in the notochord, and in the floorplate of the neural tube, i.e. in temporal windows where cell-to cell communication awaking gene expression and consequent cell movements (Drews, 1975) take place. Thus, the presence of AChE activity is related to 3 classes of developmental events: I: during gamete maturation, activation and interaction (Angelini et al., 2004; Angelini et al., 2005); II: during the early development of invertebrate and vertebrate embryos. In this case cholinergic molecules are located mainly in moving cells and tissues engaged in relevant morphogenetic events, such as gastrulation and limb bud differentiation, and are often co distributed with special extracellular matrix molecules such as fibronectin (Aluigi et al., 2005) and laminin (Johnson et al., 2003); III: during inductive communications between mesenchyme and other tissues such as the limb bud development (Falugi and Raineri, 1985). The cholinergic system thus seems to be a multifunctional cell communication system. It appeared early during evolution as a regulator of intercellular communications mediated by ion dynamics (In *Paramecium primaurelia* it is related to the mating behaviour of single eukaryotic cells: Delmonte Corrado et al., 1999), before becoming involved in highly specialized communication structures, such as synapses and nerve endings.

Vertebrate models

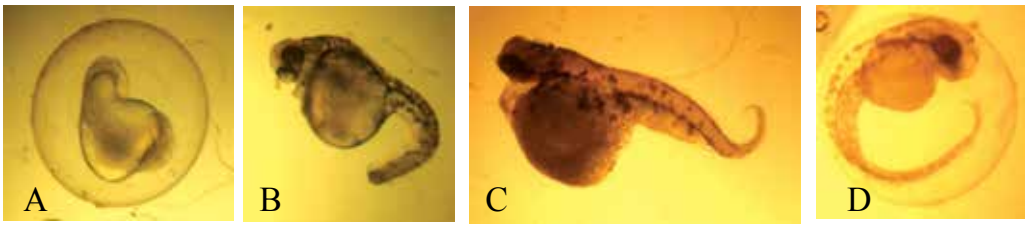

Fig. 4. Developmental anomalies in zebrafish embryos exposed at the mid-gastrula stage to different concentrations of fenthion: A: 10^{-5}M; B and C: 10^{-6}M; D: unexposed sample.

The curled trunk and tail are common features for a number of neurotoxic pesticides: the same aspects were found by interlaboratory calibration of the test by the team of Prof. Layer after exposure to chlorpyriphos and carbamates (in the frame of SENS-PESTI) (unpublished data).

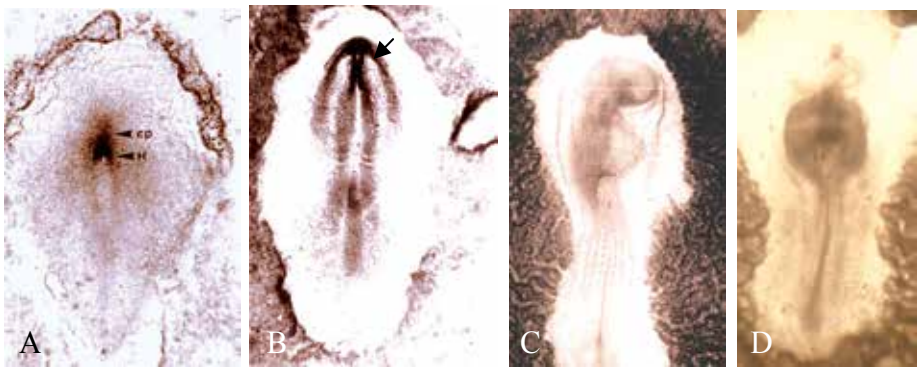

Fig. 5. A: 24 h incubated chick embryo. The Hensen's node (H) and the ridges of the primitive streak appear positive for the AChE reaction; B: 36h incubated chick embryo: the head neural fold (arrow), the first neural tube and the primitive streak are positive to the AChE reaction, revealed by dark reaction products. C: 48h control embryo; D: chick embryo, exposed to 10 µM DZN at 24 h incubation (corresponding to the A image) and sampled at 48h incubation, showing anomalies in the proximal part of the body (head did not develop nor differentiated and heart is double, because mesoderm movement was impaired, so that the two simple tubles forming heart failed to join anteriorly) This kind of anomalies was found to be caused by all the cholinergic pesticides, with high sensitivity as compared to the sensitivity of adults (see Aluigi et. Al., 2005).

4. Mechanisms of action

4.1 Apoptosis

4.1.1 Alternative models: cultured cells.

In terms of gene expression analysis, cDNA microarray studies showed that the most statistically significant pathways affected were related to cellular death and cell proliferation. (Catalano, 2007). Actually, we (Aluigi et al, 2010b) had evidence that the OP

compounds may affect differentiation and cell proliferation/death of NTERA2-D1 cells (NT2) The NT2 cell line, which was derived from a human teratocarcinoma, exhibits properties that are characteristics of a committed neuronal precursor at an early stage of differentiation. Its property to express a whole set of molecules related to the cholinergic neurotransmission system, including active acetylcholinesterase (AChE, EC 3.1.1.7) makes it a good alternative model for testing the effects of neurotoxic compounds, such as organophosphorus (OP) insecticides, whose primary target is the inhibition of AChE activity.

Non-neuromuscular AChE expression was also found in a number of cell lines upon induction of apoptosis by various stimuli (Zhang et al., 2002) The induction of AChE expression was determined by cytochemical staining, immunological analysis, affinity chromatography purification, and molecular cloning. The authors found the AChE protein in the cytoplasm at the initiation of apoptosis and then in the nucleus or apoptotic bodies upon commitment to cell death. Sequence analysis revealed that AChE expressed in apoptotic cells is identical to the synapse type AChE

Pharmacological inhibitors of AChE prevented apoptosis. Furthermore, blocking the expression of AChE with antisense inhibited apoptosis.

As the mechanisms of the relation between AChE and apoptosis are still rather obscure, we carried on bioassays, by blocking the AChE activity in cultured human cells, NTera2-D1.

NT2 cells exposed to the OP insecticide diazinon at concentrations ranging between 10^{-4} and 10^{-5}M showed a time-dependent enhancement of cell death. When exposed at 10^{-6}M diazinon showed higher cell viability than control samples up to 72 h, followed by a decreasing phase. The cell death caused by the exposures showed a number of features characteristic of apoptosis, including membrane and mitochondrial potential changes. We suggest the hypothesis that such behaviour is due to a dynamic balance between activated and blocked acetylcholine receptors that in turn trigger electrical events and caspase cascade. (Fig.6)

4.2 Calcium dynamics

4.2.1 Models for developmental effects: Invertebrates (sea urchin)

For this research, we mainly used, besides cultured cells, sea urchin early development as a model. Sea urchin is one of the few organismic models approved and validated by the European Agency for Alternative models. Actually, sea urchin embryonic development has been studied for over a century, and the complex nets of intercellular communications leading to the different events are well known, as well the possibility for environmental molecules and their residuals to interfere with such communications, causing developmental anomalies. In particular, the main goal of toxicologists since several years has been to establish a correlation between the cell-to-cell communications occurring during different developmental events and the signals occurring during neurogenesis, with the aim to pursue a mechanistic understanding of these processes and their deviations caused by stressors from different sources. By use of this model, at different developmental stages, we established that neurotoxic insecticides may affect calcium dynamics since fertilization events (Pesando et al., 2002). The biological effects of *Basudin* (an organophosphate compound containing 20% *Diazinon*), *Diazinon* (Dzn, a thionophosphate), *Carbaryl* and *Pirimicarb* (carbamates) on the early phases of sea urchin development were thus investigated. Morphological, biochemical, histochemical and immuno histochemical analyses were performed both during embryo and larval development.

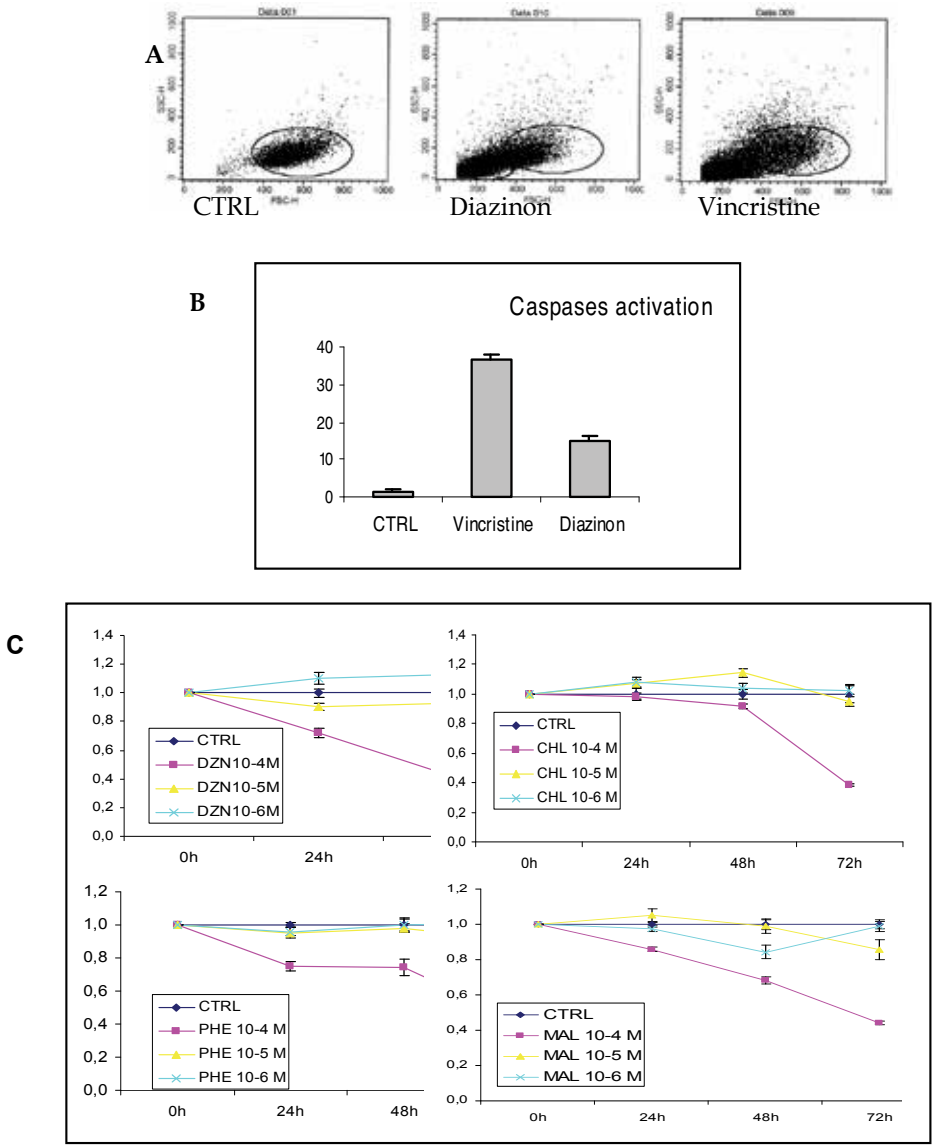

Fig. 6. (from Aluigi et al., 2010b). A: cytofluorimetry showing apoptosis of NT2 cells, controls and exposed to 10 µM DZN and vincristine, as a positive control. The amount of apoptotic cells was C<DZN<vincristine. (B) The same trend was seen in caspase expression. C shows the percentage of survival (Y axis) along time (X axis, each unit corresponds to 24 h), at concentrations ranging between 100 and 1 µM. (cell viability was measured by use of the MTT method)

4.2.2 Invertebrate models: the sea urchin, Paracentrotus lividus

For the morphological effects on fertilisation and first cleavages, the effective concentration of insecticides was found to be 10^{-4}M, while for further stages concentrations between 10^{-5} and 10^{-7}M were effective. 10^{-3}M of any of these insecticides totally arrested development.

This results depend on the fact that no cholinergic molecules are involved in fertilisation, as we demonstrated successively (Harrison et al., 2002). Thus, the high dose (that is about IC50, according to the previously shown data of Rakonczay) may cause a general toxicity effect, not related to cholinergic molecules. On the other hand, Casida and Quistad (2004) reported a number of non-cholinergic secondary targets, and this could explain the general toxicity. In contrast, effects revealed at the molecular level, such as lectin binding and AChE activity seem much more sensitive, and may reveal anomalies at the chronic exposure concentrations (10^{-7}M). At these low concentration, an effect was seen at later stages, during the larval growth, on cell proliferation and larval plasticity (Aluigi et al., 2010a), as larvae exposed to CPF and PTH low levels along the whole development showed longer perioral arms and fastened metamorphosis. Concentrations as high as 10^{-5} and 10^{-6}M blocked larval development and, when used to expose larvae next to metamorphosis, caused immature forms of juveniles, lacking skeletal structures. The effects of AChE inhibition on the skeleton formation were also seen in the early larvae (Ohta et al., 2009). As other Authors (Hoogduijn et al., 2006) found an effect on human osteogenic stem cells, we can speculate some involvement in human osteoporosis for direct toxicity on AChE that has also been reported to be present in pre-cartilage nodules of chick embryos (Falugi and Raineri, 1985). In the case of sea urchin, arm elongation, sustained by calcium carbonate skeletal rods, may be due to different causes: the first may be due to slowering of the ciliary movement, and consequent starvation of the larvae. According to Fenaux et al. (1988), perioral arms elongate for increasing the ciliated area that brings food to the mouth. The second explanation is that enhancement of arm growth could be due to a direct effect on muscarinic receptors, which are distributed along the arms at the basis of the cilia.
Exposure to diazinon in a particular developmental window (10 min after fertilization) also caused the formation of exogastrulae (Fig.7).

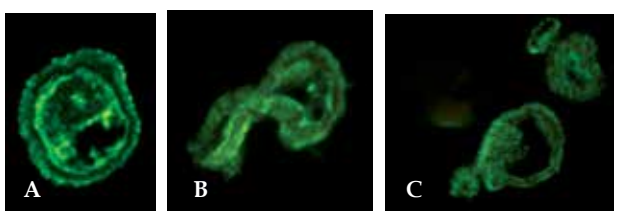

Fig. 7. A: control gastrula; B, C, different aspects of exogastrulae exposed to 10^{-5}M diazinon The green immunofluorescence shows the localization of muscarinic receptors (primary antibody obtained from Chemunex, Fr) Unpublished images.

The final target of OP poisoning, as we have seen above for other models, is the regulation/disregulation of particular genes: Also this was studied by using sea urchin as a model. In this model, we recorded the effects of OP exposure (in particular diazinon) on the localization of a regulatory protein that is immunologically related to the human OTX2. The severe anomalies and developmental delay observed after treatment at 10^{-5} M concentration are indicators of systemic toxicity, while the results after exposure to the inhibitor at 10^{-6} M concentration suggest a specific action of the neurotoxic compound. In this case, exposure to diazinon caused partial delivery of the protein into the nuclei, a defective translocation that particularly affected the blastula and gastrula stages. Therefore, the possibility that neurotoxic agents such as organophosphates may disregulate expression of outstanding proteins is taken into account.

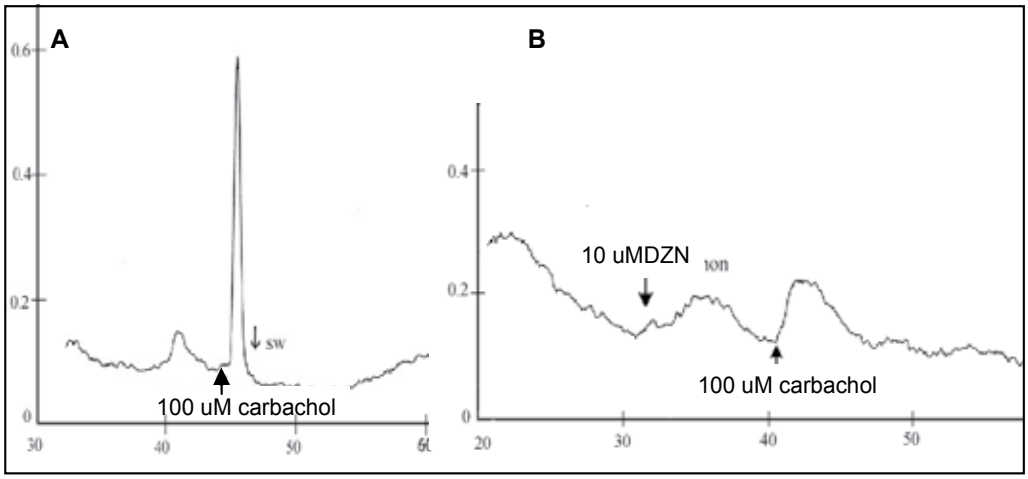

Fig. 8. A: *Litechinus pictus* zygotes were exposed to 100 µM carbamylcholine (carbachol), a cholinomimetic agonist of the muscarinic receptors. Exposure to carbachol is followed by a spike of fura2-dextran fluorescence. B: exposure to carbachol is preceded by exposure to 100 µM diazinon, no spike follows the exposure to 100 µM carbachol . Y axis represents fluorescence (scale units = 0,2) units; X axis represents time (scale units 30 sec). These experiments were performed by Dr Harrison P. in the laboratory of Prof. Whitaker, MJ, and published in the paper Harrison et al., 2002.

Actually, from the zygote stage, stimulation of ACh receptors may evoke calcium spikes, anticipating those related to the nuclear breakdown (Harrison et al., 2002). In this event, muscarinic drugs were proved to have a prominent role.). As a consequence of intracellular [Ca^{2+}] alteration, all the calcium related intracellular dynamics are altered, including delivery of transcription factors to the nuclei.

By use of sea urchin early developmental stages, and DZN exposure at different concentrations, evidence was provided that cytoplasmic dynamics were perturbed and in particular the delivery of the OTX2 protein, which in mammalians plays a role in forebrain development. We (Aluigi et al., 2008) submitted the hypothesis that this effect could be due to altered calcium dynamics, which in turn alter cytoskeleton dynamics: the asters, in fact, appear strongly positive to the OTX2 immunoreaction. (Aluigi et al., 2008). In this work, sea urchin early developmental stages were used as a model to test the effects of the organophosphate pesticide (diazinon) on the regulation of gene expression by immunohistochemical localization of the regulatory protein against the human OTX2. Egg exposure to diazinon did not affect fertilization; however, at concentrations 10^{-5}–10^{-6} M, it did cause developmental anomalies, among which was the dose-dependent alteration of the cytoskeleta. Coimmunoprecipitation experiments showed the link between cytoskeletal tubulins and the OTX2 protein, thus justifying the partial delivery to nuclei.

In addition, Pesando et al. (2003) showed that, during embryonic development, the treatment with organophosphates slowed the rate of early mitotic cycles down, affected nuclear and cytoskeletal status as well as DNA synthesis. From the gastrulation stage onwards, the main effects were exerted on the rate of primary mesenchyme cells migration, larval size, perioral arm length, and acetylcholinesterase activity distribution, thus deregulating the cholinergic system, which modulates cell-to-cell communication mediated by the signal molecule acetylcholine.

4.3 Biosensors

We found that the effects of cholinergic insecticides exposure were more drastic in developing organisms than in adult tissues. Although the experiments on developing embryos were used at concentrations of the drugs including those indicated in the labels as under threshold (NOEL), effects were found on developmental anomalies. This is because development is a multi-phase event, where each stage depends on the previous ones, thus amplifying also the small defects that in adults are easily corrected or healed.

In order to protect not only human health, but also environment and next generations, at present, a great deal of effort is concentrated on creating "biosensors", capable of perceiving neurotoxic compounds in the environment, as well as in food and water. Most of the biosensors are represented by devices that have the capacity to measure, with high sensitivity, the activity of acetylcholinesterase in the presence of suspected inhibitors and in particular OP or carbamate compounds.

These high-technology instruments can measure the presence and amount of neurotoxic compounds in environmental matrices, or in rough material and elaborated foods. The biosensors used for this purpose are generally based on highly sensitive molecular forms of AChE, immobilised in devices capable of recording changes in activity in real time, and by transferring them to screens or other recording devices (Crew et al. 2004), or by use of mutated bacteria or yeast (Wu et al. 2002).

All those biosensors are very good tools to evaluate the degree of exposure of people, by analysing blood, urine, or else. Along development of the SENS-PESTI project, in the Laboratory of by Hagen Thielecke (the Fraunhofer Institute for Biomedical Engineering (IBMT), based on micro technology), a new kind of biosensor is was studied , which is able to evaluate the effects of exposures on living organisms, and their health risks, by evaluating living cells and tissues responses to exposure. In this case, it is possible to evaluate not only the effects on the primary target AChE, but also any response evoked by secondary targets of pesticides (Abdallah et al. 1992; Sultatos 1994). Such a biosensor has the capacity to translate the effect of neurotoxic pesticides in living cells into electrical signals by using microtechnological devices for measuring e.g. the alteration of ion fluxes or their intracellular concentration. The advantages of this biosensor are represented by the fact that complex cell response is taken into account, and that the AChE molecules and the ACh receptors are in their natural environment, and follow their natural transduction cascades up to the cell response.

The employment of such devices is at present innovative, as well as complying with the International bioethical concerns. Actually, it may solve some controversial points, such as:

1. The problem of experiments on animals, which are more expensive, besides causing pain, which is particularly evident in higher organisms.
2. The improved knowledge of developmental biology, within the emerging knowledge that neurotransmitter molecules are not limited to neuromuscular structures, but are generally involved in cell-to-cell communication, leading to interaction between developing cells and tissues.
3. Exporting the results between different organisms, including man, by comparing the effects of exposures on animal tissues (zebrafish, sea urchin, xenopus early embryos, that are considered bioethical by ECVAM, ICCVAM and other International Institutions (dealing with toxicity test validation) with the effects on human cultured cells and adult stem cells.

4. Allowing us to establish conversion parameters among the different cell sources, in order to use the most suitable and available for each situation of risk assessment.

5. Present problems and possible solutions

The main problem for the use of pesticides is the confusion at present existing in this field. Very huge numbers of researches are carried out all over the world, but the results are often contradictory, and scarce information is given to the final users, i.e. the agriculturers and consumers. The number of accidents occurring every year is high, although a legislation exists about the use of safety items and safety provisions. Moreover, the Thematic Strategy on the sustainable use of pesticides adopted in 2006 by the European Commission aims at filling the current legislative gap regarding the use-phase of pesticides at EU level through setting minimum rules for the use of pesticides in the Community, so as to reduce risks to human health and the environment from the use of pesticides. For the moment, the Commission has proposed to restrict the scope of the Framework Directive to plant protection products. Directive 2009/127/EC amending Directive 2006/42/EC with regard to machinery for pesticide application: Machinery used for applying pesticides in European farms, orchards, vineyards, parks and gardens will be more environmentally friendly, thanks to an amendment to the Machinery Directive published in November 2009.

Everyone who uses pesticides, has the responsibility to ensure their correct and effective use. To help them, the EC provides guidance on best practice in the use of pesticides in a number of ways. Nevertheless, up to date, the label is the main source of information on the safe and effective use of a product. The product label must always be supplied with the container. Additional information may also sometimes be supplied as a separate leaflet within the boxes containing the products.

Actually, besides intentional self-poisoning or terrorist attacks, the more usual way to be intoxicated by neurotoxic pesticides is the practice of agriculturers, mainly in the moments when they dissolve high amounts of powders or use sprays without safety aids.

Epidemiological studies suggest that chronic exposure may increase susceptibility to neurodegeneration diseases (Betarbet et al., 2000), not only for agriculturers but also for housekeepers who take care of their clothes and safety aids. Thus all the family of agriculturers is involved in learning how to prevent exposure. Bystanders and consumers are also a target of chronic toxicity (Keifer & Mahurin, 1997), and no information is provided on the markets about the date of last application of plant protection products on vegetables. So, a new trend is emerging in consumers about the use of organic food. For pregnant women and children, the benefits are worth the higher price (Jurosek et al., 1999)

For this reason it is needed a careful information and use of safety aids in the correct way. When used responsibly, pesticide products provide many benefits such as promoting affordable and abundant food supplies. To ensure the safety of the environment and human health, pesticides are also heavily regulated. The Environmental Protection Agency (EPA) is the government body responsible for regulating pesticides and assessing risks associated with these chemicals. This includes evaluating whether pesticides pose an unreasonable risk to humans and the environment and requiring pesticide registrations when applicable

It is essential that all the information is read carefully and understood before a pesticide is used because it informs the user of the safe and proper use of the product. To this aim, it is requested a great effort in the future for training of agriculturers, to provide them with a clear picture of risks and ways to avoid them.

6. References

Aardema, H.; Meertens, J.H.J.M.; Ligtenberg, J.J.M.; Peters-Polman, O.M.; Tulleken, J.E. & Zijlstra, J.G. (2008). Organophosphorus pesticide poisoning: cases and developments- *The Nederlands Journal of Medicine* (apr 2008), Vol.66 N°4 (jan 2008) 149-153 ISSN: 0300-2977

Abdallah EAM.; Jett, DA.; Eldefrawi, ME. & Eldefrawi, A.T. (1992). Differential effects of paraoxon on the M3 muscarinic receptor and its effector system in rat submaxillary gland cells. *Journal of Biochemical Toxicology* Vol.7 (1992 summer) 125–132 pISSN: 0887-2082

Adamec, R.; Head, D.; Soreq, H. & Blundell, J. (2008). The role of the read through variant of acetylcholinesterase in anxiogenic effects of predator stress in mice. *Behavioural brain research.*Vol.189, N°1 (may 2008) 180-190, ISSN 1872-7549

Akogbéto, MC.; Padonou GG.; Gbénou D.; Irish S. & Yadouleton A. (2010). Bendiocarb, a potential alternative against pyrethroid resistant *Anopheles gambiae* in Benin, West Africa. *Malaria journal* Vol.14, N°9 (jul 2010)204, ISSN: 1475-2875

Aluigi, M.G.; Angelini, C.; Corte, G. & Falugi, C. (2008). The sea urchin, *Paracentrotus lividus,* embryo as a "bioethical" model for neurodevelopmental toxicity testing. Effects of diazinon on the intracellular distribution of OTX2-like proteins. *Cell biology and Toxicology* Vol.24, N° 6 (dec 2008) 587-601. pISSN: 0742-2091; eISSN:1573-6822

Aluigi, MG.; Angelici, C.; Falugi, C.; Fossa, R.; Genever, P.; Gallus, L.; Layer, PG.; Prestipino, G.; Rakonczay, Z.; Sgro, M.; Thielecke, H. & Trombino, S. (2005). Interaction between organophosphate compounds and cholinergic functions during development. *Chemico-biological interactions* Vol.15, N°157-158 (dec 2005) 305-316, pISSN: 0009-2797 eISSN: 872-7786

Aluigi, MG.; Falugi, C.; Mugno MG.; Privitera D.; Chiantore M. (2010a). Dose-dependent effects of chlorpyriphos, an organophosphate pesticide, on metamorphosis of the sea urchin, *Paracentrotus lividus.* Ecotoxicology Vol.19, N°3 (Mar 2010) 520-529, pISSN: 1573-3017

Aluigi, MG.; Guida, C. & Falugi, C. (2010b). Apoptosis as a specific biomarker of diazinon toxicity in NTera2-D1 cells. *Chemico-biological interactions* 187(1-3) (sept 2010) 299-303, pISSN: 0009-2797; eISSN:872-7786

Angelici, C.; Aluigi, MG.; Sgro, M.; Trombino, S.; Thielecke, H. & Falugi C. (2005). Cell signalling during sea urchin development: a model for assessing toxicity of environmental contaminants. *Progress in molecular and subcellular biology* Vol.39 (jul 2005) 45-70, ISSN 0079-6484

Angelini C.; Baccetti B.; Piomboni P.; Trombino S.; Aluigi MG.; Stringara S.; Gallus L.; Falugi C. (2004) Acetylcholine synthesis and possible functions during sea urchin development. European Journal of Histochemistry 48(3) (jul-sept 2004) 235-243 pISSN: 1121-760X

Berson, A.; Knobloch, M.; Hanan, M.; Diamant, S.; Sharoni, M.; Schuppli, D.; Geyer, BC.; Ravid, R.; Mor, TS.; Nitsch, RM & Soreq, H. (2008) Changes in readthrough acetylcholinesterase expression modulate amyloid-beta pathology. Brain Vol.131, Pt 1 (Jan 2008) 109-119, pISSN: 0006-8950, eISSN:1460-2156 .

Betarbet, R.; Sherer, TlB.; MacKenzie, G.; Garcia-Osuna, M.; Panov, AlV. & Greenamyre, JlT. (2000). Chronic systemic pesticide exposure reproduces features of Parkinson's

disease. *Nature Neuroscience* Vol.3, N°12 (Dec. 2000), 1301-1306, pISSN: 1097-6256 eISSN: 1546-1726

Biagioni, S.; Tata, AM.; DeJaco, A. & Augusti-Tocco, G. (2001). ACh synthesis and neuron differentiation. *The International journal of developmental biology* Vol.44 (Feb. 2001) 689-697, pISSN: 0214-6282; eISSN: 1696-3547

Bouchard, MF.; Bellinger, DC.; Wright, RO. & Weisskopf, MG. (2010). Attention-deficit/hyperactivity disorder and urinary metabolites of organophosphate pesticides. *Pediatrics.* 125(6) (Jun. 2010) e1270-1277 ISSN: pISSN: 0031-4005; eISSN: 1098-4275

Brimijoin, S. & Koenigsberger, C. (1999). Cholinesterases in neural development: new findings and toxicologic implications. *Environmental health perspectives*. 107(suppl 1) (Mar. 1999) 59-64, pISSN: 0091-6765; eISSN: 1552-9924

Buckley, N.A.; Roberts, D. & Eddleston, M. (2004) Overcoming apathy in research on organophosphate poisoning *BMJ* 329 (Jan. 2004) 1231-1233 pISSN: 0959-8138; eISSN: 1468-5833

Buznikov GA, Nikitina LA, Bezuglov VV, Milosević I, Lazarević L, Rogac L, Ruzdijić S, Slotkin TA, Rakić LM.(2008) Sea urchin embryonic development provides a model for evaluating therapies against beta-amyloid toxicity.Brain Res Bull. 2008 Vol.75,N°1(Jan 2008) 94-100, pISSN: 0361-9230; eISSN: 1873-2747

Buznikov, G.A. (1990). Neurotransmitters in embryogenesis. Vol. 1. (Series Ed: Turpaev, TM. *Physiology and General Biology, Section F of Soviet Scientific Reviews*.) Harwood Acad. Publ., London, Paris, New York, Victoria

Buznikov, G.A.; Shmukler, Y.B. & Lauder, J.M. (1996). From oocyte to neuron: do neurotransmitters function in the same way throughout development? *Cellular and molecular neurobiology.* 16(5) 533-559, pISSN 0272-4340; 1573-6830.

Cabello, G.; Valenzuela, M.; Vilaxa, A.; Duran, V.; Rudolph, I.; Hrepic, N. & Calaf, G. (2001). A rat mammary tumor model induced by the organophosphorus pesticides parathion and malathion, possibly through acetylcholinesterase inhibition. *Environmental health perspectives* Vol.109, N°5 (may 2001) 471-479, pISSN: 0091-6765; eISSN: 1552-9924

Casida, J.E. & Quistad, J.B. (2004). Organophosphate Toxicology: Safety Aspects of Nonacetylcholinesterase Secondary Targets. In: *Chemical Research in Toxicology* Vol.17, N°8 (aug 2004) 983-998. pISSN: 0893-228X; eISSN: 1520-5010

Catalano, J. Mechanisms of neurotoxicity of organophosphates, carbamates, and alkylating agents. *PhD dissertation*, University of Maryland, Baltimore, 2007, 294 pages; AAT 3258397

Chanda, S.M. & Pope, C.N. (1996). Neurochemical and neurobehavioral effects of repeated gestational exposure to chlorpyriphos in maternal and developing rats. *Pharmacology, biochemistry, and behavior* Vol.53 (aug 1995) 771-776, pISSN: 0091-3057; eISSN: 1873-5177.

Clavel, J.; Hermon, D.; Mandereau, L.; et al. (1996). Farming, Pesticide use and hairy cell Leukemia. *Scandinavian joural of Work, Environment and Health* Vol.22, N°4 (nov 1996), 285-293, pISSN: 0355-3140; eISSN: 1795-990X

Colosso, C.; Tiramani, M.; Brambilla, G.; Colombi, A. & Moretto A. (2009). Neurobehavioural effects of pesticides with special focus on organophosphorus

compounds: which is the real size of the problem? Neurotoxicology Vol.30, N°6 (nov 2009) 1155-1161, pISSN: 0161-813X;eISSN: 1872-9711

Cooke, J.P. & Bitterman, H. (2004) Nicotine and angiogenesis: a new paradigm for tobacco related diseases. *Annals of Medicine*, Vol.36, N°1 (2004) 33-40. pISSN: 0785; eISSN: 1365-2060

Crew A.;Wedge R.;Hart JP.;Marty JL. & Fournier D. (2004) A samperometric biosensor array to measure organophosphate concentration in raw products. *Proceedings of 8th World Congr on Biosensors,* 24–26 May, Granada, Spain

Dauberschmidt, C.; Dietrich, D.R. & Schattler, C. (1996). Toxicity of Organophosphorous Insecticides in the Zebra Mussel, *Dreissena polymorpha* P. *Archives of environmental contamination and toxicology* Vol.30 (oct 1996) 373-378, pISSN: 0090-4341: eISSN:1432-0703

Davis, KL.; Yesavage, JA. & Berger, P.A. (1978). Possible organophosphate-induced Parkinsonism. The Journal of nervous and mental disease Vol.166 (march 1978) 222-225, pISSN:0022-3018; eISSN:1539-736X

Davis, R.; Rizwani, W.; Banerjee, S.; Kovacs, M.; Haura, E.; Coppola, D. & Chellappan, S. (2009). Nicotine promotes tumor growth and metastasis in mouse models of lung cancer. PLoS One. Vol 4, N°10 (oct 2009) e7524: eISSN:1932-6203

Delmonte Corrado, MU.; Politi, H.; Trielli, F.; Angelici, C. & Falugi, C. (1999). Evidence for the presence of a mammalian-like cholinesterase in Paramecium primaurelia (Protista.; Ciliophora) developmental cycle. *Journal of Experimental Zoology*, vol.283, N°1 (feb 1999) 102-105, ISSN: 0022-104X

Dodds, HM.; Hanrhan, J. & Rivory, L.R. (2001), The inhibition of acetylcholinesterase by irinotecan and related camptothecins: key structural properties and experimental variables, Anti-cancer drug design, Vol.16, N°4-5, (aug-oct 2001) 239-246, pISSN: 0266-9536

Dori, A.; Ifergane, G.; Saar-Levy,T.; Bersudsky, M.; Mor, I.; Soreq, H. & Wirguin, I. (2007) Readthrough acetylcholinesterase in inflammation-associated neuropathies *Life Sciences* 80(24-25) (may 2007) 2369-2374: pISSN: 0090-5542

Drews, U. (1975). Cholinesterase in embryonic development. Progress in histochemistry and cytochemistry vol.7, 1-52, pISSN:0079-6336; eISSN:1873-2186.

Falugi, C. & Raineri, M. (1985). Acetylcholinesterase (AChE) and pseudocholinesterase (BuChE) activity distribution pattern in early developing chick limbs. *Journal of Embryology & Experimental Morphology*. 86, 89-108, ISSN: 0022-0752.

Falugi, C. (1993). Localization and possible role of molecules associated with the cholinergic system during "non nervous" developmental events. European Journal of Histochemistry Vol. 37, N°4 (1993), 287-294. ISSN: 1121-760X

Fenaux L.; Cellario C. & Rassoulzadegan F. (1988) Sensitivity ofdifferent morphological stages of the larva of Paracentrotuslividus (Lamarck) to quantity and quality of food. In: *Echinoderm biology*. Burke D, Mladenov PV, Lambert P, Parsley RL (eds) 259–266 A.A. Balkema, ISBN:906191 7557 Rotterdam

Filogamo, G. & Marchisio, P.C. (1971). Acetylcholine system and neural development. *Neuroscience research* Vol.4, 29-64 pISSN: 0168-0102; eISSN: 1872-8111

Fischel, F.M. (2008). Pesticide Toxicity Profile: Carbamate Pesticides. *University of Florida, IFAS- Pesticide information Office*, Publication # PI-51

Fluck, RA.; Winshaw-Boris, AJ. & Schneider, L.M. (1980). Cholinergic molecules modify the in vitro behavior of cells from early embryos of the medaka Oryzias latipes, a teleost fish. *Comparative biochemistry and physiology*. Vol.67, series C (1980) 29-34, pISSN: 0010-406X.

Frost, S.D. (2000). Gulf War syndrome: proposed causes. *Cleveland Clinic journal of medicine*. Vol. 67, N°1 (jan 2000) 17-20, pISSN: 0891-1150; eISSN: 1939-2869.

Gorell, JM, Johnson, CC, Rybicki, BA et al (1998). The risk of Parkinson's disease with exposure to pesticides, farming, wellwater, and rural living. *Neurology* Vol.50 (apr 1998) 1346-1350, pISSN: 0028-3878; eISSN: 1526-632X.

Guo, J.X.; Wu, J.J-Q.; Wright, J.B. & Lushington, J.H. (2006). Mechanistic Insight into Acetylcholinesterase Inhibition and Acute Toxicity of Organophosphorus Compounds: A Molecular Modeling Study. *Chemical research in toxicology* Vol.19, N°2 (feb 2006) 209–216, pISSN:0893-228X; eISSN:1520-5010 .

Harrison, P. K.; Falugi, C.; Angelini, C. & Whitaker, M. J. (2002) Muscarinic signalling affects intracellular calcium concentration during the first cell cycle of sea urchin embryos *Cell Calcium* Vol.31, N°6 (jun 2002) 289–297: pISSN:0143-4160; eISSN:1532-1991

Hayes, W.J.; Jr, & Laws, E.R.; Jr (1991). Organic Phosphorous Pesticides: In *Handbook of Pesticide Toxicology.* Vol. 3. Acad. Press,1-1189 San Diego, New York, Boston, London, Sydney, Tokyo, Toronto. ISBN-10: 0123341604

Hoogduijn, MJ.; Cheng A. & Genever PG. (2009). Functional nicotinic and muscarinic receptors on mesenchymal stem cells. *Stem cells and development* Vol.18, N°1 (jan-feb 2009) 103-112, pISSN:1547-3287; eISSN:1557-8534

Jamal, G.A. (1997). Neurological symptoms of organophosphorous compounds. Adverse Drug React. *Toxicological reviews*. Vol.16 (aug 1997) 133-170, pISSN: 1176-2551

Johnson, G. & Moore, S.W. (2003). Human acetylcholine esterase binds to mouse laminin-1 and human collagen IV by an electrostatic mechanism at the peripheral anionic site. *Neuroscience Letters,* Vol.337, No 1, (jan 2003) 37-44, pISSN: 0304-3940; eISSN: 1872-7972.

Juroszek, P.; Lumpkin, H.M.; Yang, R.Y.; Ledesma, D.R. & Ma, C.H. (2009). Fruit quality and bioactive compounds with antioxidant activity of tomatoes grown on-farm: comparison of organic and conventional management systems.J *Journal of agricultural and food chemistry*. Vol.57, N°4 (feb 2009) 1188-1194, pISSN:0021-8561; eISSN:1520-5118

Karczmar, AG.; Usdin, E.; & Willis, J.H. (1970). Anticholinesterase agents. In:. (*International Encyclopedia of Pharmacology and Therapeutics,* Vol.1,Chapt 13, pp.1-508 Pergamon Press, Oxford, New York, Toronto, Sydney, Brownschweig.

Keifer, MC. & Mahurin, R.K. (1997). Chronic neurologic effects of pesticide overexposure. *Journal of occupational medicine*. Vol.12 (apr-jun 1997) 291-304, pISSN: 0096-1736

Khurana, D. & Prabhakar, S. (2000) Organophosphorus Intoxication. *Archives of neurology*. 2000; Vol.57 (apr 2000) 600-602, pISSN: 0003-9942; eISSN: 1538-3687.

Mellanby, K. (1992) The DDT Story, British Crop Protection Council (BCPC), 1992

Minganti, A.; Falugi, C.; Raineri, M. & Pestarino, M. (1981). Acetylcholinesterase in the embryonic development: an invitation to a hypothesis. *Acta embryologiae et morphologiae experimentalis.* n.s. Vol.2, (sept 1981) 30-31. pISN: 0567-7416

Minna, J.D. (2003) Nicotine exposure and bronchial epithelial cell nicotinic acetylcholine receptor expression in the pathogenesis of lung cancer, *Journal of Clinical Investigation,* Vol.111, N°1, (jan 2003) 81-90, pISSN:0021-9738; eISSN:1558-8238.

Minneau, P. (1991). Cholinesterase inhibiting insecticides. Their impact on Wildlife and environment. In: *Chemicals in Agriculture,* Elsevier, Vol.2, 1-348 Amsterdam, London, New York, Tokyo

Morale, A.; Coniglio, L.; Angelini, C.; Cimoli, G.; Bolla, A.; Alleteo, D.; Russo, P. & Falugi, C. (1998). Biological effects of a neurotoxic pesticide at low concentration early development. A Teratogenic assay. *Chemosphere* Vol.37 (dec 1998) 3001-3010, pISSN:0045-6535; eISSN:1879-1298.

Nelson, L. (1990). Pesticide perturbation of sperm cell function. *Bulletin of environmental contamination and toxicology* Vol. 45 (dec 1990) 876-882, pISSN: 0007-4861; eISSN: 1432-0800.

Ofek, K.; Krabbe, K.S.; Evron, T.; Debecco, M.; Nielsen, A.R.; Brunnsgaad, H.; Yirmiya, R.; Soreq H. & Pedersen, B.K. (2007) Cholinergic status modulations in human volunteers under acute inflammation. *Journal of molecular medicine* Vol 85.; N°11 (nov 2007) 1239-1251, pISSN: 0377-046X

Ohta,K., Takahashi,C. & Tosuji,H. (2009) Inhibition of spicule elongation in sea urchin embryos by the acetylcholinesterase inhibitor serine. *Comparative Biochemistry and Physiology*, *Part B* Vol 153 (Aug 15); 310–331.pISSN: 0300-9629

O'Malley, M. (1997) Clinical evaluation of pesticide exposure and poisonings. Lancet Vol.349, N°9059 (apr 1997) 1161-1166, pISSN:0140-6736; eISSN: 1474-547X

Percivale, G (2003) Caratterizzazione funzionale in membrane modello del recettore della rianodina nelle fasi precoci dello sviluppo embrionale del riccio di mare *Paracentrotus lividus lividus*. Thesis (Prestipino G, Tutor), University of Genova, Italy, 148 pp

Pesando, D.; Huitorel, P.; Dolcini, V.; Angelini, C.; Guidetti, P. & Falugi, C. (2003). Biological targets of neurotoxic pesticides analysed by alteration of developmental events in the Mediterranean sea urchin *Paracentrotus lividus*. *Marine environmental research*. 2003 Feb; Vol.55, N°1 (feb 2003).39-57, pISSN:0141-1136; eISSN:1879-0291.

Purdey, M. (1998). High-dose exposure to systemic phosmet insecticide modifies the phosphatidylinositol anchor on the prion protein: the origins of new variant transmissible spongiform encephalopathies? *Medical hypotheses* Vol.50, N°2(feb 1998) 91-111, pISSN:0306-9877; eISSN:1532-2777.

Ragnarsdottir, K.V. (2000). Environmental fate and toxicology of organophosphate pesticides. *Journal-of-the-Geological-Society* Vol.157, N°4 (jul 2000) 859-876, pISSN: 0016-7649

Rakonczay, Z. & Papp, H. (2001). Effects of chronic metrifonate treatment on cholinergic enzymes and the blood-brain barrier. *Neurochemistry international* Vol.39, N°1 (jul 2001) 19-24, pISSN:0197-0186; eISSN:1872-9754

Reeves, M.; Schafer, K.; Hallward, K. & Katten, A. (1999). Fields of poison: *California Farmworkers and Pesticides.* (San Francisco: Californians for Pesticide Reform/Pesticide Action network-North America/United Farm Workers of America/California Rural Legal Assistance Foundation, 1999).

Romero, P.; Barnett, P.G. & Midtling, J.E. (1989). Congenital anomalies associated with maternal exposure to oxydemeton-methyl. *Environmental research.* Vol.50, N°2 (dec 1989) 256-261, pISSN:0013-9351; eISSN:1096-0953

Shapira, M.; Grant, A.; Korner, M. & Soreq, H. (2000). Genomic and transcriptional characterization of the human AChE locus: Complex involvement with acquired and inherited diseases. *The Israel Medical Association journal* Vol.2, N°6 (jun 2000), 470-473, pISSN:1565-1088.

Sherman, J.D. (1996). Chlorpyrifos (Dursban)-associated birth defects: report of four cases. *Archives of environmental health* Vol.51 (jan-feb 1996) 5-8, pISSN: 0003-9896.

Soreq, H. & Zakut, H. (1990). Amplification of butyrylcholinesterase and acetylcholinesterase genes in normal and tumor tissues: putative relationship to organophosphorus poisoning. *Pharmaceutical Research* 7(1) (mar 1990) 1–7, pISSN: 0724-8741; 1573-904X eISSN

Sultatos, L.G. (1994). Mammalian toxicology of organophosphorous pesticides. *Journal of toxicology and environmental health. Health* Vol.43, N°3 (nov 1994) 271-289, pISSN: 0098-4108.

Trombino, S.; Cesario, A.; Margaritora, S.; Granone, PL.; Motta, G.; Falugi, C. & Russo, P. (2004). α7-Nicotinic acetylcholine receptors affect growth regulation of human mesothelioma cells: role of mitogen]activated protein kinase pathway, *Cancer Research*, Vol.64 (jan 2004) 135-145, pISSN: 0008-5472; eISSN: 1538-7445

Ukegawa, JI.; Takeuchi, Y.; Kusayanagi, S. & Mitamura, K. (2003). Growth-promoting effect of muscarinic acetylcholine receptors in colon cancer cells, *Journal of Cancer Research and Clinical Oncology* Vol.129, N°5 (may 2003) 272-278, pISSN:0171-5216; eISSN:1432-1335

Underner, M.; Cazenave, F. & Patte, F (1987). Occupational asthma in the rural environment. *Revue de pneumologie clinique* Vol.43 (mar. 1987) 26-35, pISSN: 0761-8417

Wu, CF.; Valde,s JJ.; Rao, G.; Bentley, W.E. (2002) Enhancement of organophosphorus hydrolase yield in *Escherichia coli* using multiple gene fusions. *Biotechnology and bioengineering* Vol.75,N°1(oct 2001) 100–103, pISSN: 0006-3592;eISSN:1097-0290

Zhang, XJ.; Yang, L.; Zhao, Q.; Caen, JP.; He, HY.; Jin, QH.; Guo, LH.; Alemany, M.; Zhang, L.Y. & Shi, Y.F. (2002) Induction of acetylcholinesterase expression during apoptosis in various cell types. *Cell death and differentiation*. 9(8) (aug. 2002) 790-800, pISSN:1350-9047;eISSN:1476-5403

12

Organophosphorus Pesticides - *Mechanisms Of Their Toxicity*

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1. Introduction

In this rapidly developing, capitalist world, people are continually exposed to numerous **environmental pollutants** such as industrial waste, polluted air and pesticides. These invariably comprise complex mixtures of chemicals. The effects of the mixtures and their mode of action in humans are insufficiently well studied. The majority of pollutants are potentially toxic for organisms, some being connected to disease development. In this context, the increase of chronic degenerative disease including cancer in humans, is of considerable concern (Gupta, 2006).

Pesticides are a very important group of environmental pollutants used in intensive agriculture for protection against diseases and pests. The estimated annual application is more than 4 million tons, but only 1% of this reaches the target pests (Gavrilescu, 2005). Functionwise they are divided into herbicides (protection against weeds), insecticides (against insects), fungicides (against fungi), and others. While their use improves the quantity of agricultural products it potentially affects their quality, as pesticides may enter human diet (Grlić, 1988). This is a matter of major current concern.

Organophosphorus compounds or organophosphates (OPs) form a large group of chemicals used over the past 60 years for protecting crops, livestock, human health and as warfare agents. On the basis of structural characteristics they are divided into at least 13 types, including phosphates, phosphonates, phosphinates, phosphorothioates (S=), phosphonothioates (S=), phosphorothioates (S substituted), phosphonothioates (S substituted), phosphorodithioates, phosphorotrithioates, phosphoramidothioates (Gupta, 2006). OPs are the most widely used pesticides worldwide and their metabolites are widespread across different populations (Aprea, 2000; Barr, 2004; Curl, 2003). The adverse short-term effects of exposure to these chemicals have been studied mostly in the nervous system, which is their primary target (Gupta at al., 2001), but there is a growing concern about their possible toxic effects in non-target tissues and (long-term) chronic effects that have not been studied in such detail. The majority of people are continually exposed to low OP concentrations, and long-term epidemiologic studies reveal linkage to higher risk of cancer development (Brown et al., 1990; Waddell at al., 2001). The World Health Organization estimates that every year 3 million people experience acute poisoning by OPs, 200.000 people terminally (WHO, 1990). The main routes of OP exposure are shown in Figure 1. Humans are exposed to OPs via ingested food and drink and by breathing polluted air (WHO, 2001). The exposure of workers in closed areas and of agricultural workers or people living near farms, is also very important (Gupta, 2006).

The primary mechanism of OPs toxicity is well studied – they function as **inhibitors of the enzyme acetylcholinesterase** (AcHE). Human **exposure to OPs** is most frequently assessed by measurement of decrease in AcHE activity. This method is relevant for professional exposure, where OP concentrations entering to body are relatively high. However, low OP concentrations, which are present continuously, do not cause significantly decreased AcHE activity. Exposure of wider populations must lean on assessment of OP metabolites, such as alkylphosphate in urine (Gupta, 2006).

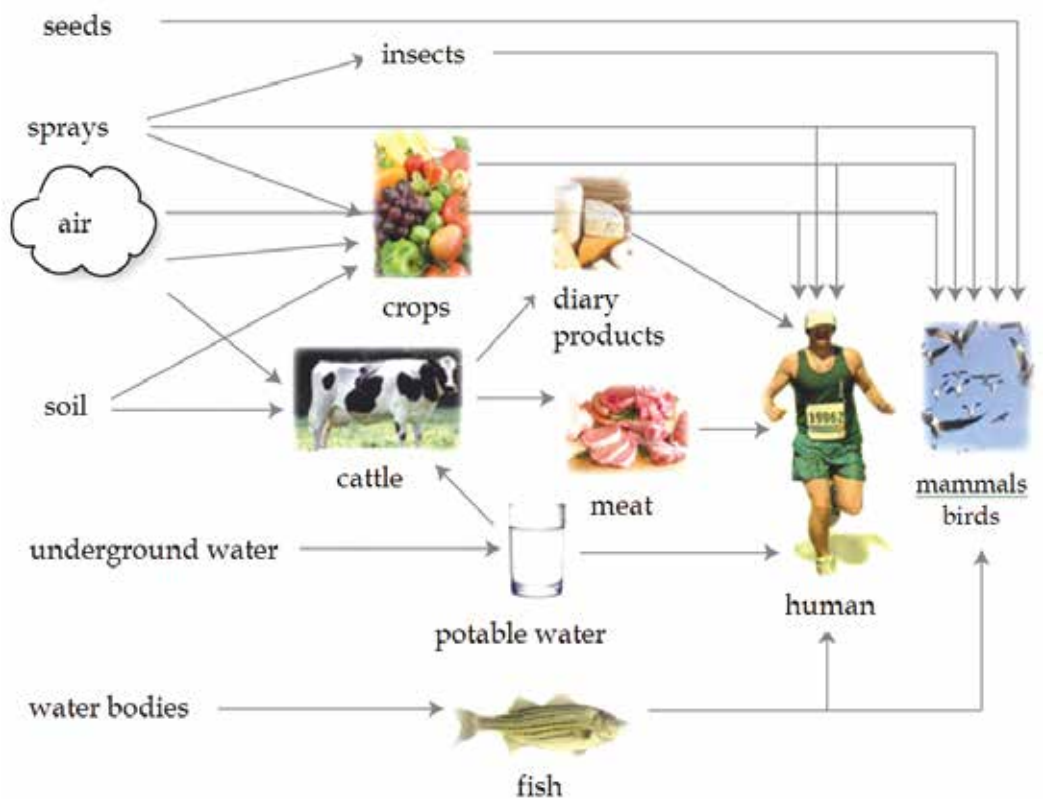

Fig. 1. Routes of exposure to OPs (adapted from WHO, 2001)

2. Short history of OPs

The first OPs were synthesised in the 19th century, but they only started to be used widely in the 1930s. The German chemist Gerhard Schrader synthesised many commercial OPs of which parathion (Figure 2) is still used as a common pesticide (Costa, 2006). At the beginning of the Second World War the development of OP substances switched to highly toxic compounds employed as nerve warfare agents, e.g. sarin, soman and tabun (Figure 3). After the War, in the 40's and 50's, the study of OPs was again oriented towards the development of less toxic compounds (Gupta, 2006). However, OP pesticide usage increased rapidly in the 70's, when the application of organochlorine pesticides such as DDT was prohibited because of their long-life persistence in the environment.

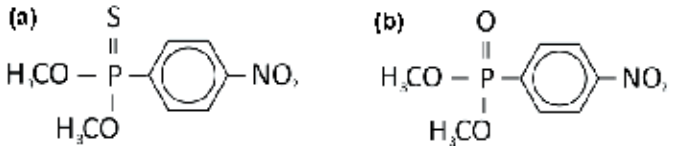

Fig. 2. Two examples of OP pesticides: (a) methyl-parathion with a bonded S atom; (b) methyl-paraoxon with a bonded O atom.

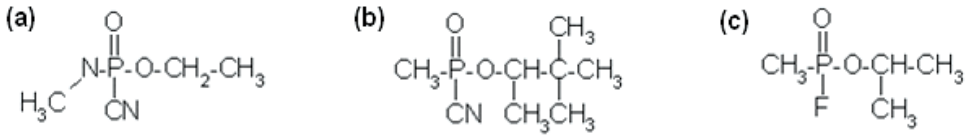

Fig. 3. The structure of toxic OP warfare agents: (a) tabun, (b) soman, (c) sarin

3. Structure – activity relationships

OPs are esters of phosphoric acid and its derivates. The general chemical structure of an organophosphate (Figure 4) comprises a central phosphorus atom (P) and the characteristic phosphoric (P=O) or thiophosphoric (P=S) bond. The symbol X represents the leaving group, which is replaced (by nucleophilic substitution) by the oxygen of serine in the AcHE active site. The rate of AcHE inhibition depends on the leaving group; higher tendency of leaving results in higher affinity of the inhibitor to the enzyme.

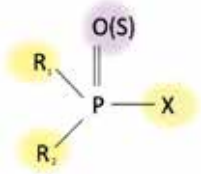

Fig. 4. General structure of OPs. X represents the leaving group, R1 and R2 the side, usually alkoxy, group (adapted from Hreljac, 2009)

In very toxic warfare agents the leaving group contains fluorine (F), which has high tendency to hydrolysis and thus extremely high AcHE inhibition. In less toxic OPs the leaving group usually contains alkyl or aryl groups. Side groups R1 and R2 are usually alkoxy groups. The active configuration of OP, which binds to the AcHE active site, is an oxono structure, with the central phosphorus linked to the oxygen atom (Figure 2b). The majority of novel OP pesticides possess the thion, P=S, linkage (Figure 2a). In this case, metabolic activation with CYP enzymes (cytochrome P450) must first metabolize the thiono to an oxono group – only then can the OP act as an AcHE inhibitor (Gupta, 2006).

4. Metabolism

Metabolism of xenobiotics takes place mostly in the liver and to a lesser extent also in the lung and intestine. It comprises two phases; the metabolic enzymes in **phase I** activate the chemical with the introduction of functional groups, on which **phase II** reactions can take

place. The phase II enzymes attach various hydrophilic groups, e.g. glucuronic acid, sulphate, glycine, glutamic acid, enabling excretion of the metabolite from the organism (Josephy & Mannervik, 2006).

In a manner similar to that of the majority of xenobiotics, OPs, after entering an organism, are metabolised with phase I and II enzymes (Figure 5). Phase I of OP metabolism involves oxidation and hydrolysis:

- **Oxidation** is the most important reaction in the activation of the OP thiono form to form active inhibitors of AcHE. With the aid of CYP enzymes, the sulphur atom in the thiono form binds one atom of oxygen, resulting in an unstable intermediate that disintegrates to the OP oxono metabolite and active sulphur atom (oxidative desulphuration). OP oxono metabolites are strong inhibitors of AcHE, so this is the key reaction for the majority of neurotoxic effects caused by OPs. The influence of the active sulphur atom, as the side product of this reaction, is still unknown. It can interact with neighbouring proteins and, for example, inactivate CYP enzymes on binding to their active sites.
- **Hydrolysis** of OPs takes place after oxidation, with the aid of the enzyme esterase A, also called paraoxonase. This reaction is important for OP detoxification processes. OP detoxification occurs when paraoxonase cleaves the OPs to dialkylphosphate and the leaving group. OPs are also hydrolysed by carboxylesterase, which differs from paraoxonase in self-inactivation on hydrolysis.

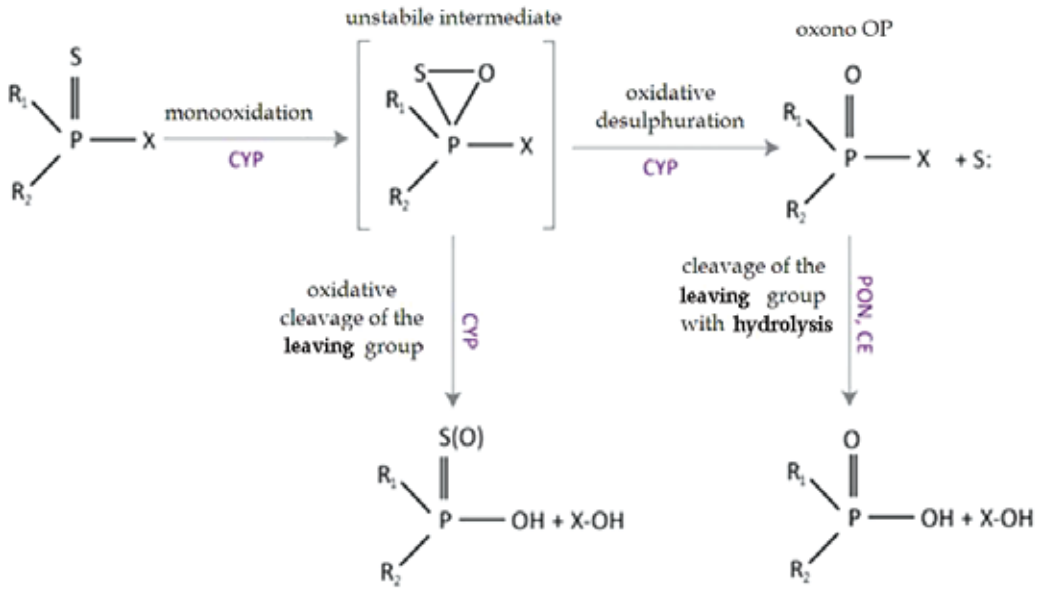

Fig. 5. Main reactions of phase I OP metabolism. CYP, cytochrome P450; PON, paraoxonase; CE, carboxylesterase (adapted from Hreljac, 2009)

In phase I metabolism, besides oxidative desulphuration and hydrolysis, reactions of oxidative removal of the side chains (dealkylation) or oxidative cleavage of the leaving group can also take place. The latter occurs after the formation of an unstable intermediate with CYP and competes with the reaction of desulphuration. Since desulphuration activates

OPs, while oxidative cleavage of the leaving group detoxifies them, the equilibrium between these two reactions is very important for the final OP toxicity outcome. The outcome of oxidation is often a more hydrophilic compound, which can be more easily conjugated in phase II metabolism, thus enabling faster excretion from the organism (Gupta, 2006).
The OP metabolites resulting from phase I metabolism are conjugated with hydrophilic groups under catalysis by enzymes of phase II, and excreted in urine. In phase II metabolism, detoxification reactions take place exclusively.
Many studies of the metabolism of model OPs have shown that the most important enzymes are cytochromes CYP1A1, CYP3A4, CYP2B6 and CYP2C19. The first three have the highest affinity for desulphuration and activation to oxon, but for oxidative cleavage of the leaving group and detoxification CYP2C19 is the most effective (Mutch & Williams, 2006). The high degree of polymorphism in the various human CYPs means that the susceptibility of individuals to toxic effects of OPs depends on the expression level of specific CYP isoforms (e.g. Buratti et al., 2007).

5. Mechanisms of toxicity

The toxicity of OPs depends on their chemical structure, metabolism in target organism, concentration (i.e. dose), mode of application, degree of decomposition, mode of entering organisms, etc. (Grlić, 1988). The best described OP toxic effects are the neurological symptoms following acute poisoning as a consequence of the primary target (AcHE). Potential secondary targets and toxic effects outside the nerve system have not been well studied, but are nevertheless very important for risk assessment.
Unlike other man-made chemicals, OP pesticides can affect a large proportion of the human population, as a result of exposure through domestic use, proximity to agricultural activities and consumption of contaminated food and water (Maroni at al., 2000).

5.1 Neurotoxicity of OPs

The primary mechanism of OPs toxicity involves **inhibition of the enzyme AcHE**. AcHE is found in synaptic membranes, where it degrades, through its hydrolytic activity, the neurotransmitter acetylcholine, producing choline and acetate, a reaction important for the regulation of synaptic activity in the central and peripheral neural system. The central neural system is composed of brain and spinal cord, and the periphery of pairs of spinal and cerebral nerves. The latter constitutes the somatic and autonomic (vegetative) nervous system. The somatic nervous system is composed of sensory fibres that flow from different receptors to the brain, and neuromuscular fibres that flow from the brain to the periphery, towards muscle. The autonomic nerve system, which controls the operation of smooth muscles (found within the walls of organs), cardiac muscle and glands, is divided into sympathetic and parasympathetic parts. OP cholinesterase inhibitors block the function of acetylcholinesterase, causing the accumulation of excessive acetylcholine in the synaptic cleft. This causes neurotoxic effects such as neuromuscular paralysis (i.e. continuous muscle contraction) throughout the entire body (Gupta, 2006).
OPs inhibits AcHE by forming covalent bond between OP and the active site of AcHE. (Figure 6). Spontaneous hydrolysis of OP from the active site is very slow, sometimes irreversible, resulting in long-term toxic effects. Novel OPs are altered in such a way that spontaneous hydrolysis of the OP-AcHE complex is accelerated (Gupta, 2006).

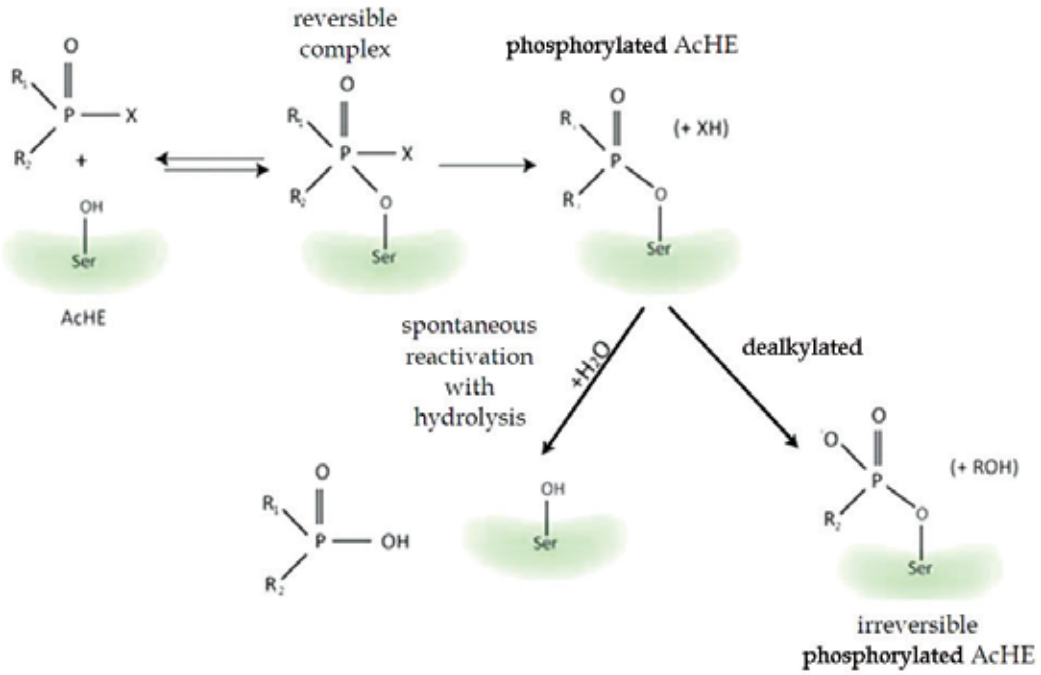

Fig. 6. The binding of OP to the active site of AcHE (adapted from Hreljac, 2009)

Neurotransmitters of the autonomic nerve system act on target organs by binding to specific receptors. Adrenergic (α, β) and cholinergic (nicotine, muscarine) receptors are affected by different types of neurotransmitter. The excess of acetylcholine in the peripheral nerve synapse causes the activation of muscarine and nicotine receptors and increases the activation of the sympathetic and parasympathetic parts. Since the synapses in the central nerve system are quite inaccessible for experimental work, compared with neuromuscular junctions in the peripheral nerve system, the mechanism of poisoning by OPs in the central nerve system has been less studied.

Symptoms of acute OP poisoning can be divided according to the site of acetylcholine accumulation in the organism (Figure 7). In addition to acute symptoms, some OPs can cause other symptoms that arise a few days after exposure or poisoning with OP. Weakness in muscles and breathing difficulties usually appear 1 – 4 days after poisoning while, after 7-21 days, weakness in peripheral muscles also occurs.

The cause of these delayed symptoms is inhibition of the neuropathy target esterase (NTE) located in the neural system, rather than AcHE inhibition. NTE belongs to the same group of serine esterases as AcHE, however its primary role in the organism is not well known (Kamanyire & Karalliedde, 2004). Several other neurotoxic symptoms that cannot be ascribed to AcHE inhibition, but act on different secondary targets inside the neural system, have been also proposed.

ACETYLCHOLINE ACCUMULATION SITE	SYMPTOMS
central neural system	headache, insomnia, vertigo, spasms, confusion, sleeplessness, speaking disorders, coma and respiratory paralysis
peripheral autonomic nerve system	
muscarine receptors	muscarine symptoms; increased elimination (salivation, perspiration, lachrymation), indigestion (spasms, vomiting, diarrhoea), lowered heart beat, visual disorders
nicotine receptors	nicotine symptoms; uncontrolled muscle contractions and paralysis

Fig. 7. Symptoms of acute OP poisoning that differ according to the site of acetylcholine accumulation.

5.2 Non-neuronal molecular targets of OPs

Although the neurotoxicity of OPs is well described, little is known about their secondary mechanisms of activity and the consequences of chronic exposure to OPs on non-target (non-neuronal) tissues and organs in humans. Recent studies have revealed several secondary targets for OPs that possibly disturb a variety of biological processes. Among the enzymes that are inhibited by OPs are **carboxylases**, which take part in xenobiotic metabolism. Their inhibition with OPs can block metabolic transformation of various substances (Hodgson & Rose, 2005). OPs can also influence xenobiotic metabolism via the active sulphur atom that arises from desulphuration in phase I of metabolism and strongly inhibits **CYP enzymes** (Hodgson & Rose, 2006). OPs also inhibit **lipases**, which play an important role in cell signalling (Quistad et al., 2006). Bomser et al. (2002) showed that OP chlorpyrifos-oxon, by inhibiting DAG-lipase indirectly, activates ERK kinases that are members of the group of mitogen-activated protein kinases (MAPK) that regulate cell proliferation and differentiation. OPs can also affect signalling pathways by activation of protein kinase (PKC) (Bagchi et al., 1997). However, this activation is probably indirect through OP mediated formation of reactive oxygen species (ROS) that activate PKC. OP induced ROS formation and oxidative stress have also been shown to be associated with apoptosis in different tissues (Oral et al., 2006; Yu et al., 2008). Kojima et al. (2004) showed that OPs can inhibit steroid androgen (AR) receptor, which can cause steroid hormone disturbances in the organism.

Further targets of OPs have been revealed, by *in vitro* and *in vivo* studies, five of which were particularly sensitive (Casida & Quistad, 2004):

- malathion and malaoxon (IC_{50} = 1-9 nM) **inhibit lysyl oxidase** in homogenates of *Xenopus* embryos, suggesting that they alter posttranslational modification of collagen, with resulting morphological defects in connective tissue (Snawder & Chambers, 1993);
- chlorpyrifos and chlorpyrifos oxone (at below 1 nM) are reported to **activate Ca^{2+}/cAMP response element binding protein** in cultured rat neurons, as a possible mechanism for neurotoxicity (Schuh et al., 2003);

- paraoxon (1-10 nM) **causes apoptotic cell death** in a leukaemia cell line by disruption of mitochondria, leading to activation of caspase-9 (Saleh et al., 2003);
- ethyl arachidonyl fluorophosphonate (at < 1 μM) and diisopropylfluorophosphate (at 100 μM**) inhibit platelet activating factor acetylhydrolase** (Kell et al., 2003);
- fenitrothion (at 22 nM) acts as an **androgen receptor antagonist** *in vitro* (Tamura et al., 2003) and **inhibits the development of androgen-dependent tissues** *in vivo* (Tamura et al., 2001).

5.3 Immunotoxicity of OPs

Toxic effects of OPs on the immune system can be direct or indirect and are reflected in different immune organ pathologies and lowered humoral and/or cell immunity.
Direct immunotoxic effects of OPs can be due to:

- inhibition of **serine hydrolases** (complement system) or **esterases** (lymphocyte and monocyte membranes) in the immune system;
- **oxidative damage** of immune system organs;
- **changes in signal transduction pathways** that control proliferation and immune cell differentiation.

Indirect immunotoxicity of OPs is expressed as changes in the nervous system or chronic effects of altered metabolism on the immune system. Selecting appropriate biomarkers and biological methods is very difficult since the physiological diversity of immune systems among organisms is very high (Galloway & Handy, 2003).

5.4 Genotoxicity and carcinogenicity of OPs

Because of the chronic exposure of human populations to low OP concentrations, it is very important to study the influence of OPs on cancer development and progress, and to elucidate the underlying mechanisms. The most informative data regarding potential human carcinogenicity come from epidemiological studies of exposed populations. **Chronic** occupational **exposure** to **OPs** has been linked to increased risk for cancer development such as non-Hodgkin lymphoma (Waddell et al., 2001) and some types of leukaemia (Brown et al., 1990). However, the major limitation of these studies is the fact that exposure has been assessed based on questionnaires and that workers were exposed not only to OPs but also to other pesticides.
Cancer development is a multi-stage process that involves initiation, promotion and progression. Many carcinogens are genotoxic and initiate cancer development by causing DNA damage and mutation, while non-genotoxic carcinogens mostly induce neoplastic cell transformation and promote cell proliferation by different mechanisms, such as avoiding apoptosis, stimulation of growth factors, and avoiding growth suppression signals.
Experimental *in vitro* and *in vivo* studies have shown that several OPs exert **genotoxic activity** (Bolognesi, 2003), and there are also reports showing that OPs can induce neoplastic transformation of cells (Cabello et al., 2001; Isoda et al., 2005). OPs have been reported

- to be **weakly mutagenic** in bacteria, but mutagenic in yeast (IARC, 1987);
- to induce **DNA damage** in peripheral lymphocytes *in vitro* (Blasiak and Kowalik, 1999; Ündeger and Basaran, 2005) and *in vivo* in occupationally exposed workers (Garaj-Vrhovac et al., 2000);
- to induce chromosomal aberrations and sister chromatid exchange (Galloway et al., 1987);

- to induce **micronuclei formation** in bone marrow (Mathew et al., 1990);
- to cause **sperm abnormalities in** OP exposed mice (Mathew et al., 1992).

Hreljac et al. (2008) have recently studied the mechanisms of genotoxicity and potential carcinogenicity of selected model OPs (parathion (PT), paraoxon (PO) and dimefox (DF)) in the *in vitro* experimental model with human hepatoma (HepG2) cells. They demonstrated that OPs act on several targets:

- low concentrations of parathion and paraoxon were **genotoxic**, while dimefox acted as a **mitogen**;
- the three model OPs induced numerous **variations of gene expression**, particularly of genes involved in stress response, inseparably connected to basic cell processes important in cancer development (cell cycle, apoptosis, xenobiotic metabolism, DNA repair);
- in addition, **changes in phosphorylation of kinases**, which are connected to stress response, were observed.

The methodologies used in these studies have involved a series of different genotoxicity assays (*Salmonella typhimurium* reverse mutation assay, and comet and micronucleus assay in HepG2 cells) and methods for measuring cell proliferation (MTT assay, cell-cycle analysis by flow cytometry and Ki-67 immunostaining), that were associated with molecular methods for gene expression analysis (quantitative real time PCR) and biochemical methods for enzyme activity measurements. Here we describe some of these methods in more detail.

The **comet assay** (also known as **Single Cell Gel Electrophoresis assay**) is a relatively simple and sensitive technique for the detection of DNA damage at the level of the individual cell (Figure 8). HepG2 cells are exposed to graded doses of model OPs and DNA damage is determined with the comet assay after 4- and 24-hr exposures to OPs. (Hreljac & Filipič, 2008). The comet assay was performed as described by Singh et al. (1988). The slides were stained with ethidium bromide and analyzed using a fluorescence microscope and image analysis software.

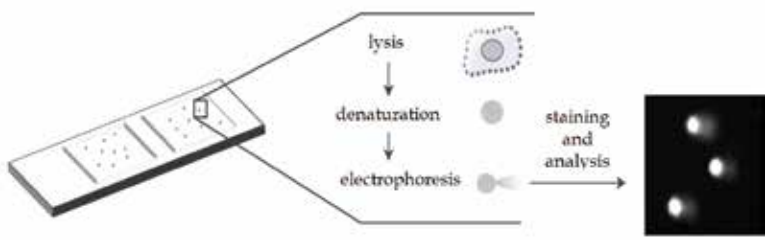

Fig. 8. The principle of the comet assay (adapted from Hreljac, 2009)

The effect of exposure of HepG2 cells to OPs on chromosomal damage was determined with the **micronucleus assay** (Figure 10). Micronucleus in this concept is the name given to the small nucleus that forms whenever a chromosome or fragment of a chromosome is not incorporated into one of the daughter nuclei during cell division, and reflects structural or numerical chromosomal aberration. The micronucleus assay (Hreljac et al., 2009) was performed according to the method optimized by Yamamoto et al. (2005) (Figure 10). HepG2 cells were seeded on chamber slides and left overnight to attach. They were treated with parathion or paraoxon alone, and also in combination with BaP, to determine the effect of combined exposure. The medium was then changed and the cells incubated for another

68 h to allow cell division to take place. The cells were then washed, incubated in hypotonic KCl solution, and fixed. The slides were dried and stained with acridine orange, and examined under the fluorescence microscope. Micronuclei were scored according to established criteria (Fenech, 2000). To determine cytotoxicity, cells were seeded in parallel, with the same treatment and recovery protocol as for micronuclei, and assayed for cell viability using the MTS assay according to the manufacturer's protocol.

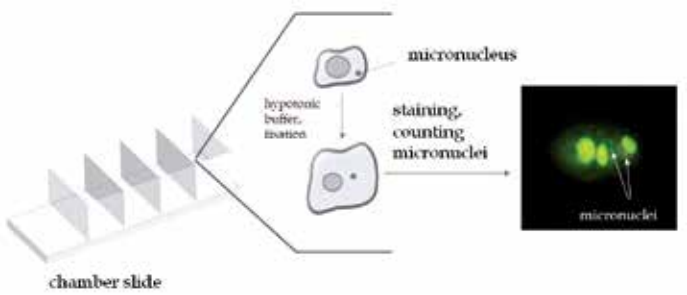

Fig. 9. The principle of the micronucleus test (adapted from Hreljac, 2009)

The effect on cell proliferation has been determined by cell cycle analysis, using flow cytometry, MTT viability and proliferation assay, and immunostaining for Ki-67 proliferation marker.

Immunostaining for Ki-67 proliferation marker (Figure 10) is a method for detecting proliferating cells. The antigen KI-67, also known as Ki-67 or MKI67, is a protein that, in humans, is encoded by the *MKI67* gene (antigen identified by monoclonal antibody Ki-67). The cells were seeded onto slides in drops, left to attach and then exposed to parathion, paraoxon or dimefox for 72 hr. The cells were then fixed, permeabilized and blocked with BSA. Primary mouse Ki-67 antibody was added to the cells, incubated and secondary anti-mouse Alexa- Fluor488 labelled antibody, together with Hoechst, was then added and incubated. Cells were scored under a fluorescence microscope. Ki-67 positive nuclei were granularly stained with intense green fluorescence. The proliferation index was determined as the ratio between Ki-67 positive nuclei and total nuclei (stained with Hoechst) and was scored for each experimental point (Hreljac et al, 2008).

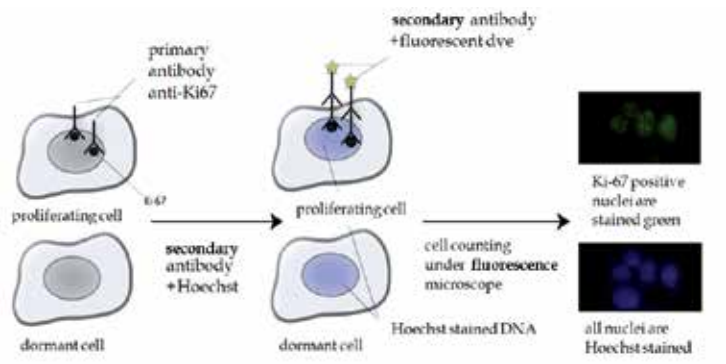

Fig. 10. The principle of immunocytochemistry for Ki-67 proliferation marker (adapted from Hreljac, 2009).

Gene expression of DNA damage responsive genes: *p53*, *MDM2*, *GADD45a* and *p21,* and of genes coding for *CYP1A1* and *AKR1C1/2* metabolic enzymes, was quantified using real-time quantitative PCR (ABI 7900 HT Sequence Detection System, Applied Biosystems, USA). The following Taqman Gene Expression Assays were used (all from Applied Biosystems): *p53* (tumour protein p53), Hs00153349_m1; *MDM2* (Mdm2, 'transformed 3T3 cell double minute 2', p53 binding protein gene), Hs00234753_m1; *GADD45a* ('growth arrest and DNA-damage-inducible gene, alpha'), Hs00169255_m1; *p21* ('cyclin-dependent kinase inhibitor 1A') Hs00355782_m1; *AKR1C1/2*- Hs00413886_m1 and *CYP1A1*- Hs00153120. Amplification of GAPDH probe was used as an internal control. The conditions for PCR were 50°C for 2 min, 95°C for 10 min and 40 cycles of 95°C for 15s and 60°C for 1 min. The obtained data were analyzed using the ΔΔCt algorithm (Hrelajc et al., 2008; Hreljac & Filipič, 2009).

The studies showed that parathion and paraoxon are genotoxic (Fig 11a), while dimefox has no genotoxic potential but induced an increase in cell proliferation (Fig. 11b), indicating mitogenic activity (Hreljac et al., 2008). The differences in genotoxic and mitogenic activities of the three OPs were confirmed at the level of mRNA expression of DNA damage response genes – the tumour suppressor gene *p53* and its downstream regulated genes, the cyclin-dependent kinase (CDK) inhibitor *p21* and E3 ubiquitin ligase *MDM2* genes, and the growth arrest and DNA damage-inducible gene *GADD45a.* While parathion and paraoxin up-regulated mRNA expression of all four genes, dimefox did not change the expression of *p53, GADD45a* and *MDM2,* but down-regulated expression of *p21. p21* is a cyclin kinase inhibitor responsible for cell-cycle arrest, which may explain the observed mitogenic activity of dimefox.

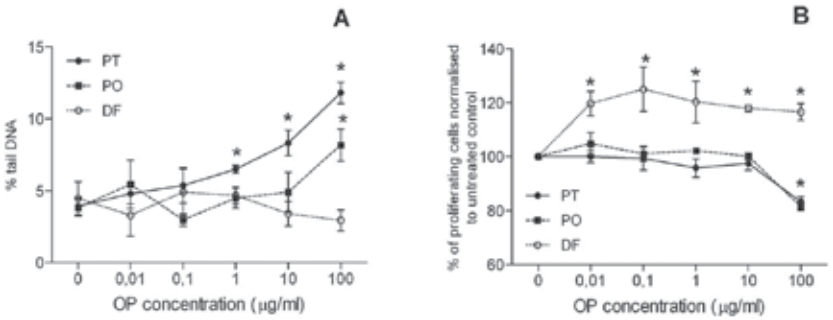

Fig. 11. DNA strand breaks (A) and increase in cell proliferation (B) induction by parathion, paraoxon and dimefox in HepG2 cells. DNA damage was assessed after 24 hour exposure using the Comet assay. The proliferation was assessed with immunostaining for the Ki-67 proliferation marker after 72 hour exposure. * Significant difference in % tail DNA or proliferation index between treated cells and the non-treated control (Student t-test $P < 0.05$).

The lowest concentration (LOEL) at which significant dimefox-mediated increase of cell proliferation was observed was 0.01 μg/mL (0.0625 μM) (Fig. 11B). The LOEL at which parathion induced significant increase in DNA damage was 1 μg/ml while paraoxon was less effective, inducing significant increase in DNA damage only at 100 μg/ml (Fig 11A). However, in HepG2 cells exposed to parathion and paraoxon, significant increase in micronuclei formation was observed for both OPs, appearing at an LOEL of 0.1 μg/ml (Fig.

12) (Hreljac & Filipič, 2009). On the basis of pharmacokinetic models and biomonitoring data, Buratti et al. (2007) recently proposed that OP concentrations lower than 10 μM (corresponding to 2.6, 2.5 and 1.6 μg/mL of methyl parathion, methyl paraoxon and dimefox, respectively) in the *in vitro* studies reflect conditions in human during environmental exposure, and that concentrations higher than 100 μM reflect acute accidental intoxication. These studies thus showed that parathion, paraoxon and dimefox can cause adverse effects at concentrations that are relevant, not only for occupational, but also general human exposure. Thiono forms of OPs have been considered safer for use as pesticides because of their lower acute toxicity mediated by direct AChE inhibition, however this study indicated that, at chronic exposure, the thiono form of OPs may pose a similar or even greater health risk than the oxono form.

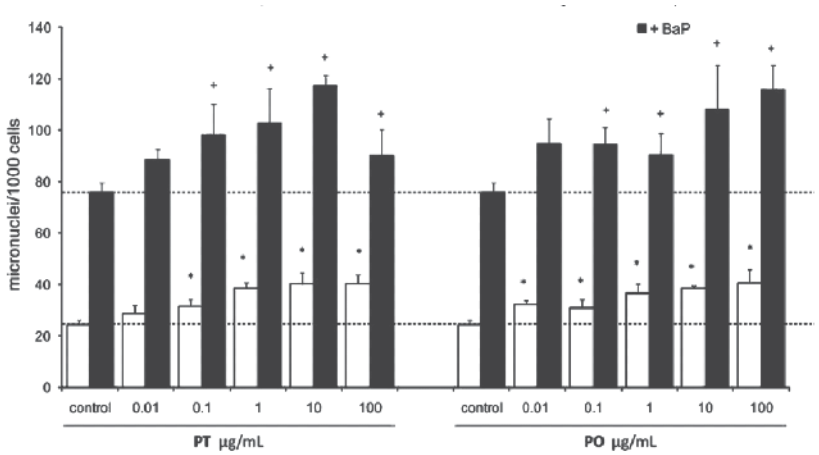

Fig. 12. Effect of OPs on BaP-induced micronuclei (MN) in HepG2 cells (Hreljac & Filipič, 2009). The cells were exposed to parathion or paraoxon alone (white bars) or together with 25 μM BaP (black bars) for 4 h and then left to recover for an additional 68 h. The dotted lines delineate background and BaP-induced levels of MN. (*) significant difference from the vehicle control; (+) significant difference from BaP treated cells (Student's t-test, $P < 0.05$).

Humans are exposed in the environment to mixtures of various pollutants and very little is known about their combined effects. Hreljac & Filipič (2009) showed co-genotoxic effects of parathion and paraoxon in combination with one of the most widespread genotoxic pollutants, benzo(a)pyrene (BaP). In the bacterial test system with *Salmonella typhimurium* microsomal reverse mutation assay, parathion and paraoxon alone did not induce mutations while, in combination with BaP, both dramatically increased BaP induced mutations, with parathion being more effective than paraoxon. In the experimental model with HepG2 cells, combined exposure to either parathion or paraoxon with BaP was studied by means of the comet assay and micronuclei induction. With the latter a clear, more than additive, co-genotoxic effect of parathion or paraoxon, each in combination with BaP, was observed (Fig. 12). In the comet assay, on the other hand, a less than additive effect of combined exposure was observed, which was surprising. This effect was then explained by experiments showing that parathion and paraoxon form DNA crosslinks, thus interfering with the comet assay results. This finding indicates that the cross linking potential of OPs should be taken into account when using the comet assay for evaluating genotoxic potential of OPs.

Formation of cross links by parathion and paraoxon may also explain why LOEC of parathion or paraoxon for DNA damage was higher than that for micronuclei formation.
Parathion and paraoxon have been shown to modulate metabolic activation of BaP (Hreljac & Filipič, 2009). BaP, like other PAHs, needs metabolic activation to exert its mutagenic and carcinogenic effects. It is readily oxidized by CYP-1A1, -1A2 and -1B1 enzymes and subsequently hydrolysed by epoxide hydrolase to yield an inactive BaP-diol intermediate. From this point onwards the carcinogenic activation can take two main routes: i) oxidation by CYP1 enzymes to the proximate carcinogen diol epoxide (BPDE) that forms DNA adducts; and ii) oxidation by human aldo-keto reductases (AKR1A1, 1C1-1C4) to yield reactive BaP *o*-quinones via BaP catechols. The latter undergo spontaneous auto-oxidation (Fig 13). BaP *o*-quinone can react with DNA and other biological molecules, and can also be reduced back to the catechol to yield futile redox cycles, thus producing reactive oxygen species (ROS) (Shimada, 2006).

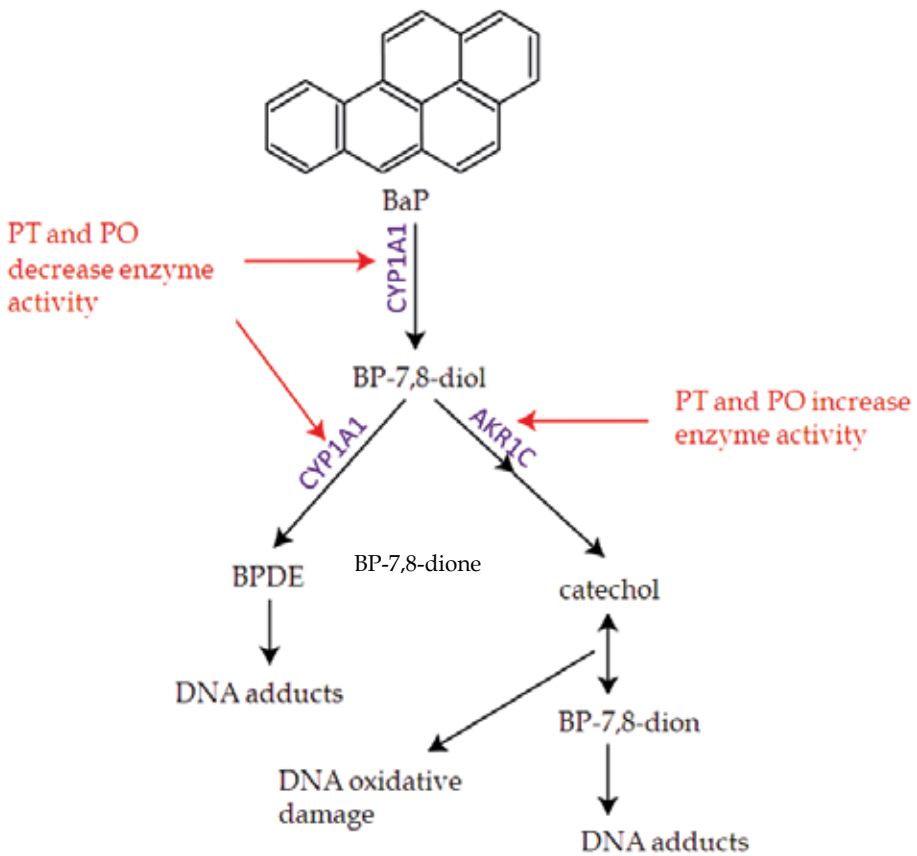

Fig. 13. Main metabolic pathways of BaP activation and the effect of OP on CYP and AKR1C enzymes. BaP is oxidized to its diol with enzymes of the CYP1 group, and may be further converted via the CYP1 activation pathway to the ultimate DNA reactive epoxide (BPDE) or via the AKR1C pathway to o-quinone (BP-7,8-dione) that forms DNA adducts and/or, via auto-oxidation, generates ROS formation (adapted after Hreljac, 2009).

Treatment of HepG2 cells with BaP in the presence of parathion or paraoxon decreased mRNA expression and enzyme activity of CYP1A, while AKR1C1/2 levels were elevated. Based on these results it was proposed that the co-genotoxicity results from OP mediated modulation of BaP metabolism. This favours the induction of AKR1C enzymes known to catalyse the formation of DNA reactive BaP *o*-quinones and the production of reactive oxygen species (Fig. 13).

6. Conclusions

Organophosphorus pesticides are the most widely used pesticides worldwide and their metabolites are widespread across different populations. The primary mechanism of OP toxicity is the inhibition of acetylcholine esterase (AChE) in the central and peripheral nervous system, leading to a variety of short-term and chronic effects such as nausea, headache, confusion, depression, memory loss and chronic fatigue syndrome. Accordingly, safety evaluation of OP pesticides is generally based on the premise that AChE inhibition is the principal cause for their acute and chronic toxicity.

Recent studies, however, have revealed a number of OP secondary targets that are not associated with the cholinergic system and may lead to immunotoxic, endocrine disrupting, genotoxic and potential carcinogenic effects.

The genotoxicity and potential carcinogenicity of OPs are of particular concern. In commercial pesticide formulations, thiono forms of OPs are usually used because their acute neurotoxicity is lower than that of oxono forms. However, a recent comparative study of genotoxic potential of parathion and paraoxon in human hepatoma HepG2 cells revealed that parathion may have even higher genotoxic and co-genotoxic potential than paraoxon. In addition, genotoxic and co-genotoxic effects of parathion and paraoxon have been observed at concentrations that are relevant for human environmental exposure. These are important points that should be further investigated and considered in evaluations of the hazards and risk of exposure to these chemicals.

Taken together, the scientific evidence supports the assertion that chronic exposure to OPs is associated with higher risk of developing diseases, including cancer, and that world usage of OPs should be reduced and better controlled. Further investigations are needed, particularly in regard to mechanisms of toxicity toward non cholinergic systems. Safety of the continuous use of OPs in agriculture and its expanding use in medicine depends on understanding the relevance of not only AChE inhibition but also of secondary targets in the effects of acute and long term exposure on health.

7. References

Aprea, C.; Strambi, M.; Novelli, M.T.; Lunghini, L. & Bozzi, N. (2000) Biologic monitoring of exposure to organophosphorus pesticides in 195 Italian children. *Environ Health Perspect* 108 (6): 521-5

Bagchi, D.; Bagchi, M.; Tang, L. & Stohs, S.J. (1997) Comparative in vitro and in vivo protein kinase C activation by selected pesticides and transition metal salts. *Toxicology Letters* 91 (1): 31-37

Barr, D.B.; Bravo, R.; Weerasekera, G.; Caltabiano, L.M.; Whitehead, R.D. Jr.; Olsson, A.O.; Caudill, S.P.; Schober, S.E.; Pirkle, J.L. & Sampson, E.J. (2004) Concentrations of dialkyl phosphate metabolites of organophosphorus pesticides in the U.S. population. *Environ Health Perspect* 112 (2):186-200

Blasiak, J. & Kowalik, J. (1999) Effect of paraoxon-methyl and parathion-methyl on DNA in human lymphocytes and protective action of vitamin C. *Pestic Sci* 55: 1182-86

Bolognesi, C. (2003) Genotoxicity of pesticides: a review of human biomonitoring studies. *Mutat Res* 543 (3): 251-72

Bomser, J.A.; Quistad, G.B. & Casida, J.E. (2002) Chlorpyrifos oxon potentiates diacylglycerol-induced extracellular signal-regulated kinase (ERK 44/42) activation, possibly by diacylglycerol lipase inhibition. *Toxicology and Applied Pharmacology* 178 (1):29-36.

Brown, L.M.; Blair, A.; Gibson, R.; Everett, G.D.; Cantor, K.P.; Schuman, L.M.; Burmeister, L.F.; Van Lier, S.F. & Dick, F. (1990) Pesticide exposures and other agricultural risk factors for leukemia among men in Iowa and Minnesota. *Cancer Res* 50 (20): 6585-91

Buratti, F.M.; Leoni, C. & Testai, E. (2007) The Human Metabolism of Organophosphorothionate Pesticides: Consequences for Toxicological Risk Assesment. *J Verbr Lebensm* 2: 37-44

Cabello, G; Valenzuela, M; Vilax, A; Duran, V; Rudolph, I; Hrepic, N & Calaf, G. (2001) A rat mammary tumor model induced by the organophosphorous pesticides parathion and malathion, possibly through acetylcholinesterase inhibition. *Environ Health Perspect* 109: 471-9

Casida, J.E. & Qistad, G.B. (2004) Organophosphate Toxicology: Safety Aspects of Nonacetylcholinesterase Secondary Targets: *Chemical Research in Toxicology* 17 (8): 983-998

Costa, L.G. (2006) Current issues in organophosphate toxicology. *Clinica Chimica Acta* 366: 1-13

Curl, C.L.; Fenske, R.A. & Elgethun, K. (2003) Organophosphorus pesticide exposure of urban and suburban preschool children with organic and conventional diets. *Environ Health Perspect* 111 (3): 377-82

Fenech, M. (2000) The in vitro micronucleus technique. *Mutat. Res.* 455: 81–95

Galloway, T. & Handy, R. (2003) Immunotoxicity of organophosphorus pesticides. *Ecotoxicology* 12: 345-363

Galloway, S.M.; Armstrong, M.J.; Reuben, A.; Colman, S.; Brown, B.; Cannon, C.; Bloom, A.D.; Nakamura, F.; Ahmed, M.; Duk, S.; Rimpo, J.; Margolin, B.H.; Resnick, M.A.; Anderson, B. & Zeiger, E. (1987) Chromosome aberrations and sister chromatid exchanges in Chinese hamster ovary cells: evaluations of 108 chemicals. *Environ Mol Mutagen* 10 (10): 1-175

Garaj-Vrhovac, V. & Zeljezic D. (2000). Evaluation of DNA damage in workers occupationally exposed to pesticides using single-cell gel electrophoresis (SCGE) assay: pesticide genotoxicity revealed by comet assay. *Mutat Res* 469: 279–285

Gavrilescu, M. (2005) Fate of pesticides in the environment and its bioremediation. *Eng Life Sci* 5 (6): 497-526

Grlić, L. (1988) Mali kemijski leksikon Napried, Zagreb

Gupta, R.C. (2006) *Toxicology of Organophosphate & Carbamate Compound*. Elsevier Academic Press

Gupta, S.; Stravitz, R.T.; Dent, P. & Hylemon, P.B. (2001) Down-regulation of cholesterol 7alpha-hydroxylase (CYP7A1) gene expression by bile acids in primary rat hepatocytes is mediated by the c-Jun N-terminal kinase pathway. *J Biol Chem* 276 (19): 15816-22

Hodgson, E. & Rose, R.L. (2005) Human metabolism and metabolic interactions of deployment-related chemicals. *Drug Metabolism Reviews* 37 (1): 1-39

Hodgson, E. & Rose, R.L. (2006) Organophosphorus chemicals: Potent inhibitors of the human metabolism of steroid hormones and xenobiotics. *Drug Metabolism Reviews* 38 (1-2): 149-162

Hreljac, I. & Filipic, M. (2009) Organophosphorous pesticides enhance the genotoxicity of benzo(a)pyrene by modulating its metabolism. *Mut Res* 671: 84-92

Hreljac, I.; Zajc, I.; Lah, T. & Filipic, M. (2008) Effects of model organophosphorous pesticides on DNA damage and proliferation of HepG2 cells. *Environ Mol Mutagen* 49 (5): 360-7

Hrejac, I. (2009) Genotoxic, cogenotoxic and potential carcinogenic activity of model organophosphorous pesticiedes. Doctoral thesis, University of Ljubljana, Ljubljana, Slovenia 2009.

IARC. (1987) Evaluation of the carcinogenic risk of chemicals to humans: overall evaluations of carcinogenicity. IARC Monograph Supp 7., pp. 392. , Lyon: International Agency for Research on Cancer.

Isoda, H.; Talorete, T.P.; Han, J.; Oka, S.; Abe, Y. & Inamori, Y. (2005) Effects of organophosphorous pesticides used in china on various mammalian cells. *Environ Sci* 12: 9-19

Josephy, P.D. & Mannervik, B. (2006) *Molecular Toxicology*. New York: Oxford University Press

Kamanyire, R. & Karalliedde, L. (2004) Organophosphate toxicity and occupational exposure. *Occupational Medicine-Oxford* 54 (2): 69-75

Kell, P.J.; Creer, M.H.; Crown, K.N.; Wirsig, K. & Mchowat, J. (2003) Inhibition of platelate-activating factor (PAF) acetylhydrolase by methyl arachidonyl fluorophosphonate potentiates PAF synthesis in thrombin-stimulated human coronary artery endothelial cells. *J. Pharmacol. Exp. Ther*. 307: 1163-1170

Kojima, H.; Katsura, E.; Takeuchi, S.; Niiyama, K. & Kobayashi, K. (2004) Screening for estrogen and androgen receptor activities in 200 pesticides by in vitro reporter gene assays using Chinese hamster ovary cells. *Environmental Health Perspectives* 112 (5): 524-531

Maroni, M.; Colosio, C.; Ferioli, A. & Fait, A. (2000) Chapter 1 – Organophosphorous pesticides, In: *Toxicology* 143: 5-37

Mathew, G.; Rahiman, M.A. & Vijayalaxmi, K.K. (1990) In vivo genotoxic effects in mice of Metacid 50, an organophosphorus insecticide. *Mutagenesis* 5: 147-9

Mathew, G.; Vijayalaxmi, K.K. & Abdul-Rahiman, M. (1992) Methyl parathion-induced sperm shape abnormalities in mouse. *Mutat Res* 280 (3): 169-73

Mutch, E. & Williams, F.M. (2006) Diazinon, chlorpyrifos and parathion are metabolised by multiple cytochromes P450 in human liver. *Toxicology* 224 (1-2): 22-32

Oral B.; Guney, M.; Demirin, H.; Ozguner, M.; Giray, S.G.; Take, G.; Mungan, T. & Altuntas, I. (2006) Endometrial damage and apoptosis in rats induced by dichlorvos and ameliorating effect of antioxidant vitamins E and C. *Reprod Toxicol* 22(4): 783-90

Quistad, G.B.; Liang, S.N.; Fisher, K.J.; Nomura, D.K. & Casida, J.E. (2006) Each lipase has a unique sensitivity profile for organophosphorus inhibitors. *Toxicological Sciences* 91 (1): 166-172

Saleh, A.M.; Vijayasarathy, C.; Masound, L.; Kumar, L.; Shahin, A. & Kambal, A. (2003) Paraoxon induces apoptosis in EL4 cells via activation of mitochondrial pathways. *Toxicol Appl Pharmacol* 190: 47-57

Schuh, R.A.; Lein, P.J.; Beckles, R.A. & Jett, D.A. (2002) Noncholinesterase mechanisms of chlorpyrifos neurotoxicity: altered phosphorylation of Ca2+/cAMP response element binding protein in cultured neurons. *Toxicol Appl Pharmacol* 182: 176-185

Shimada, T. (2006) Xenobiotic-metabolizing enzymes involved in activation and detoxification of carcinogenic polycyclic aromatic hydrocarbons. *Drug Metab Pharmacokinet* 21: 257-276

Singh, N.P.; McCoy, M.T.; Tice, R.R. & Schneider, E.L. 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp Cell Res* 175 (1): 184-91

Snawder, J.E. & Chambers, J.E. (1993) Osteolathyrogenic effects of malthion in Xenopus embryos. *Toxicol Appl Pharmacol* 121: 210-216

Tamura, H.; Maness, S.C.; Reischmann, K.; Dorman, D.C.; Gray, L.E. & Gaido, K.W. (2001) Androgen receptor antagonism by the organophosphate insecticide fenitrothion. *Toxicol Sci* 60, 56-62

Tamura, H.; Yoshikawa, H.; Gaido, K.W.; Ross, S.M.; DeLisle, R.K.; Welsh, W.J. & Richard, A.M. (2003) Interaction of organophophate pesticides and related compounds with the androgen receptor. *Environ Health Perspect* 111: 545-552

Ündeger, U. & Basaran N. 2005. Effects of pesticides on human peripheral lymphocytes in vitro: induction of DNA damage. *Arch Toxicol* 79: 169-76

Waddell, B.L.; Zahm, S.H.; Baris, D.; Weisenburger, D.D.; Holmes, F.; Burmeister, L.F.; Cantor, K.P. & Blair, A. (2001) Agricultural use of organophosphate pesticides and the risk of non-Hodgkin's lymphoma among male farmers (United States). *Cancer Causes and Control* 12 (6): 509-517

WHO (1990) *Public health impact of pesticides used in agriculture*. Geneva: WHO

WHO (2001) *Organophosphorous pesticides in the environment*- Integrated Risk Assessment, Geneva: WHO

Yamamoto, M.; Motegi, A.; Seki, J. & Miyamae, Y. (2005) The optimized conditions for the *in vitro* micronucleus (MN) test procedures using chamber slides, *Environ Mut Res* 27: 145–151

Yu, F.; Wang, Z.; Ju, B.; Wang, Y.; Wang, J. & Bai, D. (2008) Apoptotic effect of organophosphorus insecticide chlorpyrifos on mouse retina in vivo via oxidative stress and protection of combination of vitamins C and E. *Exp Toxicol Pathol* 59 (6): 15-23

13

Acute Toxicity of Organophosphorus Pesticides and Their Degradation By-products to *Daphnia magna*, *Lepidium sativum* and *Vibrio fischeri*

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1. Introduction

Organophosphorus pesticides (OPPs) attained the growing importance in pests control because of their rapid decomposition and less likely accumulation in environment. They are still of great concern however, for water sources contamination because of their high solubility in water and excessive usage. Their usage amounts were elevated after they were introduced as replacements for the highly persistent organochlorine pesticides.

They are classified into two main groups, organophosphates (P=O) and organothiophosphates (P=S) depending on whether oxygen or sulphur forms a double bond with the central phosphorous atom. They were found in environment with enough frequency (Ballesteros and Parrado, 2004) to constitute an ecotoxicological risk. Their concentration in water sources (Barcelo et al., 1990; Konstantinou et al., 2006), in air (Tuduri et al., 2006) and food (Bai et al., 2006; Darko and Akoto, 2008) can vary between a few ppb to ppm levels.

The presence of these pesticides can directly affect the health of aquatic and terresterial organisms and may present a threat to humans through contamination of drinking water supplies. OPPs always pose acute toxicity but not chronic toxicity on organisms because of their quick degradation (Ye et al., 2010).

OPPs are known to cause inhibition of acetylcholinesterase (AChE) in target tissues which leads to accumulation of acetylcholine. According to its key physiological role in nerve transmission, AChE is the target of various insecticides. AChE is an enzyme vital for normal nerve function and AChE inhibition leads to over stimulation of the central and peripheral nervous systems, resulting in neurotoxic effects in organisms. OPPs also produce oxidative stress in different tissues (Possamai et al., 2007) and shows genotoxic (Bolognesi, 2003; Cakir and Sarikaya, 2005, Arredondo et al., 2008) and immunotoxic (Yeh, et al., 2005; Day et al., 1995) effects. The majority of OPPs give rise to only slight inhibition of AChE by themselves, unless they undergo oxidative activation. This process involves the substitution of the sulfur atom in the P=S bond of the organophosphate pesticide with an oxygen atom resulting with formation of oxon derivatives (OPPs-oxons) (Fig. 1).

This substitution is a result of advanced oxidation processes such as O_3, O_3/UV, H_2O_2/UV, fenton, photo-fenton, TiO_2/UV, etc. in water treatment and natural oxidation processes such as UV radiation and microbial degradation. Combined oxidation systems decreases toxicity effects of by-products via enhancing mineralization. Kim et al. (2006) used *Vibrio fischeri* and

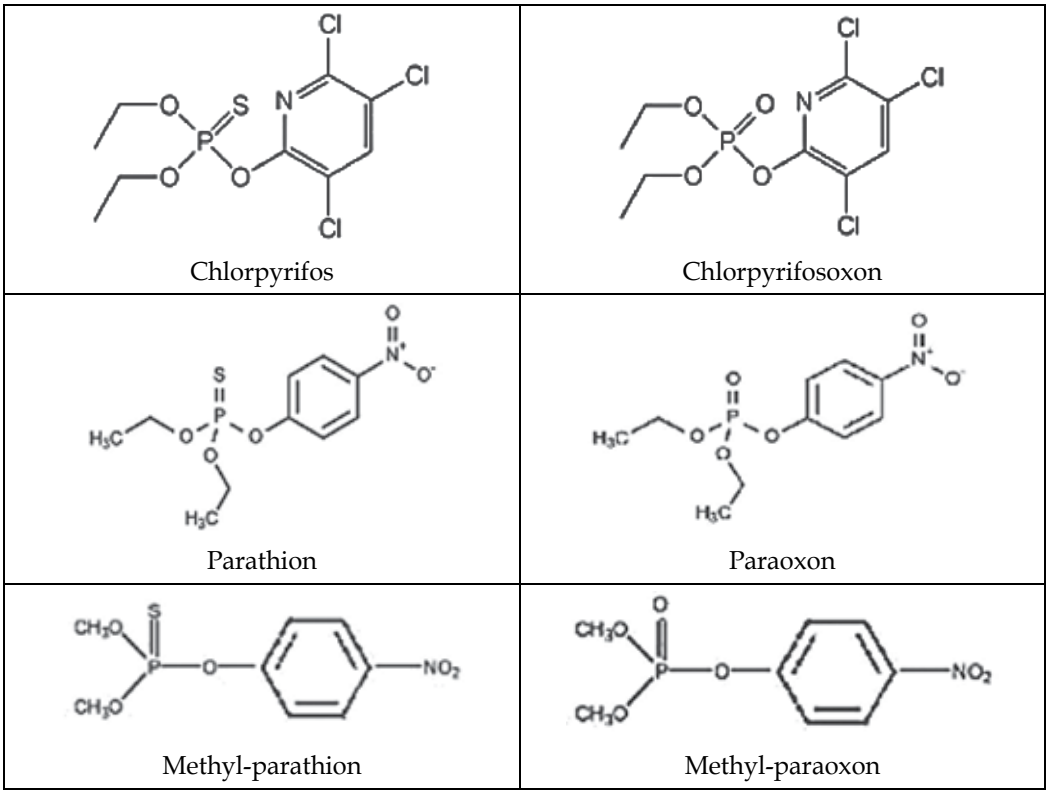

Fig. 1. Chemical structures of OPPs and OPPs-oxons

Daphnia magna bioassays to test acute toxicity of methyl parathion solutions treated by photocatalysis and photolysis. Test results showed that relative toxicity was reduced almost completely under photocatalysis for two of the tested organisms, whereas an 83% reduction for *Vibrio fischeri* and 65% reduction for *Daphnia magna* was achieved with photolysis alone.

There are some studies dealing with the ecotoxicity of OPPs (Burkepile et al., 1999; Zhang et al., 2008) but few provide data about the hazards of the degradation products (Kim et al., 2006; Kralj et al., 2007; Virag et al., 2007). According to Sparling and Fellers (2007) oxon analogs of chlorpyrifos, malathion, diazinon are 10 to 100 times more toxic for foothill yellow-legged frog (*Rana boylii*) than their parental forms. Kralj et al. (2007) studied degradation kinetics, toxicity, and degree of mineralization of malathion, malaoxon, isomalathion, and Radotion, during UV photolysis and TiO_2 photocatalysis. Formation of malaoxon, isomalathion or trimethyl phosphate esters correlated well with the induced toxicity (inhibition of acetylcholinesterase), which was observed in photocatalysis of malathion and Radotion, and in photolysis of malaoxon and Radotion.

Tsuda et al. (1997) reported that the 48 h LC_{50} values for killifish (*Uryzias latipes*) are 4.4 mg L^{-1} for diazinon and 1.8 mg L^{-1} for malathion while 0.22 mg L^{-1} for diazoxon and 0.28 mg L^{-1} for malaoxon.

Three pesticides, chlorpyrifos, parathion, methyl parathion, and their oxon derivatives; chlorpyrifosoxon, paraoxon, methyl paraoxon were involved in our study. Physical and chemical properties of parent compounds are given in Table 1. When compared with organochlorine pesticides their solubility in water is quite high.

Compound	Molecular Formula	Molecular Weight	Melting Point ^{0}C	Solubility in Water mg L^{-1}	Vapor Pressure	pKow
chlorpyrifos	$C_9H_{11}Cl_3NO_3PS$	350.59	41	1.4 (25 ^{0}C)	2.02X10^{-5}	4.96
parathion	$C_{10}H_{14}NO_5PS$	291.26	6.1	11 (20 ^{0}C)	6.68X10^{-6}	3.83
methyl parathion	$C_8H_{10}NO_5PS$	263.21	35	55 (20 ^{0}C)	1.72X10^{-5}	2.86

Table 1. Physical and chemical properties of OPPs (HSDB, 2010)

Parathion and its methyl analog are probably the most widely used organophosphorus insecticides in agriculture. Methyl parathion is a persistent pesticide commonly found in trace levels in the environment. According to a study about occurrence and temporal distribution of 49 pesticides and pesticide metabolites in air and rain samples conducted in Mississippi, the pesticide with the highest concentration in rain was methyl parathion (Coupe et al., 2000).

Methyl parathion is expected to have moderate to low mobility in soil. In moist soils, greater than 40% degrades to carbon dioxide or bound residues in 14 days, while in dry soils degradation is slower. Hydrolysis is expected to be an important process in moist soils, since methyl parathion hydrolyzes in natural water with half-lives ranging from 6.5 to 13 days at 40 ^{0}C and pH values less than 8. Half-lives in non-sterile sediment/water slurries range from 2.3 to 30 days. Aqueous photolysis half-lives range from 8 to 38 days; products include p-nitrophenol, O-methyl-O'-p-nitrophenylthiophosphoric acid, and methyl paraoxon (HSDB, 2010).

Parathion is 2-3 times more persistent than methyl parathion in natural water systems. Parathion is expected to have moderate to no mobility in soil. The primary oxidative pathway involves an initial hydrolysis to p-nitrophenol and diethylthiophosphoric acid; a second oxidative pathway involves oxidation to paraoxon (HSDB, 2010).

Chlorpyrifos is mainly used to control grain, cotton, fruit, and vegetable pests. Chlorpyrifos is acutely toxic to invertebrates and aquatic organisms (Pablo et al., 2008; Zhou et al., 2007; Gul, 2005). In soil, chlorpyrifos has half-lives of 33 to 56 days for soil-incorporated applications and 7-15 days for soil surface applications. Chlorpyrifos is expected to adsorb to suspended solids and sediment in aqueous media. The hydrolysis half-life of chlorpyrifos in distilled water at 25 ^{0}C was reported as 62 days (pH 4.7), 35 days (pH 6.9) and 22 days (pH 8.1) (HSDB, 2010). Microbial degradation contributes significantly to the dissipation of chlorpyrifos in freshwater, but is inhibited in seawater, leading to increased persistence (Bondarenko, 2004). Main oxidation by-products of chlorpyrifos are O,O-diethylphosphorothioate, TCP and chlorpyrifos-oxon (Kralj et al., 2007).

Ecotoxicological studies with a broader spectrum of aquatic organisms are needed to determine whether currently applied OPPs and their transformation products may constitute a potential risk to ecosystem. The potential utility of biomarkers for monitoring both environmental quality and the health of organisms inhabiting polluted ecosystems has received increasing attention during the recent years.

Three different biotests from different trophic levels were chosen in this study. For the trophic level, producers, the terrestrial plant *Lepidium sativum* was used. As representatives of the primary consumers the crustacea *Daphnia magna* was chosen. Representative for the decomposer was *Vibrio fischeri*. Since one simple bioassay never provides a safety estimation of the environmental hazard of a chemical, these three test organisms, which represent three different trophic levels, are incorporated.

For our knowledge no studies about OPPs-oxon's phtotoxicity have been reported. After agricultural applications of OPPs, natural effects, such as UV irradiation and microbial transformation, may cause decomposition of pesticides and formation of oxon derivatives. For phytotoxic evaluation of OPPs-oxon's *Lepidium sativum* test organism was selected. *Lepidium sativum*, known as garden cress, is a fast growing annual herb widely cultivated in temperate climates throughout the world for various culinary and medicinal uses (Moser et al., 2009).
Water flea *Daphnia magna* is a standardized test organism and has been widely used in toxicity tests (Jemec et al., 2007; Palma et al., 2008). *Daphnia magna* is very sensitive to OPPs (Barata et al., 2001). It is often inhabits in small water bodies around agricultural fields receiving OPPs treatments. In this study, acute effects of OPPs and their oxon derivatives on *Daphnia magna* was determined.
Luminescence bacteria test with *Vibrio fischeri,* commonly called Microtox test, is a convenient test to perform in a short time. The photo luminescent bioassay uses a suspension of *Vibrio fischeri* bacteria and measures the reduction in light output of its natural luminescence on exposure to the toxicant of interest (Kaiser, 1998). Bacterial bioluminescence is related with cell respiration, and any inhibition of cellular activity because of toxicant results in a decreased rate of respiration and a corresponding decrease in the rate of luminescence.

2. Aim of the study

The widespread use of OPPs for pest control poses a risk of contamination to aquatic and terrestrial environments. OPPs are transformed into degradation by-products both in biotic and abiotic processes. These compounds are toxic especially for the organisms in lower trophic levels. Toxicity assessments of both parent OPPs and their degradation by-products are necessary for safety consideration of OPPs applications.
The objective of this study is to investigate the toxicity of OPPs and their main degradation by-products; OPPs-oxons by using *Daphnia magna*, *Lepidium sativum* and *Vibrio fischeri*. For the trophic levels, the terrestrial plant *Lepidium sativum* was chosen as producer while representative for the consumers the crustacea *Daphnia magna* was selected. Representative for the decomposer was *Vibrio fischeri*. Conducted data in this study can be used for environmental risk assessment, and guide further use of pesticides correctly and appropriately.

3. Materials and methods

3.1 Chemicals

All chemicals used were of analytical grade. Chlorpyrifos, parathion, methyl parathion, and their oxon derivatives; chlorpyrifosoxon, paraoxon, methyl paraoxon were obtained from Accustandard Co. (USA). Stock solutions were prepared with dimethyl sulfoxide (DMSO) obtained from Merck (Palma et al., 2008). All solutions were stored in the dark at 4 ^{o}C. Working solutions were prepared by dilution of standard stock solution with distilled water.
Daphtoxkit was obtained from MicroBioTest Inc (SOP, 2009). The *Vibrio fischeri* bioassay used was LCK 480, obtained from Dr. Lange (ISO, 1998).

3.2 Bioassay tests

To determination of the toxicity of OPPs and their main degradation by-products; OPPs-oxons by using *Daphnia magna*, *Lepidium sativum* and *Vibrio fischeri*, three different biotests

from different trophic levels were chosen in this study. For the trophic level of producers the terrestrial plant *Lepidium sativum* were used. The microtox test using luminescent bacteria was employed as representative for the decomposers. As representatives of the primary consumers the crustacea *Daphnia magna* was used. Properties of the selected tests protocols are described in Table 2.

Test	Trophic level	Group of organisms/plants	Type of test	Test duration	Test criterion	Test principles
Microtox* (*Vibrio fischeri*)	Decomposer	Bacteria	Acute	30 min	Inhibition of luminescence	Measure of luminescence reduction with luminometer
Daphtox* (*Daphnia magna*)	Primary consumer	Crustaceans	Acute	48 h	Immobility/ Mortality	Counting of dead and alive crustacean
*Lepidium sativum***	Producer	Garden cress	Chronic	3 day	Root length	Measurement of root length

*Aquatic test, **Terrestrial test

Table 2. Properties of the selected ecotoxicological tests

Water blank analyses without adding pesticides but including the solvents were carried out for controlling solvent effect to the toxicity. Quality control tests with potassium dichromate for all of the tests were performed. These tests should be repeated regularly to check the correct execution of the test procedure and the good physiological condition of the test organisms.

3.2.1 *Lepidium sativum* toxicity test

Garden cress test with *Lepidium sativum* was carried out according to Devare & Bahadir (1994). Phytotoxicity of OPPs were assayed by adding 25 seeds of *Lepidium sativum* onto 90 mm of two filter papers placed in a petri dish filled with 5 mL of sample. In the experiment, for the control six replicates and for the test samples three replicates were carried out. The dishes were covered and incubated in darkness for 72 hours. The lengths of the roots were measured after 72 h exposure duration and the inhibition of the root growth in the test solutions were calculated in comparison to the control. *Lepidium sativum* and test pictures are given in Fig. 2

3.2.2 *Daphnia magna* toxicity test

The toxicity tests on *Daphnia magna* bioassay were performed following the standard operational procedures of the respective Daphtoxkit F™ toxkits microbiotest (Fig. 3).
Standard freshwater solution for *Daphnia magna* toxicity tests was prepared from salt solutions provided in the test kit and aerated prior to use. For hatching of the *Daphnia magna* ephippia, they were transferred into a petri dish with 15 mL pre-aerated standard freshwater. *Daphnia magna* were hatched from eggs (ephippia) for 72 hours under continuous illumination (11000 lux) at 20-22 °C. 2 h prior to the test, the neonates were fed with a suspension of Spirulina micro-algal. Different concentrations and a control in five

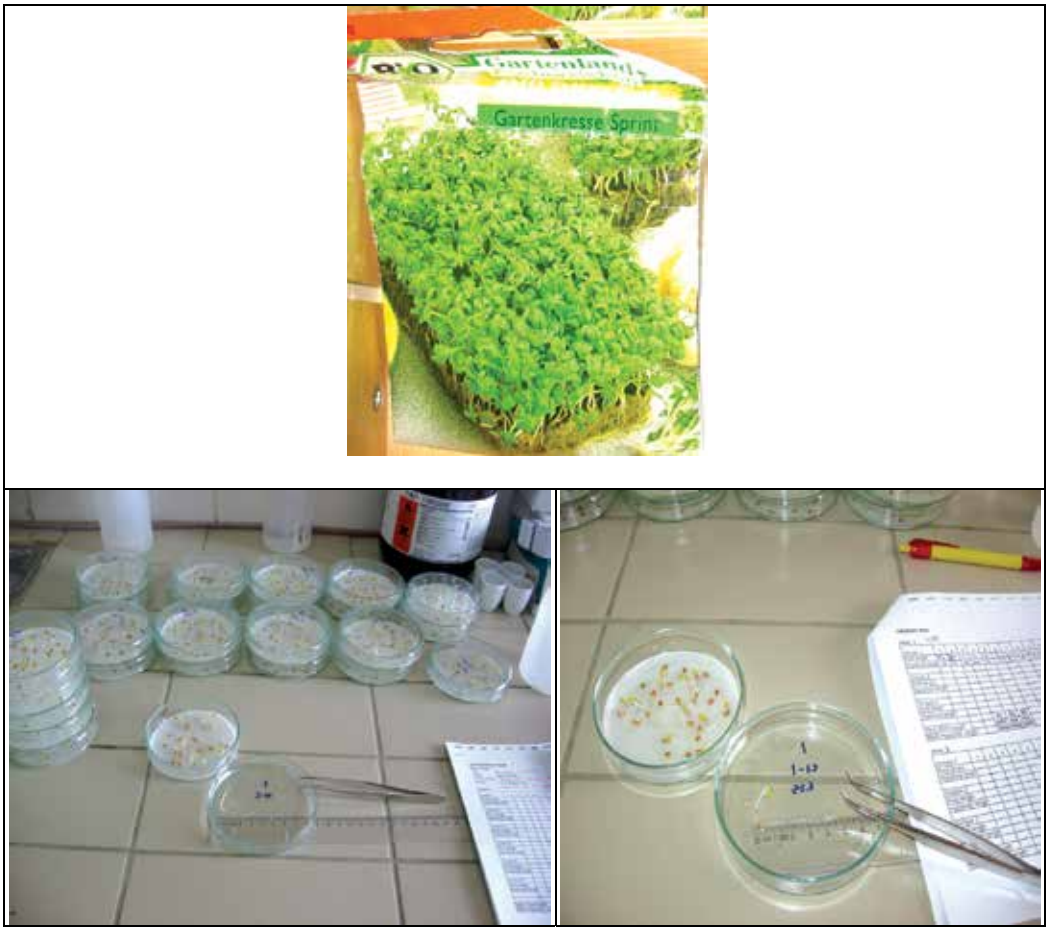

Fig. 2. *Lepidium sativum* and test pictures

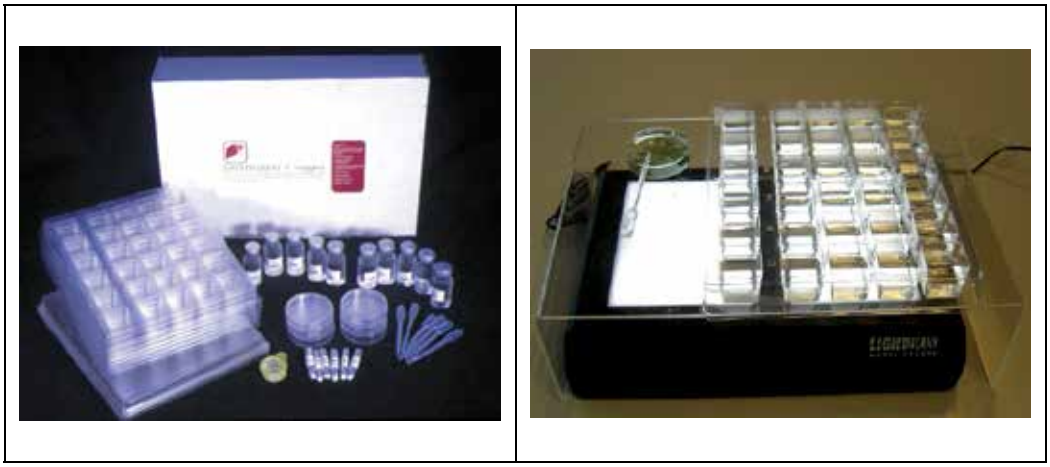

Fig. 3. Daphtoxkit pictures (www.microbiotests.be)

replicates were tested. Five *Daphnia magna* neonates were transferred into each cell Daphnids were exposed to samples of the tested agent for 48 h at a temperature of 20 °C in darkness. The immobile and dead daphnids were counted after 48 h of exposure. The percentage of dead and immobilized organisms and the EC_{50} values for samples were calculated.

3.2.3 *Vibrio fischeri* toxicity test

Luminescence bacteria test with *Vibrio fischeri* was carried out according to DIN/EN/ISO 11348-2 (Luminescent bacteria test LCK 482) measured with Dr. Lange LUMIStox 300 Luminometer (Fig. 4). The pH of the sample was adjusted to pH 7. Since *Vibrio fischeri* is a marine organism, an adjustment of the osmotic pressure of the samples was applied to obtain samples with 2% salinity, using a concentrated salt solution.

A dilution series of the sample was prepared directly in the glass cells according to DIN/EN/ISO 11348-2 and including the so called "G1" dilution of 80 %. Bioluminescence bacteria *Vibrio fischeri* were in freezedried form and activated prior to use by a reconstitution solution. For that, 12 mL reactivation solution for the luminescence bacteria was added into the reactivation tube and tempered to 15 °C for 30 min. Frozen luminescence bacteria were thawed in a water bath at room temperature for 2 min. 0.5 mL reactivation solution were added into the luminescence bacteria tube and tempered to 15 °C for 15 min.

Toxicity was assessed by measuring the inhibition of the luminescence bacteria after 30 min of incubation at 15 °C and the EC_{50} values and validation data were calculated according to DIN/EN/ISO 11348-2.

Fig. 4. Dr. Lange LUMIStox 300 Luminometer

3.3 EC_{50} and toxic unit

In order to assess the acute toxicity, test results are expressed in EC_{50}. EC_{50} values are the concentration responsible for the inhibition/mortality in 50% of the tested population in the different volumes of sample. The data expressed as EC_{50} were transformed into Toxic Units (TU) to reveal the direct relationship between toxic effects and the test system used. TU was calculated according to equation (1).

$$TU = \left[{}^{1}\!/_{EC_{50}} \right] x100 \quad (1)$$

4. Discussion

Toxicity of the reference compound, potassium dichromate was observed 0.9 mg L^{-1} for Daphtox with *Daphnia magna*, 4.1 mg L^{-1} for Microtox with *Vibrio fischeri*, 15 mg L^{-1} (root length) for *Lepidium sativum,* which is within the limits accepted by the ISO methods. According to their responses for reference tests, order of the trophic levels is the primary consumer, the decomposer and the producer, respectively.

The 72 h EC_{50} values of the OPPs and OPPs-oxons obtained for *Lepidium sativum, Daphnia magna* and *Vibrio fischeri* in our research were given in Table 3 and Table 4 respectively.

Lepidium sativum test results show that chlorpyrifosoxon, paraoxon and methyl paraoxon inhibit root growth. Negative inhibition effect was observed up to 50 mg L^{-1} concentration for parent compounds, so upper concentrations were not investigated. Straw et al. (1996) reported negative inhibition effect of chlorpyrifos on 2–4 year old Sitka spruce. Trees treated with chlorpyrifos showed a 25% increase in height growth and a 13% increase in side shoot extension growth after 2 years compared with control trees.

		Chlorpyrifos	Parathion	Methyl Parathion	Ref.
Lepidium sativum	EC_{50} (µg L^{-1})	n.d	n.d	n.d	In our study, 2010
	TU (µg L^{-1})	n.d	n.d	n.d	In our study, 2010
Daphnia magna	EC_{50} (µg L^{-1})	0.37	6.35	1.14	In our study, 2010
		0.74	-	-	Palma et al. (2008)
		-	2.2	-	Guilhermino et al. (1996)
	TU (µg L^{-1})	270	15	87	In our study, 2010
Vibrio fischeri	EC_{50} (µg L^{-1})	23190	12650	1187	In our study, 2010
		2840	-	-	Palma et al. (2008)
	TU (µg L^{-1})	0.004	0.008	0.08	In our study, 2010
WHO Class.	LD_{50} (mg kg^{-1})	Moderately hazardous 135	Extremely Hazadous 13	Extremely Hazadous 14	WHO (2005)

n.d. not determined

Table 3. EC_{50}, TU and LD_{50} values of the OPPs investigated against three tested organisms

According to toxic effects on root growth, toxicity order of the investigated compounds were paraoxon (EC_{50}: 0.634 mg L^{-1}), methyl paraoxon (EC_{50}: 1.599 mg L^{-1}) and chlorpyrifosoxon (EC_{50}: 2.048 mg L^{-1}) respectively. The toxicity of pesticides is investigated during their registration process, but the toxicity of their degradation products to the plant is unexplored. However, pesticides sprayed on the plant and soil surface are exposed to effect of microbial degradation and UV photons resulting with decomposition of the molecule, so inhibition effects of the transformation products should be noted.

		Chlorpyrifosoxon	Paraoxon	Methyl Paraoxon	Ref.
Lepidium sativum	EC_{50} (µg L^{-1})	2050	630	1600	In our study, 2010
	TU (µg L^{-1})	0.048	0.158	0.062	In our study, 2010
Daphnia magna	EC50 (µg L^{-1})	0.31	0.76	0.28	In our study, 2010
		-	0.2	-	Guilhermino et al. (1996)
	TU (µg L^{-1})	322	131	357	In our study, 2010
Vibrio fischeri	EC50 (µg L^{-1})	4380	4140	4404	In our study, 2010
	TU (µg L^{-1})	0.022	0.024	0.022	In our study, 2010

n.d. not determined

Table 4. EC_{50} and TU values of the OPPs-oxons investigated against three tested organisms

By comparing the results reached with the toxicological response of test organism *Daphnia magna*, toxicity order of the tested pesticides were chlorpyrifos (EC_{50}: 0.37 mg L^{-1}), methyl parathion (EC_{50}: 1.14 mg L^{-1}) and parathion (EC_{50}: 6.35 mg L^{-1}), from most to least. This order changes when oxon derivatives are investigated. Toxicity order of the tested OPPs-oxons is methyl paraoxon (EC_{50}: 0.28 mg L^{-1}), chlorpyrifosoxon (EC_{50}: 0.31 mg L^{-1}) and paraoxon (EC_{50}: 0.76 mg L^{-1}).

According to the acute toxicity classification system reported by Persoone et al. (2003), one can consider as class I (no acute toxicity, TU<0.4), class II (slightly acute toxicity, 0.4<TU<1), class III (acute toxicity, 1<TU<10), class IV (high acute toxicity, 10<TU<100), class V (very high acute toxicity, TU ≥100). Considering the toxic unit classification, chlorpyrifos is in class V (very high acute toxicity) for *Daphnia magna* and class I (no acute toxicity) for *Vibrio fischeri*. Parathion is in class IV (high acute toxicity) for *Daphnia magna and* class I (no acute toxicity) for *Vibrio fischeri*. Methyl parathion is similarly in class IV (high acute toxicity) for *Daphnia magna and* class I (no acute toxicity) for *Vibrio fischeri. Chlorpyrifosoxon, paraoxon and methl paraoxon are in class V* (very high acute toxicity) for *Daphnia magna* and in class I (no acute toxicity) for *Lepidium sativum* and *Vibrio fischeri.*

OPPs impairs the nerves function and consequently the normal mobility of organisms, which is the most frequent observed endpoint of the acute toxicity test with water fleas (Tisler et al., 2009). Neurotoxic effects in organism result with mortality.

In this study, mortality/immobility was the evaluated endpoint of the acute toxicity. Among parent OPPs chlorpyrifos was the most toxic insecticide to Daphnia magna. Guzella et. al. (1997) reported similar results in a study on acute toxicity of 11 OPPs to two marine invertebrates *Artemia sp.* and *Brachionus plicatilis*. Chlorpyrifos was the most toxic insecticide to both species while parathion and methyl parathion showed lower toxic responses. Palma et. al. (2009) reported developmental abnormality for *Daphnia magna* embryos exposed to a concentration of 0.01 μg L^{-1} chlorpyrifos. Besides, chlorpyrifos possesses an inhibitory effect on soil bacteria during the initial periods after its treatment (Xiaoqiang et al., 2008).

Evaluating the calculated EC_{50} values, acute effects of chlorpyrifos and chlorpyrifosoxon on survival parameters of *Daphnia magna* are very close to each other. On the other hand, paraoxon is nearly eight times and methyl paraoxon is nearly four times more toxic than their parental forms. Dzyadevych and Chovelon (2002) used Lumistox test for toxicity determination of methyl parathion and methyl paraoxon. The inhibition levels for methyl parathion and methyl paraoxon were roughly 2% and 25%, respectively.

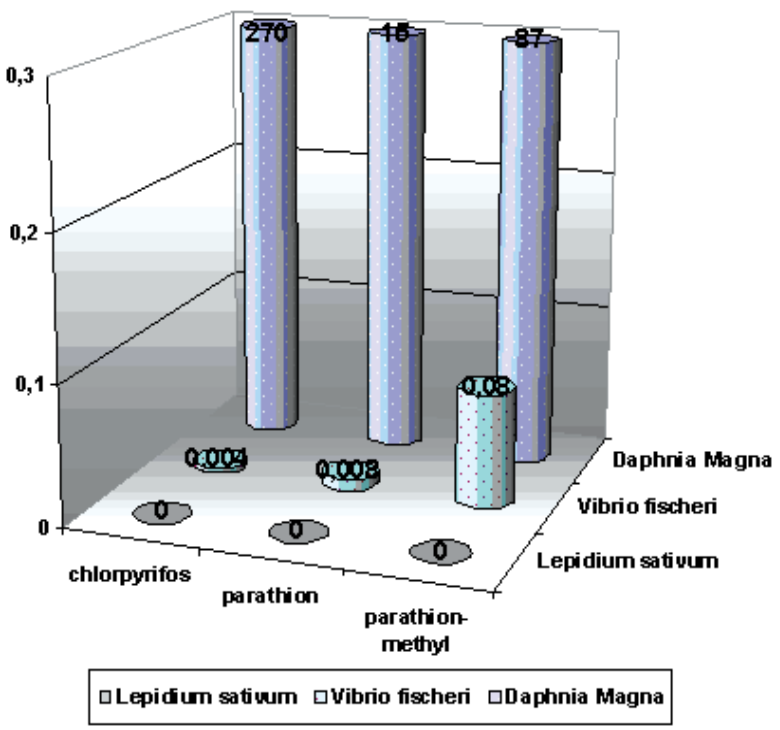

Fig. 5. TU values for test organisms; *Daphnia Magna, Lepidium sativum* and *Vibrio fischeri* in case of OPPs treatment

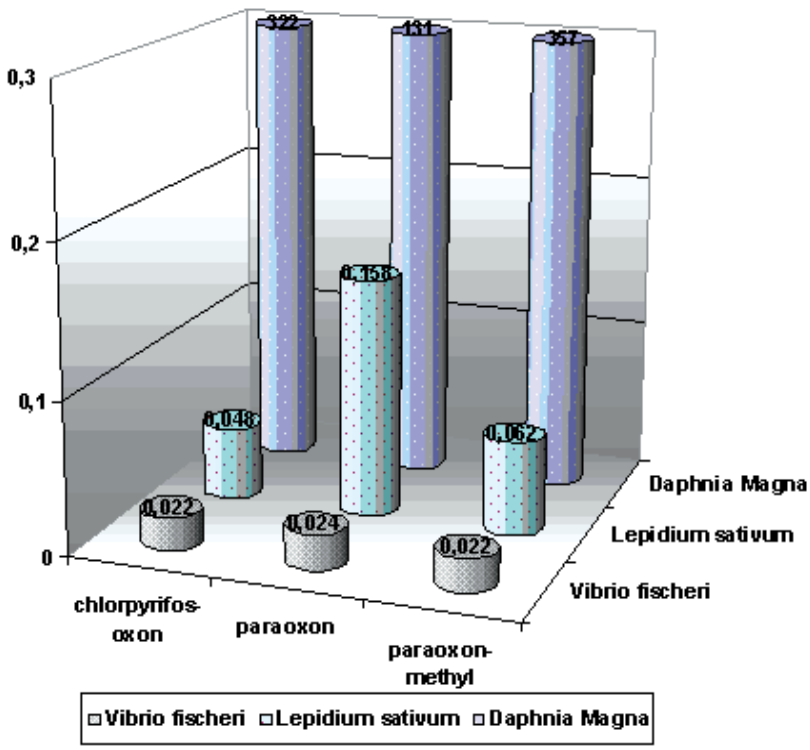

Fig. 6. TU values for test organisms; *Daphnia Magna, Lepidium sativum* and *Vibrio fischeri* in case of OPPs-oxons treatment

EC_{50} values are in ppm levels for *Daphnia magna* and OPPs can be detected in these levels in aquatic environments. TU values clearly demonstrate that *Daphnia magna* test organism is much more sensitive to OPPs and OPPs-oxons than *Vibrio fischeri* and *Lepidium sativum* and this difference is shown in Figure 5 and Figure 6. TU values for test organisms; *Daphnia Magna, Lepidium sativum* and *Vibrio fischeri* in case of OPPs treatment is given in Figure 5 and in case of OPPs-oxons treatment is given in Figure 6.

Oxon derivatives of chlorpyrifos, parathion and methyl parathion increase inhibition effect on *Daphnia magna* and *Vibrio fischeri* test organisms. For *Lepidium sativum* test organism, parent compounds did not show any inhibition effect up to 50 mg L^{-1} concentration, so TU values are accepted as zero. On the other hand OPPs-oxons inhibition effect on *Lepidium sativum* test organism is bigger than *Vibrio fischeri*.

EC_{50} values for *Daphnia magna* can also be used to understand toxic effects of analytes on other aquatic organisms. Daphnids are more sensitive to toxicants than fish; therefore, the EC_{50} values for *Daphnia magna* can be used as an upper threshold for fish tests (Zvinavashe et al., 2009).

There are studies using acetylcholinesterase (AChE) bioassays that rely on inhibiting AChE activity (Kralj et al., 2007). Oxon derivatives have higher AChE inhibition capacity, however AChE inhibition does not explain all the symptoms of OPPs intoxication. Dzyadevych and Chovelon (2002) reported that effect of methyl parathion and methyl paraoxon on the luminescent bacteria is not related to cholinesterase activity. Xuereb et al.(2009) investigated relations between whole-body acetylcholinesterase (AChE) inhibition and changes in locomotor behaviours in adult male *Gammarus fossarum* exposed to chlorpyriphos and reported that significant mortality was observed from 50% AChE inhibition This result suggests that, for chlorpyriphos, the observed mortality was not directly related to AChE inhibition but that an additional toxic mode of action occurred.

Biotransformation of OPPs to oxon derivatives in the body of the organism may also occur. Metabolic activation occurs by monooxygenase enzymes to form the active oxon analogues This metabolic activation occurs especially in crustacean species compared to fish and mollusc species (Takimoto et al., 1987). Some species have an elimination capacity. According to the results of the study conducted by Ashauer et al. (2006) accumulated chlorpyrifos in *Gammarus pulex* is rapidly eliminated, suggesting a biotransformation capacity in this species. This capacity of species may be used for bioremediation of contaminated environments. Cycon et al. (2009) used three bacterial strains *Serratia liquefaciens, Serratia marcescens and Pseudomonas sp* to determine their capacity to use in bioremediation of contaminated soil and reported that isolated bacterial strains may have potential for use in bioremediation of diazinon-contaminated soils.

Additional studies are required to test combined toxicity of OPPs and OPPs-oxons, since the compounds usually applied simultaneously or one after another for crop protection. According to the results of the study conducted by Xiaoqiang et al. (2008) the inhibitory effect of chlorpyrifos on soil microorganisms was increased by its combination with chlorothalonil.

Since biological techniques are inefficient for the treatment of pesticide containing wastewaters chemical water treatment processes are preferred. A number of different Advanced Oxidation Processes are widely used for water treatment for sanitation and oxidation purposes. In many cases total mineralization is not possible. OPPs transformation to more toxic products during the application of these processes make it essential to use more convenient treatment methods and measurement of toxicity. According to the results conducted in our study, *Daphnia magna* appears to be the most sensitive method.

5. References

Arredondo, E.S., Heredia, M.J.S, Garcia, E.R., Ochoa, I.H. & Vega, B.Q. (2008). Sperm chromatin alteration and DNA damage by methyl-parathion, chlorpyrifos and diazinon and their oxon metabolites in human spermatozoa. *Reprod. Toxicol*., 25, 55–460.

Ashauer, R., Boxall, A., Brown, C. (2006). Uptake and elimination of chlorpyrifos and pentachlorophenol into the freshwater amphipod Gammarus pulex. *Arch. Environ. Contam. Toxicol*., 51, 542–548.

Bai, Y., Zhou, L., Wang, J. (2006) Organophosphorus pesticide residues in market foods in Shaanxi area, China. *Food Chem.,* 98, 240–242.

Ballesteros, E., Parrado, M.J. (2004). Continuous solid-phase extraction and gas chromatographic determination of organophosphorus pesticides in natural and drinking waters. *J. Chromatogr. A,* 1029, 267–273.

Barata, C., Baird, D.J., Soares, A.M.V.M. & Guilhermino, L. (2001). Biochemical factors contributing to response variation among resistant and sensitive clones of Daphnia magna straus exposed to ethyl parathion. *Ecotoxicol. Environ. Saf,.* 49, 155–163.

Barcelo, D., Porte, C., Cid, J. & Albaiges, J. (1990). Determination of organophosphorus compounds in Mediterranean coastal waters and biota samples using gas chromatography with nitrogen–phosphorus and chemical ionization mass spectrometric detection. *Int J Environ Anal Chem.,* 38, 199–209.

Bolognesi, C. (2003). Genotoxicity of pesticides: a review of human biomonitoring studies. Mutat. Res., 543, 251–272.

Bondarenko, S., Gan, J., Haver, D.L., Kabashima, J.N. (2004). Persistence of selected organophosphate and carbamate insecticides in waters from a coastal watershed, *Environ. Toxicol. Chem.* 23, 2649–2654.

Burkepile, D.E., Moore, M.T., Holland, M.M. (2000). Susceptibility of five nontarget organisms to aqueous diazinon exposure. *Bull. Environ. Contam. Toxicol.* 64, 114–121.

Cakir, S., Sarikaya, R. (2005). Genotoxicity testing of some organophosphate insecticides in the Drosophila wing spot test, *Food and Chem. Toxicol.*, 43, 443–450.

Coupe, R. H., Manning, M. A., Foreman, W. T., Goolsby, D. A. & Majewski, M. S. (2000). Occurrence of pesticides in rain and air in urban and agricultural area of Mississippi, April–September 1995. *Sci. Total Environ.,* 248, 227–240.

Cycon, M., Wojcik, M., Seget, Z.P. (2009). Biodegradation of the organophosphorus insecticide diazinon by Serratia sp. and Pseudomonas sp. and their use in bioremediation of contaminated soil, *Chemosphere,* 76, 494–501.

Darko, G., Akoto, O. (2008). Dietary intake of organophosphorus pesticide residues through vegetables from Kumasi, Ghana *Food and Chem. Toxicol.* 46, 3703–3706.

Day, B., Walse, M.M., Sharma, J.M. & Andersen, D.E. (1995). Immunopathology of 8-week-old ring-necked pheasants (Phasianus colchicus) exposed to malathion. *Environ. Toxicol. Chem*., 14, 1719-1726.

Devare, M. & Bahadir M. (1994). Biological monitoring of landfill leachate using plants and luminescent bacteria. *Chemosphere,* 28, 261-271.

Dzyadevych, S.V. & Chovelon,J.M.A. (2002) Comparative photodegradation studies of methyl parathion by using Lumistox test and conductometric biosensor technique Mater. Sci. and Eng. C. 21, 55–60.

Guilhermino, L, Lopez, M.C., Carvalho, A.P. & Soared, A.M.V.M. (1996). Inhibition of acetylcholinesterase activity as effect criterion in acute tests with juvenile Daphnia Magna. *Chemosphere,* 32, 727-738.

Gul, A. (2005). Investigation of acute toxicity of chlorpyrifos-methyl on Nile tilapia (Oreochromis niloticus L.) larvae. *Chemosphere,* .59, 163–166.

Guzzella, L., Gronda, A., Colombo, L. (1997). Acute toxicity of organophosphorus insecticides to marine invertebrates. *Bull. Environ.Contam. Toxicol.*, 59, 313–320.

Hazardous Substances Data Bank (HSDB), Toxicology Data Network, 2010. http://toxnet.nlm.nih.gov/cgi-bin/sis/search

Jemec A., Drobne, D., Tisler, T., Trebse, P, Rosa, M. & Sepcic, K. (2007). The applicability of acetylcholinesterase and glutathione S-transferase in Daphnia magna toxicity test. *Comp. Biochem. Physiol. C*, 144, 303–309.

Kaiser, K.L.E. (1998). Correlations of *Vibrio fischeri* Bacteria Test Data with Bioassay data for Other Organisms. *Environ. Health Perspect.* 106, 583-591.

Kim, T.S., Kim, J.K., Choi, K., Stenstrom, M.K. & Zoh, K.D. (2006). Degradation mechanism and the toxicity assessment in TiO_2 photocatalysis and photolysis of parathion. *Chemosphere,* 62, 926–933.

Konstantinou, I.K., Hela, D.G. & Albanis, T.A. (2006). The status of pesticide pollution in surface waters (rivers and lakes) of Greece. Part I. Review on occurrence and levels. *Environ. Pollut.* 141, 555-570.

Kralj, M.B., Trebse, P. & Framno, M. (2007). Applications of bioanalytical techniques in evaluating advanced oxidation processes in pesticide degradation. *Trends Analyt Chem,* 26, 1020-1031.

Moser, B.R., Shah, S. N., Moser, J.K.W., Vaughn, S.F. & Evangelista, R.L. (2009). Composition and physical properties of cress (Lepidium sativum L.) and field pennycress (Thlaspi arvense L.) oils, *Ind Crops Prod,* 30, 199–205.

Pablo, F., Krassoia, F.R., Jones, P.R.F., Colville, A.E., Hose, G.C & Lim, R.P. (2008). Comparison of the fate and toxicity of chlorpyrifos—Laboratory versus a coastal mesocosm system. *Ecotoxicol. Environ. Saf.,*.71, 219–229.

Palma, P., Palma, V.L., Fernandes, R.M., Soares, A.M.V.M. & Barbosa, I.R. (2008). Acute Toxicity of Atrazine, Endosulfan Sulphate and Chlorpyrifos to Vibrio fischeri, Thamnocephalus platyurus and Daphnia magna, Relative to Their Concentrations in Surface Waters from the Alentejo Region of Portugal. *Bull Environ Contam Toxicol.,* 81, 485–489.

Palma, P., Palma, V.L., Fernandes, R.M., Bohn, A., Soares A.M.V.M. & Barbosa I.R. (2009). Embryo-toxic effects of environmental concentrations of chlorpyrifos on the crustacean Daphnia magna. *Ecotoxicol. Environ. Saf.*, 72, 1714-1718.

Persoone, G., Marsalek, B., Blinova, I., Törökne A., Zarina, D., Manusadzianas, L., Jawecki, G.N., Tofan, L., Stepanova, N., Tothova, L., Kolar, B. (2003) A practical and user-friendly toxicity classification system with microbiotests for natural waters and wastewaters, *Wiley InterScience,* 395-402.

Possamai, F.P., Foryunato, J.J., Feier, G., Agostinho, F.R., Quevedo, J., Filho, D.W., Pizzol, F.D. (2007). Oxidative stress after acute and sub-chronic malathion intoxication in wistar rats, *Environmental Toxicology and Pharmacology*, 23, 198–204.

ISO 11348-2, Water quality – Determination of the inhibitory effect of water samples on the light emission of *Vibrio fischeri* (Luminescent bacteria test), Part 2: Method using liquid-dried bacteria, Geneva, Switzerland: International Organisation for Standardisation, 1998.

Sparling D.W. & Fellers, G. (2007). Comparative toxicity of chlorpyrifos, diazinon, malathion and their oxon derivatives to larval Rana boylii. *Environ. Pollut*.,.147, 535-539.

Standart Operational Procedure (2009) Daphtoxkit F™ magna, Crustacean Toxicity Screening Test for Freshwater, Nazareth, Belgium.

Straw, N.A., Fielding, N.J., Waters, A. (1996). Phytotoxicity of insecticides used to control aphids on Sitka spruce, Picea sitchensis (Bong.) Carr. *Crop Protection*, 15, 451-459.

Takimoto, Y., Ohshima, M.,Miyamoto, J. (1987). Comparative metabolism of fenitrothion in aquatic organisms, III. Metabolism in the crustaceans, *Daphnia pulex* and *Palaemon paucidens*. *Ecotoxicol. Environ. Safe.* 13, 126–134.

Tisler, T, Jemec, A., Mozetic, B. & Trebse, P. (2009). Hazard identification of imidacloprid to aquatic environment. *Chemosphere,* 76, 907–914.

Tsuda, T., Kojima, M., Harada, H., Nakajima, A. & Aoki, S. (1997). Acute toxicity, accumulation and excretion of organophosphorous insecticides and their oxidation products in killifish, *Chemosphere,* 35, 939-949.

Tuduri, L., Harner, T., Blanchard, P., Li, Y.F., Poissant, L., Waite, D.T., Murphy, C., Bezler, W. (2006) A review of currently used pesticides (CUPs) in Canadian air and precipitation. Part 2: Regional information and perspectives, *Atmos. Environ.,* 40, 1579–1589.

Virag, D.,Naar, Z. & Kiss, A. (2007). Microbial Toxicity of Pesticide Derivatives Produced with UV-photodegradation. *Bull Environ Contam Toxicol.,* 79, 356–359.

WHO, 2005. The WHO Recommended Classification of Pesticides by Hazard http://www.who.int/ipcs/publications/pesticides_hazard/en/

Xiaoqiang, C., Hua, F., Xuedong, P., Xiao, W., Min, S., Bo, F. & Yunlong, Y. (2008). Degradation of chlorpyrifos alone and in combination with chlorothalonil and their effects on soil microbial populations. *J Environ. Sci.,* 20, 464–469.

Xuereb, B., Lefevre, E., Garric, J., Geffard, O. (2009). Acetylcholinesterase activity in Gammarus fossarum (Crustacea Amphipoda): Linking AChE inhibition and behavioural alteration, *Aquat. Toxicol.* 94, 114–122.

Ye, J., Zhao, M., Liu, J., Liu, W. (2010). Enantioselectivity in environmental risk assessment of modern chiral pesticides. *Environ. Pollut*., 158, 2371-2383.

Yeh, S.P., Sung, T.G., Chang, C.C., Cheng, W. & Kuo, C.M. (2005). Effects of an organophosphorus insecticide, trichlorfon, on hematological parameters of the giant freshwater prawn, Macrobrachium rosenbergii (de Man). *Aquaculture,* 243, 383–392.

Zhang, Y.H., Liu, S.S, Song, X.Q., Ge, H.L. (2008) Prediction for the mixture toxicity of six organophosphorus pesticides to the luminescent bacterium Q67, *Ecotox. Environ. Safe.,* 71, 880–888.

Zhou, S., Duan C., Fu, H., Chen, Y., Wang, X. & Yu, Z. (2007). Toxicity assessment for chlorpyrifos-contaminated soil with three different earthworm test methods. *J Environ Sci (China),* 19, 854–858.

Zvinavashe, E., Du, T., Griff, T., Hans H.J. Berg, V.D., Soffers, A.E.M.F., Vervoort J., Murk, A.J., Rietjens, I.M.C.M. (2009) Quantitative structure-activity relationship modeling of the toxicity of organothiophosphate pesticides to Daphnia magna and Cyprinus carpio, *Chemosphere*, 75, 1531–1538.

14

Novel Approaches in Genetic Toxicology of Pesticides Applying Fluorescent in Situ Hybridization Technique

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1. Introduction

The use of chemical substances in the pest control has been known since ancient times. Records have been found indicating that 2.500 years BC ancient Sumerians applied sulphur in mite control (Price, 1973). In ancient China, some 1.200 years BC inorganic mercury and arsenic compounds have been used in lice and bug treatment (Smith & Kennedy, 2002). First records of primitive fungicide and rodenticide use reach back to the time of Roman Empire where copper compounds were used in plant protection against moulds, and hellebore (lat. *Helleborus nigra*) containing poisonous baits against mice and rats (Smith & Secoy, 1975). Pyrethrium, which has remained in use as insecticide till nowadays, and is derived from *Chrysanthemum cinerariaefolium* flowers was brought to Europe from Persia by Crusaders (Wandahwa et al., 1996). However, the real revolution in use of chemicals in pest management started in early 1940-ties with the discovery of insecticidal properties of DDT by Paul Müller and the beginning of its massive production. Within next decade pesticidal properties of various synthetic chemical compounds had been discovered (hexachlorocyclohexane, 2,4-D, dithiocarbamates, chloradane, organophosphorous compounds, etc.) and they have been introduced in agricultural practice and household pest control (Ware & Whitacre, 2004). In the year 2009, more than 1.500 different active ingredients in more than 2.500 formulations were present in the world market (BCPC, 2009) with annual use exceeding 1.000.000 tones on global scale.

It was in 1962, after the publication of three parts serial in The New Yorker magazine and book entitled „Silent Spring" by Rachel Carson, when the general public became aware of possible adverse effects of unsustainable use of pesticides. The book has been based on records of the adverse effects of DDT on birds reproductive system and deaths of adults resulting from pesticide exposure. Furthermore, Carson pointed out the potential of pesticides to circulate within the ecosystems, accumulate within the organisms and affect all links in the food chain including humans (Carson, 2002). It has been ten years later when Environmental Protection Agency (EPA) estimated that evidences of adverse effect of DDT to the environment are sufficient to ban its use in the USA. Soon its prohibition was extended to European countries.

2. Carcinogenic risk of long-term pesticide exposure

Acute effects of pesticide poisoning are being recorded since their commercial use began (Green, 1949). However, more concern has been raised regarding the adverse effects that

long-term exposure to pesticides might have on human organism, specifically genomic material. The damage may be accumulating over the years without any noticeable health effects, but silently mediating cancer development. First studies indicating connection between exposure to arsenic insecticides in regular application and increased incidence of melanoma and bronchial cancer among European grape growing farmers appeared in late 1960-ties (Jungmann, 1966). However, since carcinogenic potential of arsenic had been documented already at the beginning of 20th century, these epidemiological studies did not raise much concern. In 1968, Klayman published first article suggesting association between chemically synthesized active ingredients and risk of carcinoma. The author reported several cases of larynx carcinoma in "never-smoking, never- or rarely-alcohol drinking" men with the record of more than 10 years of exposure to insecticides malathion or lindane working in either greenhouse or landscape (Klayman, 1968). Later, a case report indicating connection between lindane exposure and leukemia has been published (Hoshizaki et al., 1969). First more complex descriptive epidemiological studies aiming to evaluate carcinogenic risk arising due to occupational exposure to pesticides started in middle 1970-ties. In a study comprising the railroad workers applying herbicides amitrol or phenoxy acids, elevated occurrence of tumor deaths from stomach and lung cancer was observed (Axelson et al., 1974). With advances in knowledge on carcinogenesis it became evident that many life-style factors may elevate the risk of developing neoplasia by inducing genome damage. The profound effect on human cancer risk of smoking habits, alcohol consumption, diet, diagnostic procedures involving ionizing radiation or ultrasound, some medications, exposure to dyes and solvents may additionally pronounce or even prevail over the effect of pesticide exposure. Thus, beside simple recording of the type of pesticide exposure, all possible confounding factors should be considered in statistical analysis of data obtained by the study. Furthermore, the study should include population of individuals without any record of pesticide exposure that matches examinees by age, residence region, and life-style factors (Becher, 2005). In descriptive epidemiological studies there are three major epidemiological measures of cancer risk that are calculated. 1) Risk ratio or relative risk (RR) is the ratio of percentages of individuals developing cancer among the examinees and controls. If RR is above 1 then exposure increases the risk. 2) Odds ratio (OR) representing the ratio of the odd of cancer for the examinees and control. 3) Confidence interval (CI) represents sampling error inherent thus, statistical uncertainty of extrapolating the risk observed at the level of study group to the true population. These epidemiological measures should be adjusted for potential confounders by using Mantel-Haenszel estimation, regression methods (Cox, Poisson, multiple), or fractional polynomials. To overcome the problem of reduced statistical power in studies with small sample sizes meta-analysis is performed. It combines the results of several studies that address a set of related research hypotheses by using a form of meta-regression models. Additionally, to deduce the exact contribution of pesticides to the observed incidence of malignant disease multivariate approach in comparison between exposed and control group should be applied taking into consideration all confounding factors (e.g. MANOVA with post hoc comparison). Multiple regression analysis will identify the effect of each of confounding factors on the observed effect and canonical correlations analysis could be helpful (Ahrens et al., 2005). However, most of the individuals comprised by epidemiological studies are subjected to multiple pesticide exposures making impossible to separate the contribution of each individual pesticide to observed adverse health effect. Nevertheless, based on results of epidemiological studies, higher risk from developing neoplasia has been suspected for long-

term exposure to EPTC, pendimethalin, aldicarb, alachlor, chlorpyrifos, cyanazine, carbofuran, glyphosate and others (Dich et al., 1997). Short overview of indicated associations between pesticide exposures and increased risk of developing neoplasia published within last 3 years is presented in Table 1.

Exposure	Population	Cancer type/site	OR/RR (95% CI)	Reference
EPTC	Farmers	Colon	RR 2.09 (1.26–3.47)	van Bemmel et al., 2008
Trifluralin	Farmers	Colon	RR 1.76 (1.05–2.95)	Kang et al., 2008
Phenoxy herbicides	Plant workers	Myeloid leukemia	OR 6.99 (1.96–24.90)	van Maele-Fabry et al., 2008
EPTC	Farmers	Pancreas	OR 1.8 (1.0–3.3)	Andreotti et al., 2009
Pendimethalin			OR 1.7 (0.8–3.3)	
Metribuzin	Farmers	NHL	RR 2.42 (0.82-7.19)	DeLancey et al., 2009
Butylate	Farmers	Prostate	RR 2.09 (1.27–3.44)	Lynch et al., 2009
		NHL	RR 3.44 (1.29–9.21)	
Triazole fungicides	Farmers	HL	OR 8.4 (2.2–32.4)	Orsi et al., 2009
Urea herbicides			OR 10.8 (2.4–48.1)	
Organochlorines		Hairy cell leukemia	OR 4.9 (1.1–21.2)	
Phenoxy herbicides			OR 4.1 (1.1–15.5)	
Organophospha-tes	Residentials	Acute lymphoblastic leukemia	OR 2.5 (0.4-14.8)	Rull et al., 2009
Triazines			OR 4.1 (1.5-11.1)	
Permethrin	Farmers	Multiple myeloma	RR 5.72 (2.76–11.87)	Rusiecki et al., 2009
Terbufos	Farmers	Leukemia	RR 2.38 (1.35-4.21)	Bonner et al., 2010
		NHL	RR 1.94 (1.16-3.22)	
		Lung	RR 1.45 (0.95-2.22)	
Phenoxy herbicides	Plant workers	Urinary cancers	RR 4.20 (0.99-17.89)	Boers et al., 2010
		Genital cancers	RR 2.93 (0.61-14.15)	

Table 1. Most recent studies indicating association between pesticide exposure and cancer.

However, there are certain shortcomings of epidemiological studies that may lead to a possible bias in estimation of exposure risk and which results in studies reporting inconsistent results due to exposure to a specific pesticide (Eastmond & Balakrishnan, 2001). Due to poorly defined exposure levels, combinatorial exposure to other potentially carcinogenic agents, small study groups, or maladjustment of data for possible confounders, regulatory agencies and organizations authorized for carcinogenic classification of chemicals consider most of the reported associations of pesticide exposure with risk of cancer inconclusive. Because of these, in addition to epidemiological studies they turn to the results of long- and short-term animal and in vitro assays. Consequently, International Agency for Research on Cancer (IARC) and Environmental Protection Agency (EPA) have recognized

less than 10 active ingredients as proved human carcinogens. Yet, many substances have been assigned as likely to be carcinogenic to humans or with suggestive evidence for carcinogenic potential.

2.1 Relevance of epidemiological studies: pro and con

There is much controversy about the relevance of epidemiological studies in risk assessment of pesticide exposure. Since individuals occupationally or residentially exposed to pesticides are simultaneously affected by several substances it is not possible to determine contribution of the specific agrochemical to the observed health effect. As already mentioned this issue has been recognized by regulatory agencies. Their decisions mostly rely on surrogate short- and long-term testing performed under controlled laboratory conditions, and results of epidemiological studies may provide supportive information. There are potential endogenous confounding factors such as gender, age, genetic polymorphism, and exogenous such as smoking, alcohol intake, medications that may significantly influence observed adverse health effect and have to be considered in analysis and interpretation of the results (Anderson, 2000). Additional bias in estimation of risk may evolve due to poorly described exposure conditions, inconsistent level of exposure with time, lack of air quality measurements and data on personal protective equipment usage. Also, lot of skepticism has been raised about the value of short-term genotoxicity tests. As the results of acute testing had accumulated over the past years less and less correlation with results of chronic carcinogenicity tests on rodents has been observed (Casciano, 2000). Furthermore, though testing of active substances under strictly controlled conditions using different cell lines may provide valuable knowledge on adverse effect toward human genome, interpretation of obtained results is challenging. There is the likelihood of (a) false positive results mediated by rather high concentrations tested (which may also lead to increase in osmolality), pH value decline, or (b) false negative results mediated by neglecting the need for metabolic transformation or by testing highly pure active ingredients. The latest may also diminish the relevance of results obtained on animal models. Moreover, the results of carcinogenicity testing obtained on animals may not always univocally be extrapolated on humans since there are some distinctive differences in metabolic pathways between animals and humans. That may be crucial in activation of carcinogenic potential of substance of concern as it was reported for insecticide carbosulfan (Abass et al., 2009) and also raised controversy over saccharine risk assessment (Chappel, 1992). Major discrepancy between results obtained by using different short- and long-term experiments on both cell and animal models, and effects occurring in real-case exposures arises from the fact that conditions used in laboratory may differ significantly from those likely to be encountered in occupational and residential exposure to pesticides. By using active ingredients of high purity in experimental evaluations two important factors that occur in real exposure of humans to pesticide are completely omitted:

a. possible effect of impurities contained within pesticide formulations that are byproducts of active substance synthesis,
b. possible effect of "inert" ingredients (solvents, potentiators, surfactants, emulsifiers, stabilizers) as standard components of pesticide formulations.

There are several examples of contaminants with adverse effect on human genome exceeding those of active ingredients in whose synthesis they are by-produced. Low levels of 2,3,7,8-tetrachlorodibenzo-para-dioxin (TCDD) known as a potent human carcinogen, are

present in herbicide formulations containing chlorophenoxyacetic acids (Eastmond & Balakrishnan, 2001). Carbendazim may be found in sulphur pesticide formulations that are approved for application in organic agriculture (Balayiannis et al., 2009). It further contains 2,3 diaminophenazine (DAP) that is suspected to be responsible for carbendazim's carcinogenic effect in mice. Malaoxon impurities contained within malathion formulations are associated with its genotoxic effect (Blasiak et al., 1999) while pyrethroids contain contaminants that exhibit more sever toxic effects than active ingredient itself (Hadnagy et al., 1999). Inert ingredients are routinely added to pesticide formulation in order to facilitate the application and to secure rapid and efficient transport of the active ingredient to the target site within the pest organism. Organic solvents used as inert ingredients have been associated with elevated risk of non-Hodgkin's lymphoma (Blair & Zahm, 2008). It has been shown that toxic effects of herbicide formulations with atrazine, glyphosate, and fungicide vinclozoline may be increased by present inert ingredients (Cox & Surgan, 2006).
Consequently, as stated by Hill (2010), since detecting the effect of multiple chemical exposure epidemiological studies provide us with limited knowledge on the genotoxic mechanism of a single pesticide. However, their importance lies in the fact that they look directly at human risk in situ, and estimate impact of a specific exposure type on cytogenetic status of the population in question. Epidemiological studies of pesticide exposure and experimental evaluations mutually supplement each other, and both approaches represent inevitable segments of risk assessment mosaic of exposure to pesticide.

3. Genetic toxicology

Findings that cancer is triggered and promoted by occurrence and accumulation of genome damage induced by a series of physical and chemical agents from residential and occupational environment, genetic toxicology as a subfield of toxicology developed. Pioneer assays aiming to detect changes at the level of cell genome as the consequence of exposure to pesticides were conducted in 1970-ies. Though Kaszubiak (1968) was first to isolate Rhizobium mutants from the cultures treated with herbicides linuron, dinoseb, and dimethylurea, the first experiment intended to evaluate mutagenic effect was conducted in 1970 and it gave positive results for herbicide 3',4'-dichloropropionanilide (Prasad, 1970). The exponential growth of genotoxicity testing occurred in the middle of 1970-ties with the introduction of Ames test on mutant strains of bacterium *Salmonella typhimurium* and mouse lymphoma assay (MLA) on L5178Y cell line. Within a decade more than 100 genotoxicity tests for evaluation of genetic potential of chemicals emerged. Since most of them were flawed in providing results relevant for carcinogenic risk assessment they gradually disappeared (Casciano, 2000). The term "genetic toxicology" appeared already in 1975 in the title of the review paper of Legator & Zimmering (1975). This toxicological discipline aims to:

a. identify biomarkers of the exposure or effect at the level of the cell genome that would be affected by exposure to carcinogen in a dose-response manner and would highly correlate with risk of cancer development, and
b. interpret obtained results to deduce the mechanism of chemical-genome interaction, role of the observed effects in carcinogenesis induction, and their impact on human health.

Adverse effects that are subject of study of genetic toxicology arise at the exposure levels far beyond the concentrations that would induce observable toxic effects on cells, organs or

organism, which is somehow in disagreement with other fields of toxicology. These effects could even hardly be classified as toxic since they do not have any short-term impact on human health, and in most cases they are efficiently repaired. Nevertheless, under conditions of long-term exposure their occurrence may prevail over the repair or they may be misrepaired. Accumulation of these genomic lesions may induce cell transformation, immortalization and neoplastic growth.

Nowadays less than 15 assays are commonly applied in biomonitoring and even less are officially accepted for regulatory genotoxicity testing. Basically, assays applied in genetic toxicology may be classified in three major groups:

a. assays detecting mutagenic potential (Ames test, MLA, Drosophilla wing-spot test, mouse dominant lethal assay)
b. assays detecting genotoxic potential (micronucleus assay, structural chromosomal aberration analysis, sister chromatid exchange assay)
c. assays detecting nonspecific primary DNA lesions (alkaline comet assay).

Over the last decade, chromosomal aberration analysis, micronucleus, and comet assay have emerged as most reliable in evaluation of genotoxic effect of human exposure to pesticides. Among them, chromosomal aberrations and micronuclei as biomarkers of the effect were also proved to be good predictors in cancer risk assessment (Fenech, 2007; Rossi et al., 2009).

3.1 Chromosomal aberration analysis

Alterations in chromosome structure as the consequence of exposure to external agents has been known for more than 50 years. Their occasional application in health surveillance programs of individuals occupationally exposed to potential carcinogens started in 1960-ies. Soon structural chromosomal alterations in peripheral blood lymphocytes have been accepted as surrogate effect that reflects events triggered in the precursor cells for carcinogenesis under the exposure conditions of the issue (Hagmar et al., 2000).

Formation of structural aberrations of chromosomes is a rather complex event, involving DNA replication process in S phase of the cell-cycle, and misrepair of induced DNA strand breaks in post-replication phases. Under physiological conditions the lymphocytes are mainly in "resting" G_0 phase. Most of the genomic lesions induced by chemical agents will be efficiently repaired, especially if they occurred in transcriptionally active regions of chromosomes. Nevertheless, unrepaired lesions will interfere with DNA replication process forming DNA strand breaks thus, chromatid breaks, but also chromosome breaks which may result from DNA breaks due to additional topoisomerase II impairment (Maynard et al., 2009). Post-replicate repair mechanisms, through specific error prone pathways may convert chromosome breaks that occurred within "rejoining distance" into more complex rearrangements in chromosome structure such as chromatid exchanges and dicentric chromosomes (Obe et al., 2002). Insect chemosterilants tepa and apholate were first pesticides proved to affect morphology of human chromosomes in 1968 (Chang & Klassen, 1968). They were followed by insecticides propane sultone, aldicarb, malathion, fungicides ziram, thiram, herbicides 2,4-D, symazine and others.

Due to their good correlation with the level of exposure to chemicals in dose-dependent manner, structural chromosomal aberrations have been accepted as valuable cytogenetic biomarker of effect in epidemiological studies and risk assessment. Additional support of their application in human biomonitoring lies in their predictive value in cancer epidemiology – elevated frequency of aberrations indicates a population in increased cancer risk (Rossner et al., 2005).

3.2 Micronucleus assay

Within the last 5 years micronucleus assay has been recognized as most reliable and efficient cytogenetic test in detection of potential carcinogens. It took over the primacy of being the most relevant biomarker of effect from chromosomal aberrations (Cavallo et al., 2009). Micronuclei as manifestations of adverse effect of physical and chemical agents on cell genome have been known for more than 40 years (Matter & Schmid, 1971). They are small chromatin structures visible in cytoplasm of interphase cells, with maximum of 1/3 of nuclear diameter in size.

Micronuclei originate from:

a. chromosomal fragments formed as the result of induced DNA strand breaks (as discussed in 3.1) that lagged in anaphase for not possessing the centromere to be attached to mitotic spindle and pulled to one of the mitotic poles, or
b. whole chromosomes that lagged in the anaphase due to spindle or kinetochore protein damage and that remained unsegregated (Fenech, 2007).

Following mitosis, one of the newly formed cells will be deficient in the genetic information within the lagged chromosome/fragment, while the other micronuclei containing cell will be in surplus. Micronucleus assay owes its preference over chromosomal aberration analysis to the ability to detect two different mechanisms of genotoxicity:

a. clastogenic mechanism meaning direct interaction of genotoxic agent with DNA molecule. It mostly results in micronuclei harboring chromosomal fragments
b. aneugenic mechanism meaning interaction of genotoxins with mitotic spindle proteins not DNA molecule itself, leading to genomic instability by loss and malsegregation of chromosomes. It results in micronuclei harboring whole chromosomes (Muller et al., 2008).

Additional efficiency of micronucleus assay in detecting genotoxic chemical has been gained by implementation of scoring criteria proposed by HUman MicroNucleus (HUMN) project group (Fenech et al., 2003). Beside micronuclei (MN), other aberrant chromatin structures such as nuclear buds (NB) and nucleoplasmic bridges (NPB) are considered in evaluating genotoxic potential. NBs are chromatin structures abserved as nuclei extensions. They are formed in the process of elimination of (a) genomic regions harboring the genes related to metabolism of or resistance to exogenous chemical substance that have been amplified due to chronic exposure, or (b) DNA-repair complexes. NPBs, as chromatin structures connecting newly formed nuclei in telophase, are mostly manifestations of dicentric chromosomes, telomeric fusion of chromosomes, or union of sister chromatids (Fenech, 2007). In 1974, metepa was one of the first pesticides reported to induce micronuclei formation (Richardson, 1974). Soon, potential for micronuclei induction was reported for herbicidal phenylalkylureas, fungicide thiram, insecticide malathion etc.

Hence, micronuclei formation has been accepted as valuable cytogenetic biomarker of exposure to chemicals and there are ever more scientific evidences of its possible applicativity in epidemiological studies estimating the cancer risk of exposure to chemicals (Fenech, 2007; El-Zein et al., 2008).

3.3 Fluorescent in situ hybridization

In 1969 for the first time hybridization of small radioactively labeled RNA fragments has been successfully applied in microscopic localization of specific genes (Buongiorno-Nardelli & Amaldi, 1969). Some 20 years later, RNA molecules have been replaced with DNA probes, and radioactive labeling with antigen labeling and immunocytochemical detection of

sequences. Finally, when in early 1990-ties a fluorochrome labeled DNA probe has been produced, fluorescent in situ hybridization (FISH) as a powerful cytogenetic technique with high potential of discovering new biomarkers of effect in epidemiological studies of exposure to carcinogens was introduced. Basically, FISH technique enables visualization of specific genes, chromosome regions (centromeres, telomeres) or whole chromosomes within the cell genome in both, interphase nuclei and metaphase chromosomes. Thus, it provides us with the information regarding

a. the copy number of a specific gene or chromosome in detection of aneuploidy that may arise as the result of chromosome malsegregation or loss of broken fragments in exposure to genotoxic chemical,
b. the chromosome regions, or a specific chromosome harbored by micronuclei,
c. the occurrence of translocations as stable chromosomal rearrangements (Raap, 1998).

First attempts to categorize content of micronuclei occurred in 1990 (Becker et al., 1990). Several years later began its occasional application in human biomonitoring (Titenko-Holland et al., 1994), but it has not been before 2003 that micronuclei content has been indicated as a valuable parameter in characterization of the effect of exposure to chemicals on human genome (Norppa & Falck, 2003). The presence of whole chromosomes in micronuclei may be detected by anti-kinetochore antibodies that will target kinetochore proteins in centromeric region. However, it has been deduced that many micronucleated chromosomes may possess disrupted kinetochore or it may be detached thus, exhibiting no signal following immunocytochemical detection. Therefore detection of pan-centromeric regions within the micronuclei by hybridization of fluorescent DNA probe will render more relevant results in determining the micronuclei content. A year after the first application of pan-centromeric FISH to analyze the content of micronucleus in evaluation of the effect of exposure to pesticides was reported, and three years later according to the HUMN recommendations other aberrant chromatin structures in relation to pesticide exposure have been categorized regarding the centromere content.

Translocations are persistent alteration in chromosome morphology that significantly affect genome integrity. They are of similar ethiology as dicentric chromosomes, originating from double strand DNA breaks (DSB) that are misrepaired mostly by non-homologous end joining (NHEJ) and ectopic homologous repair. Translocations are considered as most valuable biomarker in cancer risk assessment (Obe et al., 2002). High translocation frequencies have been observed in all tumor cells, some type of them being highly associated with a specific type of cancer thus, being etiologic for the neoplasia in question (Mitelman et al., 1997). Due to their high correlation with risk of developing cancer, translocations are considered as a valuable cytogenetic biomarker of effect in evaluating human exposure to genotoxic agents. Their application in biomonitoring began in early 1990-ties, but it has been restricted to studies of exposure to ionizing radiation (Tucker et al., 1993). Several years later, Steenland et al. (1997) introduced application of translocations as biomarkers of effect in occupational exposure to pesticides by evaluating the effect of ethylenebis(dithiocarbamate) (EBDC) fungicides. However, until 2009 no further use of chromosome painting by FISH in evaluation of pesticide genotoxicity has been reported. The application of FISH technique in translocation analysis and in revealing the content of aberrant chromatin structures provides us with more precise knowledge regarding the extent of genome affected by pesticide-induced damage and its relevance to carcinogenicity.

4. FISH in genetic toxicology of pesticides

4.1 Background epidemiological studies

Concept of using structural chromosomal aberrations in peripheral blood lymphocytes in cytogenetic biomonitoring of subjects occupationally or residentially exposed to potential carcinogens has been based on the finding that level of genetic damage in lymphocytes reflects the effects occurring in precancer cells of target tissue. Until the late 1990-ties it has been generally accepted that occupational exposure to chemicals may influence chromosome structure mostly by inducing chromatid-type of aberrations such as gaps and chromatid breaks. It has been assumed that chromosome breaks may also occur but at much lower frequency than chromatid breaks, while more complex rearrangements as dicentric and ring chromosomes have been considered as cytogenetic biomarkers of exposure to ionizing radiation or a small group of radiomimetic chemicals (e.g. antineoplastic drug irinotecan) which interact with DNA in pathways that resembles the one of ionizing radiation (IAEA, 2001). Accordingly, epidemiological studies evaluating cytogenetic effects of occupational exposure to pesticides have reported increased level of alterations in chromosome morphology, but chromosome breaks being the most serious reported lesion. As discussed in section 2.1, all such studies published within last 2 decades comprised groups of examinees in multiple pesticide exposure. Significant effect on induction of chromatid and chromosome breaks in farmers has been published by Hoyos et al. (1996) due to exposure to mixture of dithiocarbamates, carbamates and organophosphates, by Garry et al. (1996) in applicators of broad spectrum of insecticides and herbicides, by Antonucci & de Syllos Cólus (2000) in applicators of organophosphates, carbamates, and some herbicides (Mann-Whitney *U*-test, $P<0.05$). Although Hoyos et al. (1996) presented detailed information regarding exposure conditions and adjusted their statistical analysis for confounders, none of that has been done by Garry et al. (1996) and Antonucci & de Syllos Cólus (2000). In the later study on chlorophenoxy herbicide applicators and exposed foresters Garry et al. (2001) showed significant increase in chromatid breaks but only in lymphocytes of applicators using more than 3,785 liters of herbicides per season (Wilcoxon rank-sum test, $P=0.017$). Again, no adjustment for confounders has been done. Conversely, Lander et al., (2000) analyzed results using a multiple log-linear Poisson regression model for smoking, age, and coffein intake as possible confounders. The authors monitored chromosomal aberrations in greenhouse workers exposed to residues of 10 different insecticides, 6 herbicides, and 3 growth regulators prior to and after the spraying season. Number of cells harboring structural chromosomal aberrations significantly increased after the spraying season ($P=0.05$). However, among all recorded structural alterations the effect has been significant only for chromatid gaps ($P=0.001$), and most prominent increase has been observed for non-glove using smokers ($P=0.04$). In spite of excellent study design, aberration nomenclature used by the authors does not follow the IAEA (2001) recommendations nor has been elaborated, which bias comparison of results with those reported in previously discussed studies.

Studies reporting complex rearrangements are summarized in Table 2. Contrary to previously cited papers, Kourakis et al. (1996) were first to report the presence of dicentric and even ring chromosomes in the absence of significant increase in number of chromatid-type aberrations, in group of both, outdoor and greenhouse spraying workers exposed to complex mixture of pesticides (organophosphoric and organochlorinic compounds, carbamates, dithiocarbamates). However, occurrence of complex alterations was not

statistically significant. Since the workers did not use any personal protection equipment the complex cytogenetic effects may be attributed to high level of exposure due to direct pesticide intake by inhalation, through the skin and eye contact. Non-significant presence of complex structural alterations accompanied by significantly increased chromatid breaks was observed in vineyard growers mostly exposed to insecticide diazinon and fungicide dithiocarbamate (Joksic et al., 1997). Unfortunately, though results were presented at the level of subgroups regarding smoking habits and gender, in both studies statistics has been done without considering life-style factors as possible confounders. Although Amr (1999) did report significant increase of dicentrics (Student's t-test, $P<0.001$) among pesticide handling workers exposed mostly to mix of DDT, chlorinated hydrocarbons, organophosphates, pyrethroids and carbamates, due to major lack of data regarding the group characteristics, and inclusion criteria, reported results are considered inconclusive. For instance, of 300 included examinees that were subjected to biochemical analyses and medical diagnostic procedures, only 30 of them were chosen for chromosomal aberration analysis without any explanation of criteria for selecting them. Furthermore, most of the examinees had medical history of suffering from various disorders. Again, no multivariate analysis of obtained data has been applied. Other study reporting elevated frequency of unstable chromosome rearrangements comprised pesticide plant workers primarily exposed to atrazine and cyanazine, with minor exposure to alachlor, 2,4-D, and malathion (Zeljezic & Garaj Vrhovac, 2001). Production of pesticides was organized seasonally and workers have been exposed to pesticides during 8 months of their production. Until next production season they were transferred to the working places out of the exposure zone. For the first time it has been reported that following exposure period, beside chromatid and chromosome breaks, dicentric chromosomes may also be significantly increased (MANOVA, $P_{ScheffePostHoc}<0.01$). Further, significant occurrence of chromatid exchanges in the form of quadriradials has been observed (MANOVA, $P_{ScheffePostHoc}<0.01$).

Exposure type	Chromosome rearrangements exposed vs. control			Reference
	Dicentric	Ring	Exchange	
Organophosphates, carbamates, dithiocarbamates, organochlorines	0.07±0.03 N/R	0.03±0.02 N/R	0.00±0.00 N/R	Kourakis et al., 1996
Ethofumesate, diazinon, vinclozolin, 2,4-D, dithiocarbamate, metalaxyl etc.	0.02±0.00 0.00±0.00	0.19±0.00* 0.02±0.00	N/R	Joksic et al., 1997
Mancozeb, methamidophos, captan, chlorpyrifos	0.08±0.30 0.05±0.09	0.02±0.05 0.03±0.07	N/R	Steenland et al., 1997
Atrazine, cyanazine, alachlor, 2,4-D, malathion	0.42±0.95** 0.00±0.00	0.00±0.00 0.00±0.00	0.10±0.40* 0.00±0.00	Zeljezic & Garaj Vrhovac, 2001
Captan, mancozeb, endosulfan, methiocarb, glyphosate, linuron etc.	Dicentric and ring chromosomes reported to be observed without providing quantitative data			Costa et al., 2006

Table 2. Studies reporting complex alterations in chromosome structures in pesticide exposed subjects. Results presented as average per 100 cells ± S.E., N/R data not reported by authors, *$P<0.05$, ** $P<0.01$.

At the end of the non-exposure period frequency of dicentric chromosomes significantly decreased, and no quadriradials were observed confirming the unstable nature of those chromosome-type aberrations (IAEA, 2001). Residual dicentrics were only detected in individuals with more than 18 years of employment in pesticide production. Conversely to previously cited studies, the authors applied multivariate analysis of variances considering smoking, gender, and age. None of them did significantly influence intergroup variations in aberration frequency. Furthermore, to avoid possible effect of x-ray diagnostics on induction of chromosome-type aberrations only workers without the record of being subjected to such procedures were allowed to participate in the study. Still, alcohol intake, nutrition, medications and other life-style factors have not been considered. Publishing of those results on pesticide genotoxicity coincided with the review article of Obe et al. (2002). The authors proposed the mechanism by which chemical genotoxins including pesticides may give rise to chromosome-type aberrations that had been previously considered as cytogenetic biomarkers of exposure to ionizing radiation. Accordingly, chemicals may induce DNA lesions such as single-strand breaks as the consequence of interaction with DNA that would result in hindered DNA replication, alkylations, bulky adducts formation, or oxidative DNA damage. During DNA replication or by error-prone DNA repair pathways, these lesions may be transduced into DSB. Further, improperly repaired DSB may give rise to complex alterations in chromosome structure such as dicentric chromosomes and exchanges. As stated by Obe et al. (2002) there are three major pathways of DSB repair: (a) ectopic homologous recombination repair (EHRR), (b) non-homologous end joining (NHEJ), and (c) single-strand annealing. Error-prone activity of two of them, NHEJ and EHRR are responsible for dicentric formation. Conversely to NHEJ that requires two initial DSB within "rejoining distance", for EHRR to form a dicentric a single DSB is needed. Furthermore, EHRR may involve homologous sequences of different chromosomes resulting in dicentric or translocation. However, it has to be stated that the repair of chemically induced DNA damage may be slower than that of ionizing radiation; thus, the probability of misrepair induced aberrations would be low (Preston, 2000) which could also be observed from the results summarized in Table 2.

More recent studies also reported appearance of dicentrics and quadriradials in lymphocytes of workers exposed to pesticides. Costa et al. (2006) detected complex structural alterations in lymphocytes of sprayers applying 33 different active ingredients without specifying the type. Ergene et al., (2007) detected dicentrics detected in residentially exposed subjects living in region contaminated mostly with organochlorines, organophosphates, carbamates, pyrethroids and benzoyl ureas. Both groups of authors did adjust statistical analysis for confounding factors. The incidence of chromosomal aberrations has been significantly affected (MANOVA, $P<0.01$) by pesticide exposure only in the study of Ergene et al., (2007) indicating that residential exposure to pesticides may also adversely affect genome integrity.

Nevertheless, findings of studies documenting the presence of dicentric chromosomes and other complex chromosome-type aberrations in lymphocytes showed necessity of using novel cytogenetic approaches that may provide more detailed knowledge regarding the potential of pesticides to affect genome integrity and induce complex genome rearrangements considered as a driving force for carcinogenicity.

4.2 FISH in analysis of pesticide induced aberrant chromatin structures

Generally, micronuclei represent the most known aberrant chromatin structure, and within the last 3 decades they have been used in evaluation of cytogenetic effect of exposure to

pesticides (Bull et al., 2006). Etiologically, they may originate either from chromosomal fragments as result of unrepaired chromosome breaks or whole chromosomes that lagged in anaphase due to damage of mitotic spindle or kinetochore. Identifying each of these two types of micronuclei provides us more detailed knowledge regarding the mechanism of genotoxicity and enables distinguishing between clastogenic pesticides that directly interact with genome and aneugenic that give rise to genomic instability by damaging protein structures. The latest leads to malsegregation of chromosomes and may result in their loss and aneuploidy. Application of fluorochrome labeled DNA probes that would hybridize in pan-centromeric region of each of 46 chromosomes makes it possible do recognize micronuclei harboring whole chromosome. When analyzing lymphocyte preparations under epifluorescence microscope, such micronuclei will contain one or more fluorescent signals. Conversely, micronuclei harboring chromosomal fragments remain without any signal.

Bolognesi et al. (2004) were among first to hybridize all-chromosome pan-centromeric probes to micronucleus slides in elucidating origin of micronuclei induced by exposure to mixture of pesticides. Study comprised floriculturists mostly exposed to organophosphates, carbamates, organochlorines, benzimidazoles, thiophtalimides, pyrethroides, and bupirimate, exposure duration ranging form 2 to 70 years. Although no statistically significant difference has been observed between pesticide users and control subjects, MN frequency increased with amount of pesticides applied, number of formulations individuals were exposed to, involvement in pesticide preparation, years of exposure, and non-use of personal protective equipment. While in the control group ratio of centromere containing micronuclei (61.9%) did not significantly differ from the reference values (±60%), in floriculturists the ratio of whole chromosome harboring micronuclei (C+MN) prevailed over the fragment harboring ones (C-MN). Though not associated with pesticide exposure duration, ratio of C+MN positively correlated with non-use of gloves in pesticide preparation. As expected, proportion of C+MN increased in both groups with age, which could be attributed to micronucleation of X and Y chromosomes associated with ageing. The effect of lagging sex chromosomes is more pronounced in females (Norppa & Falck, 2003). The most interesting conclusion made by Bolognesi et al. (2004) is higher ratio of C+MN in benzimidazolic fungicide appliers (66.52±16.11) compared to floriculturists applying other pesticide classes (63.78±14.02), which does not surprise knowing that benzimidazoles are proved as spindle microtubule poisoning agents. Single limitation of the study arises from the lack of multivariate statistical analysis of obtained data that would adjust them for age, wide range of exposure duration, and other possible confounders.

Though it renders deeper insight in mechanism of genotoxicity, revealing micronuclei content does not provide any information regarding the ability of pesticides to affect genome integrity by inducing complex rather unstable chromosome rearrangements as it has been indicated by application of chromosomal aberration analysis in section 4.1. To obtain that kind of knowledge, HUMN recommendations for considering other aberrant chromatin formations, especially nucleoplasmic bridges, had to be implemented in biomonitoring study coupled with all-chromosome pan-centromeric FISH analysis (Fenech et al., 2003).

Such an approach has been applied in evaluation of cytogenetic effect of occupational pesticide exposure in carbofuran production workers (Zeljezic et al., 2007). Though the use of carbofuran had been banned in the EU countries, in 2008 the application for its inclusion in Annex I of Council Directive 91/414/EEC concerning the placing of plant protection products on the market thus, reallowance of its use, has been resubmitted. In 2003 in some

EU countries carbofuran was still among top 5 active substances applied to vegetable crops. Epidemiological studies reported that individuals exposed to carbofuran may have increased risk for lung cancer (Usmani et al., 2004) and non-Hodgkins lymphoma (Zheng et al., 2001). In study of Zeljezic et al. (2007) lymphocytes of carbofuran production plant workers micronuclei, nuclear buds and nucleoplasmic bridges were analyzed for centromere content. Acethylcholinesteraze (AChE) activity in both, whole blood and plasma also has been recorded, as the biomarker of acute exposure to carbamate and organophosphorus insecticides. As shown in the Fig. 1. considering smoking, alcohol intake, gender, difference in age, medical procedures and exposure duration as possible covariates the incidences of MN, NBs, and NPBs have been significantly increased ($P_{DuncanPostHoc}$=0.0040; 0.0089; 0.046, respectively) in carbofuran exposed examinees. Although the ratio of centromere harboring MNs (69.6±12.5%) did not exceed referent values suggested by Norppa & Falck (2003), it has been increased compared to the unexposed group (54.3±10.3%). Statistical significance in the difference ($P_{DuncanPostHoc}$=0.0084) suggests that carbofuran, beside interacting with DNA to induce breaks in chromosome structure may also exhibit aneugenic effect by damaging mitotic spindle, and consequently resulting in chromosome loss and aneuploidy. Evident increase in the presence of heteromorphic sites of chromosomes 1, 9, 15, 16, and Y in C+MN of carbofuran handling workers ($P_{DuncanPostHoc}$=0.0036), suggests that micronucleation of whole chromosomes is not restricted to sex chromosomes as it may be expected in elderly subjects. Rather, it may indicate higher susceptibility of genomic regions rich in A-T base pairs toward aneugenic effect of carbofuran and/or its metabolites. Additional support for both clastogenic and aneugenic activity of carbofuran could be found in literature data. Stehrer-Schmid & Wolf (1995) reported adverse effect of the insecticide on formation of microtubuli and impairment of their function during chromosome segregation. Clastogenic effect of carbofuran observed as induction of C-MN may find its support in the paper of Zhang et al. (2005) who deduced carbofuran's ability to intercalate between base pairs and produce DNA-carbofuran adducts. Contrary to general perception that ACh-sensitive receptors are specific for cells of nervous tissue, they are also present on the membrane surface of most non-neuronal cells, including the periferal blood lymphocytes. Their specific physiological role has mostly remained unclear though they are confirmed to be involved in immune functions, control of gene expression, cytoskeletal organization, secretion, absorption etc. Consequently, as discussed by Rull et al. (2009) changes in AChE activity due to chronic exposure to anti-ChE compounds may induce amplification of AChE gene, and may affect cell proliferation and differentiation (Vidal, 2005). Both processes are closely associated with carcinogenesis, which may also provide a support for the results of previously cited epidemiological studies suggesting a correlation between carbamate exposure and increased risk of lymphoma. Possible amplification of AChE and butyrylcholinesterase (BuChE) genes under the chronic burden of exposure to carbamates may be the reason of elevated occurrence of NBs in lymphocytes of pesticide plant workers ($P_{DunvanPostHoc}$=0.0089). Amplification of AChE gene under chronic exposure conditions may be the reason of increased NB formation. Observed NBs might be also formed as the result of the elimination of DNA-repair complexes that have been overrepresented due to increased level of DNA damage induced (a) directly by genotoxic activity of pesticide or (b) indirectly by endogenous reactive oxygen species formed in the cells as the result of carbofuran-produced oxidative stress (Calviello et al., 2006). Although we did observe sporadic centromere signals in NBs of exposed subjects (5.8±2.21%) C+NB/C-NB ratio did not differ from the control subjects ($P_{DuncanPostHoc}$=0.72).

Unlike for C+MN, centromere signal in NBs would indicate budding of epicentric interstitial fragments or joining of chromosome harboring MNs with nucleus. Less likely scenario would be that whole chromosome may be expelled from the nucleus forming the NBs by triggering aneusomy rescue mechanism, which would be possible for highly aneugenic pesticides. Lagged chromosomes that have been incorporated within daughter nucleus instead of forming MN may cause distortion in chromosome territories and be eliminated in the budding process (Lindberg et al., 2007). However, this theory has not been proved yet nor did carbofuran exposure exhibit such prominent aneugenic potential considering observed C+MN/C-MN ratio. Finally, ratio of centromere signals has been significantly elevated ($P_{DuncanPostHoc}$=0.046) in NPBs of pesticide plant workers (20.3±12.3%) compared to the control subjects where none of detected bridges harbored the pancentromeric region. Elevated occurance of NPBs as biomarkers of dicentric and ring chromosomes, additionally supports results discussed in section 4.1 indicating that long-term exposure to pesticides is capable of inducing complex alterations in chromosome morphology. Since detected NPBs were mostly not accompanied by the presence of micronuclei (98.2±1.5%) it may be suggested that they are formed by misrepair of DSB by NHEJ or by telomere-end fusion (TEF). The latter occurs as a consequence of telomere shortening which has been commonly associated with aging, but also may be prematurely mediated by chronic influence of chemicals affecting cell proliferation and is observed in precancer cells (Fenech, 2006). Both NHEJ and TEF are characterized by the lack of acentric formation that would result in micronucleus formation. Further, increased frequency of NPBs has been recognized as a biomarker of elevated risk of lung cancer (Multivariate logistic regression analysis OR=29.05, CI=7.48-112.80, P<0.001; El-Zein, et al. 2006), for which an association has been indicated with occupational carbofuran exposure (Usmani et al., 2004).

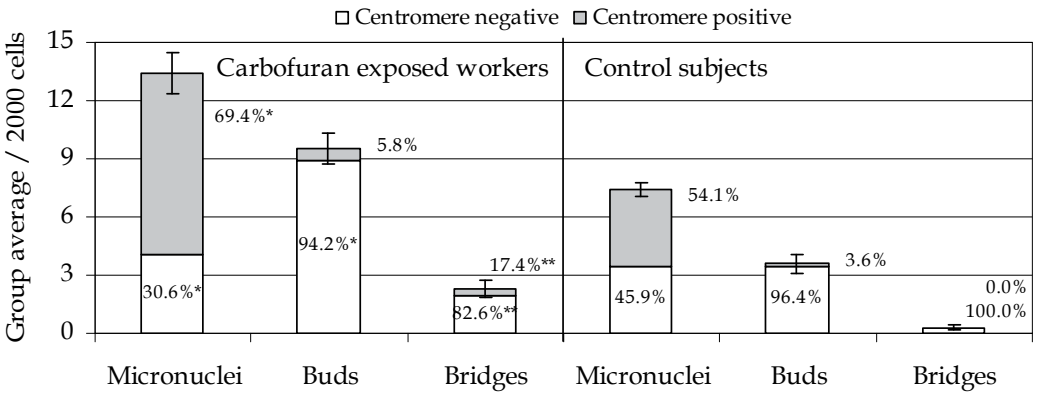

Fig. 1. Aberrant chromatin structures regarding to presence of FISH centromere signals in carbofuran production line workers and controls. *P<0.05, **P<0.01 compared to the control.

AChE activity, as a biomarker of exposure to carbamates, will not reflect possible cumulative effect of pesticides in evaluation of their adverse impact on human health. Consequently in presented study (Zeljezic et al., 2007) we did not find any correlation between plasma and whole blood AChE activity and years spent handling carbofuran formulations as assessed by multiple regression analysis (R=0.023). Average whole blood AChE activity was 94.5±1.33% (81-100%), and plasma activity 99.2±0.55% (86-100%).

Thus, to relevantly assess potential carcinogenic risk of long-term exposure to low-doses of carbamate and organophosphorous compounds, appropriate cytogenetic biomarker of exposure that will reflect cumulative effect should be determined. Interestingly, centromere containing NPBs (Fig. 2.) correlated with employment/exposure years (R=0.77, β=0.68), confirming previously reported finding that long-time occupational exposure to pesticides is capable of inducing complex chromosomal rearrangements. Additionally, multiple regression analysis considering age, gender, medical procedures and life-style factors as possible confounders revealed significant influence of carbofuran exposure duration on incidence of C+MN (R=0.83, β=0.76), NBs (R=0.86, β=0.79) suggesting that aberrant chromatin structures may be applied as nonspecific biomarkers in evaluation of cytogenetic effect of long-term carbamate exposure.

Fig. 2. Binucleated lymphocyte of a carbofuran plant worker with nucleoplasmic bridge indicated by arrow: A) DAPI staining, B) centromeric DNA dyed red (Texas Red-conjugated DNA probe), and nuclei blue (DAPI). Objective 100x, bar 1 μm.

Dynamics by which discussed cytogenetic biomarkers are being affected by pesticide exposure has been reported based on a case of acute carbofuran intoxication (Zeljezic et al., 2009). A single male worker on Furadan production line who also participated in previously discussed cytogenetic monitoring, has been transferred to medical facility with symptoms of acute anti-ChE poisoning after accidental inhalation of pesticide containing dust. The patient experienced cephalalgia, disorientation, suffocation, perspiration, weakness, fatigue, abdominal pain, and vomited. Measured whole blood AChE activity was 57% which has been significantly decreased (χ^2=16.10, P=0.0001) compared to the earlier records obtained for the same worker (83%). As shown in the Fig. 3., cytogenetic analysis revealed that 180 minutes upon intoxication frequencies of MN, C+MN, and NPBs, remained unaffected compared to the records obtained in the study prior to intoxication (P_{χ^2}=0.847; 0.683; 0.180, respectively). Only the number of C-NBs has been elevated significantly (χ^2=11.93, P=0.0006). It seems unlikely that observed NBs represent amplified AChE or BuChE genes that are being extruded from the cells. More reasonable explanation would be that these early NBs harbor expelled DNA repair complexes or which is more likely, broken chromatid/chromosome fragments as the result of genotoxicity. Latest theory might be supported by results of the alkaline comet assay. The level of primary DNA damage detected 3 hours following intoxication has been significantly increased compared to referent value for the same worker detected prior to the accident ($P_{Mann\text{-}Whitney}$=0.0019). Nevertheless, C-NBs may be indicated as possible early cytogenetic biomarkers of the effect of exposure to carbamate insecticides. In the next 72 hours, frequency of C-NB continued to increase insignificantly (χ^2=2.50, P=0.114), followed by significant occurrence of C+NBs

(χ^2=15.25, P=0.00009). Simultaneously, total MN, and proportion of MN originating from whole chromosomes were significantly increased (χ^2=10.40, P=0.0013; χ^2=10.30, P=0.0013) which categorizes them as late biomarkers of the effect.

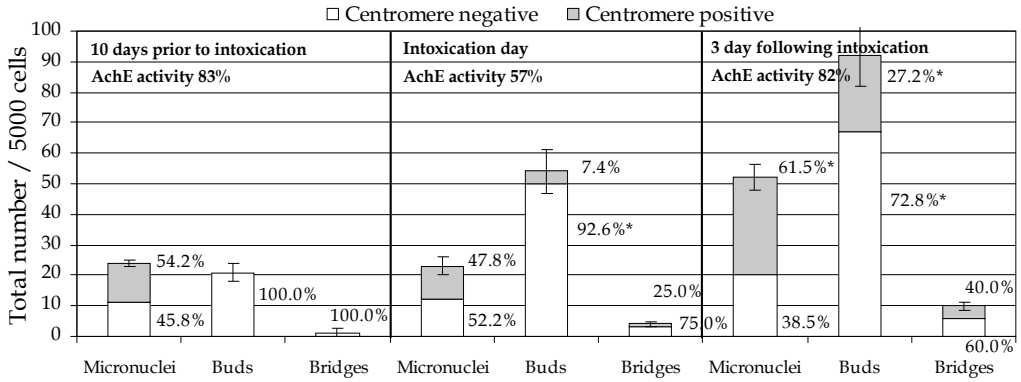

Fig. 3. Dynamics of formation of aberrant chromatin structures regarding to presence of FISH centromere signals in carbofuran intoxicated production plant worker. *P<0.05 compared to values prior to intoxication.

Conversely, even 3 days upon intoxication the level of NPBs was not significantly elevated (χ^2=2.58, P=0.1086). As already discussed, NPBs did significantly correlate with exposure to carbamate. However, NPBs are formed by dicentric or ring chromosomes whose formation is mediated through a cascade of misrepairs or significant telomere shortening. These cytogenetic events need longer period of time to occur and to form a critical pool of aberrant chromosomes that will be manifested as NPBs. Time consuming aspect of formation of chromosomes aberrant in their structure is even more pronounced in chemical than ionizing radiation genotoxicity. Some bridges will be lost due to their breakage when the daughter cells separate which also negatively reflects on their manifestation as NPBs. Thus, NPBs might be only considered as biomarkers of the effect in long-term exposure to pesticides. However, their presence in lymphocytes of individuals under pesticide exposure together with the reports indicating their presence in lymphoma patients stressed the need to look after the possible induction of translocations due to pesticide exposure. Their increased presence would indicate populations with elevated risk of tumor development.

4.3 Pesticide exposure and translocation yield assessed by FISH chromosome painting

Chromosomal translocations indicate severe impairment of genome integrity and are associated with the carcinogenic transformation of the cell, even more used as specific biomarkers in cancer diagnostics, classification, prognosis, deciding on the treatment and evaluating its efficacy. Thus, evaluation of translocation yield in population of concern is applied as a surrogate approach in epidemiological studies for estimation of cancer risk.

First attempts aiming to assess the level of translocations in peripheral blood lymphocytes of individuals exposed to pesticides started in early 1990-ties by applying G-banding technique. Method is based on treatment of metaphase spreads with trypsine and Giemsa staining that reveals a pattern of bands being specific for each of chromosome pairs. Any change in the band pattern indicates chromosomal rearrangement. Based on the

determination of patterns proceeding and following the rearrangement site chromosomes involved in the translocation may be identified. By using G-banding technique, Garry et al. (1996) detected increased (Wilcoxon rank sum test, P=0.003) frequency of rearrangements in phosphine mixing or applying subjects (1.7±0.5 per 100 metaphases) compared to the control group (0.5±0.1). However, 12 months upon some subjects ceased using the phosphine lymphocyte translocation frequency decreased to the control values. Conversely, it remained increased in individuals who continued with pesticide application. Translocations at 1p13, and 14q32 were observed in majority of applicators. Since these rearrangements are accepted to be related to non-Hodgkin's lymphoma (NHL) the authors assumed that long-term exposure to phosphine poses a risk of developing NHL. Similar results have been reported for mixed pesticide applicators (Garry et al., 1996). In applicators of multiple insecticides significantly elevated rearrangement frequency (Wilcoxon rank sum test, P=0.005), has been reported (1.4±0.3) compared to the control individuals (04±0.1). Though elevated (1.0±0.3) translocation yield in mixed herbicides applicators was not significantly affected (P=0.096). Again, 14q32 position has been involved in translocations common to most examinees indicating elevated risk of NHL. Unfortunately, both studies are characterized by rather poor pesticide exposure characterization and lack of multivariate statistical approach considering life-style factors as possible confounders; though exposed and control groups were matched by age and smoking habits. In their third study, Garry at el. (2001) applied G-banding in detection of translocations in forest and areal pesticide applicators with most prominent exposure to chlorophenoxy herbicides. Translocation, deletion and insertion yield (exposed 2.22±0.38 vs. control 0.65±0.30) has been significantly affected only in subjects applying more than 3.785 liters of herbicides per season (Wilcoxon rank sum test, P=0.003). Since Poisson regression analysis adjusted for smoking status revealed insignificant negative correlation between rearrangement frequency and measured 2,4-D urinary concentrations observed impact on induction of translocations could should be attributed to other than chlorophenoxy herbicides.

To obtain relevant results in assessing translocation frequency by analyzing G-band patterns on metaphase chromosomes requires well trained and experienced scorer, able to recognize any change in the banding sequence or width of bands that may indicate chromosomal rearrangement. Even then, due to significant variability in banding quality between metaphases of the same donor, and different donors, as well as due to terminal interchromosomal rearrangements that may not affect banding pattern, many translocations remain undetected which bias sensitivity and reliability of the technique. All these limitations classify G-banding technique laborious and inadequate for detection of cytogenetic effects resulting from low level pesticide exposures. Referred drawbacks may be circumvented by the use of FISH where single, several or all chromosomes are hybridized with DNA probes specific for each of them, and labeled with various combinations of flourochromes. As the result, each of the 22 of pairs of autosomes and 2 sex chromosomes are dyed in different color which makes easier to spot interchromosomal rearrangements and enhances the sensitivity of the method in detection of translocations. Table 3. provides an overview of studies applying FISH in translocation analysis in pesticide exposed subjects. More thorough evaluation of translocation frequency by applying chromosome painting FISH in regards to duration of pesticide exposure considering various confounders has been conducted on a group of pesticide plant workers exposed to mix of carbofuran, chlorpyrifos, metalaxyl and dodine (Zeljezic et al., 2009). In this study approach used in ionizing radiation biodosimetry has been applied, painting chromosomes 1, 2, and 4 by FISH in red,

Exposure type	FISH probe	Translocations per 100 cells vs. control	Significance	Correlation to exposure years	Reference
Mancozeb, methamidophos, captan, chlorpyrifos	Chr #1,2,4	1.21±0.97 0.92±0.82	P=0.05	N/A	Steenland et al., 1997
Carbofuran, chlorpyrifos, metalaxyl, dodine	Chr #1,2,4	1.63±0.78 0.51±0.23	P=4x10^{-5}	R^2=0.356, P=0.0003	Zeljezic et al., 2009
Dieldrin, toxaphene, lindane, atrazine	LSI IGH/BCL2 genes loci on Chr #14 & 18	N/A; OR($CI_{95\%}$) 2.1 (0.8-5.4) 1.0 (referent)	P=0.04	Yes; only descriptive	Chiu & Blair, 2009

Table 3. FISH in detection of translocations associated with long-term pesticide exposure.

green, and yellow and analyzing their mutual rearrangements, and those with other chromosomes (IAEA, 2001). Pairs of chromosomes 1, 2, and 4 represent 22.34% of the DNA content of the female genome and 22.70% of the male translocations detected painting them in 3 colors will represent 39.4% of total translocations involving all other chromosomes. Due to different gene densities along these chromosomes efficiency of DNA repair varies between them classifying translocations involving Chr 1 among most persistent, and those involving Chr 4 among least stable. Formula derived by Lucas & Sachs (1993) is used to extrapolate observed translocations frequencies for painted chromosomes to total genomic translocation frequency. In lymphocytes of pesticide plant workers genomic translocation frequency was significantly higher than in matching controls ($P_{ScheffePostHoc}$=0.000004), being higher in females (0.0062±0.0027 per cell) than in males (0.0043±0.0016) regardless of adjustment for the age difference. Most of detected translocations were reciprocal (2 bicolor chromosomes in metaphase; Fig. 4.), though their frequency in examinees (67.8±1.34) has been lower than among the controls (80.1±1.83) indicating involvement of rather small chromosomal fragments into rearrangements that are beyond resolution of the technique. Furthermore, while among the controls no complex rearrangements have been detected, among pesticide handling workers 8.5±0.51% translocations involved 3 or more chromosomes. Multiple regression analysis adjusted for confounders showed significant correlation between translocation yield and age (R^2=0.274, P= 0.021) and pesticide exposure duration (R^2=0.356, P=0.0003) the effect of latest being more prominent (exposure β=0.623, age β=0.524). Although distribution of translocations between chromosomes was random, involvement of chromosome 4 in rearrangements positively correlated with years of employment in pesticide production (R^2=0.48, p=0.0008). Since no significance in dependence of translocations upon age has been detected among controls (R^2=0.10, P=0.088), and since age and years of exposure significantly correlated among examinees (R^2=0.50, P=0.0000), and finally due to higher impact of exposure it may be concluded than translocations detected in pesticide plant workers are a consequence of impaired genomic stability due to long-term exposure. No cytogenetic effect of dodine or metalaxyl was revealed thus, observed genotoxicity may be attributed mainly to mixed carbofuran and chlorpyrifos exposure. As discussed in section 4.1, chemically induced single-strand breaks, abasic sites, oxidative damage lesions and alkylated bases may be transferred into DSBs by

error-prone base excision repair (BER; Maynard et al., 2009). Both, strand breaks induced in the course of BER, and oxidized DNA bases due to their topoisomerase II poisoning activity may be converted into DSBs (Khan et al., 2009). By error-prone activity either of original or non-classic pathways of NHEJ in its standard or modified repair, translocations may be formed (Weinstock et al., 2006). Indications for such cascade of error-prone damage transformation may be indicated by results of multiple regression analysis revealing high correlation of translocations with chromatid-type aberrations (R^2=0.26, *P*=0.003) in pesticide manufacturing workers being highly correlated with years of employment (R^2=0.479, *P*=0.00001).

Steenland et al. (1997) also reported significant increase in translocations by FISH painting of chromosomes 1, 2 and 4 in EBDC fungicide applicators (age adjusted Poisson regression *P*=0.05). Analyses restricted to reciprocal translocations nonsignificant exposure effect (p = 0.24) which may be mediated by significantly lower resolution of earlier FISH applications. However, total, and reciprocal translocations significantly correlated (R=0.28, *P*=0.02; R=0.27, *P*=0.02, respectively) with incidence of sister-chromatid exchanges that are manifestations of DSB repair.

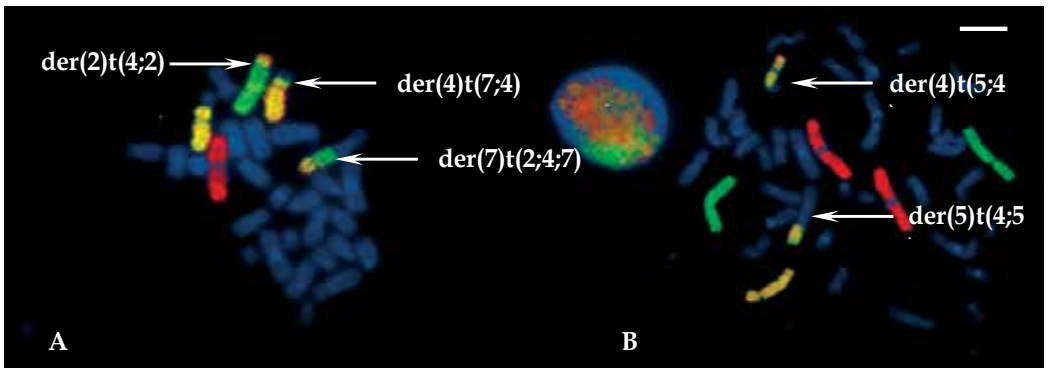

Fig. 4. Translocations in lymphocytes of the pesticide plant workers: A) complex translocations, B) reciprocal translocation t(4;5). Chr 1 – red; Chr 2 – green; Chr 4 – yellow; Objective 100x, bar 1 µm.

Similar but gene specific approach of FISH use in estimation of cancer risk due to pesticide exposure has been reported by Chiu & Blair (2009). Gene locus specific LSI IGH/BCL2 dual-fusion probe has been applied to evaluate association of t(14;18) rearrangement in NHL patients with their previous long-term pesticide exposure. Translocation t(14;18) represents the hallmark of follicular lymphoma as one of the most common adult NHLs. Exposure to crop and animal insecticides and herbicides have been associated with t(14;18) positive NHL (multivariate regression *P*=0.01). The risk of NHL for dieldrin applicators has been estimated to OR=2.4 (CI_{95}= 0.8–7.9), toxaphene OR=3.2 (CI_{95}=0.8–12.5), lindane OR=3.5 (CI=1.4–8.4), and atrazine OR=1.7 (CI_{95}=1.0–2.8) (Chiu & Brian, 2009).

5. Conclusion

Presented data indicate that within the last decade by applying the FISH technique in monitoring studies of subjects exposed to pesticides, a new set of cytogenetic biomarkers has been introduced providing us with more detailed insight in the effect of long-term

occupational pesticide exposure on genome integrity. Some biomarkers such as chromosomal rearrangements and translocation had been over a long period of time considered irrelevant in chemical carcinogenesis and cancer risk assessment due to pesticide exposure. Valuable knowledge regarding the error-prone effect of distinct DNA repair mechanisms helped us to understand how primary genome lesions induced by agrochemicals may be transformed into chromosomal rearrangements. The use of FISH in evaluation of the impact of occupational exposure to pesticides on human genome may provide us with more precise insight in the extent and type of genome damage, indicate possible specificity of pesticides of concern toward specific chromosomes or regions and help us in evaluation of pesticide exposure relevant for cancer risk.

6. References

Abass, K.; Reponen, P.; Mattila, S. & Pelkonen, O. (2009). Metabolism of carbosulfan. I. Species differences in the in vitro biotransformation by mammalian hepatic microsomes including human. *Chemico-biological Interactions*, Vol. 181, No. 2, pp 210-219, 00092797

Ahrens, W.; Krickeberg, K. & Pigeot, I. (2005). An Introduction to Epidemiology, In: *Handbook of epidemiology*, Ahrens, W. & Pigeot, I. (Ed.), pp 1-42, Springer Verlag, 354000568, Berlin

Amr, M.M. (1999). Pesticide monitoring and its health problems in Egypt, a Third World country. *Toxicology Letters*, Vol. 107, pp 1-13, 03784274

Anderson, D. (2000). Examination of various biomarkers after occupational exposure to different chemicals, In: *Human Monitoring after Environmental and Occupational Exposure to Chemical and Physical Agents*, Anderson, D.; Karakaya, A.E. & Šrám, R.J. (Ed.), pp 94-100, IOS Press, 9051995958, Amsterdam

Andreotti, G.; Beane Freeman, L.E.; Hou, L.; Coble, J.; Rusiecki, J.; Hoppin, J.A.; Silverman, D.T. & Alavanja, M.C.R. (2009). Agricultural pesticide use and pancreatic cancer risk in the Agricultural Health Study. *International Journal of Cancer*, Vol. 124, 2495–2500, 0020-7136

Antonucci, G.A. & de Syllos Cólus, I.M. (2000). Chromosomal aberrations analysis in a Brazilian population exposed to pesticides. *Teratogenesis, Carcinogenesis, and Mutagenesis*, Vol. 20, pp 265–272, 02703211

Axelson, O.; Rehn, M. & Sundell, L. (1974). Exposure to herbicides--mortality and tumor incidence: An epidemiologie investigation of Swedish railroad workers. *Lakartidningen*, Vol. 71, No. 24, pp 2466-2470, 00237205

Balayiannis, G.P.; Anagnostopoulos, H. & Kellidou, I (2009). Facile and rapid determination of contamination in sulphur pesticide formulations by liquid chromatography-tandem mass spectrometry. *Bulletin of Environmental Contamination and Toxicology*, Vol. 82, No. 2, pp 133-136, 00074861

BCPC - The British Crop Protection Council (2009). *The pesticide Manual (Incorporating the Agrochemicals Handbook) a world compendium*, Tomlin, C., (Ed.), pp 21, Datix International, 1901396188 , Bungay

Becher, H. (2005). General Principles of Data Analysis, In: *Handbook of epidemiology*, Ahrens, W. & Pigeot, I. (Ed.), pp 595-624, Springer Verlag, 354000568, Berlin

Becker, P.; Scherthan, H. & Zankl, H. (1990). Use of a centromere-specific DNA probe (p82H) in nonisotopic in situ hybridization for classification of micronuclei. *Genes Chromosomes Cancer*, Vol. 2, No. 1, pp 59-62, 10452257

Blair, A. & Zahm, S.H. (2008). Agricultural exposures and cancer. *Environmental Health Perspectives*, Vol. 103, pp 205-208, 00916765

Blasiak, J.; Jaloszynski, P.; Trzeciak, A. & Szyfter, K. (1999). In vitro studies of the organophosphorous insecticide malathion and its two analogues. *Mutation Research*, Vol. 445, No. 2, 275-283, 00275107

Bolognesi, C.; Landini, E.; Perrone, E. & Roggieri, P. (2004). Cytogenetic biomonitoring of a floriculturist population in Italy: micronucleus analysis by fluorescence in situ hybridization (FISH) with all-chromosome centromeric probe. *Mutation Research*, Vol. 557, pp 109-117, 00275107

Boers, D.; Portengen, L.; Bueno-de-Mesquita, H.B.; Heederik, D. & Vermeulen, R. (2010). Cause-specific mortality of Dutch chlorophenoxy herbicide manufacturing workers. Occupational and Environmental Medicine, Vol. 67, No. 1, pp 24-31, 13510711

Bonner, M.R.; Williams, B.A.; Rusiecki, J.A.; Blair, A.; Beane Freeman, L.E.; Hoppin, J.A.; Dosemeci, M.; Lubin, J.; Sandler, D.P. & Alavanja, M.C.R. (2010). Occupational exposure to terbufos and the incidence of cancer in the Agricultural Health Study. *Cancer Causes and Control*, Vol. 21, No. 6, pp 871-877, 09575243

Bull, S.; Fletcher, K.; Boobis, A.R. & Battershil, J.M. (2006). Evidence for genotoxicity of pesticides in pesticide applicators: a review. *Mutagenesis*, Vol. 21, No. 2, pp 93–103, 02678357

Buongiorno-Nardelli, M. & Amaldi, F. (1969). Autoradiographic detection of molecular hybrids between tRNA and DNA in tissue sections. *Nature*, Vol. 225, pp 946-947, 00280836

Calviello, G.; Piccioni, E.; Boninsegna, A.; Tedesco, B.; Maggiano, N.; Serini, S.; Wolf, F.I. & Palozza, P. (2006). DNA damage and apoptosis induction by Mancozeb in rat cells: Involvement of the oxidative mechanism. *Toxicology and Applied Pharmacology*, Vol. 211, pp 87-96, 0041008X

Carson, R. (2002). *Silent Spring*, Mariner Books, 0618249060, New York, NY

Casciano, D.A. (2000). Introduction: Historical Perspectives of Genetic Toxicology, In: *Genetic Toxicology*, Li, A.P. & Heflich, R.H. (Ed.), pp 1-12, CRC Press, 0849388153, Boca Raton, FL

Cavallo, D.; Ursini, C.L.; Rondinone, B. & Iavicoli, S. (2009). Evaluation of a suitable DNA damage biomarker for human biomonitoring of exposed workrs. *Environmental and Molecular Mutagenesis*, Vol. 50, No. 9, pp 781-790, 08936692

Chang, T.H. & Klassen, W. (1968). Comparative effects of tretamine, tepa, apholate and their structural analogues on human chromosomes in vitro. *Chromosoma*, Vol. 24, No. 3, pp 314-323, 00095915

Chappel, CI.. (1992). A review and biological risk assessment of sodium saccharin. *Regulatory Toxicology and Pharmacology*, Vol. 15, No. 3, pp 253-270, 02732300

Chiu, B.C.-H. & Blair A. (2009). Pesticides, chromosomal aberrations, and Non-Hodgkin's lymphoma. *Agromedicine*, Vol. 14, No. 2, pp 250–255, 1059-924X

Costa, C.; Teixeira, J.P.; Silva, S.; Roma-Torres, J.; Coelho, P.; Gaspar, J.; Alves, M.; Laffon, B.; Rueff, J. & Mayan, O. (2006). Cytogenetic and molecular biomonitoring of a

Portuguese population exposed to pesticides. *Mutagenesis*, Vol. 21, No. 5, pp 343–350, 02678357

Cox, C. & Surgan, M. (2006). Unidentified Inert Ingredients in Pesticides: Implications for Human and Environmental Health. *Environmental Health Perspectives*, Vol. 114, No. 12, pp 1803-1806, 00916765

DeLancey, J.L.; Alavanja, M.C.R.; Coble, J.; Blair, A.; Hoppin, J.A.; Austin, H.D. & Beane Freeman, L.E. (2009). Occupational exposure to metribuzin and the incidence of cancer in the Agricultural Health Study. Annals of Epidemiology, Vol. 19, No. 6, pp 388-395, 10472797

Dich, J.; Hoar Zahm, S.; Hanberg, A. & Adami, H-O. (1997). Pesticides and cancer. *Cancer Causes and Control*, Vol. 8, pp 420-443, 09575243

Eastmond, D.A. & Balakrishnan, S. (2001). Genetic Toxicity of pesticides, In: *Handbook of Pesticide Toxicology*, Krieger, R. (Ed.), pp 747-767, Academic Press, 0124262627, San Diego, CA

El-Zein, R.A.; Fenech, M.; Lopez, M.S.; Spitz, M.R. & Etzel, C.J. (2008). Cytokinesis-blocked micronucleus assay biomarkers identify lung cancer amongst smokers. *Cancer Epidemiology, Biomarkers & Prevention*, Vol. 17, No. 5, pp 1111-1119, 10559965

El-Zein, R.A.; Schabath, M.B.; Etzel, C.J.; Lopez, M.S.; Franklin, J.D. & Spitz, M.R. (2006). Cytokinesis-blocked micronucleus assay as a novel biomarker for lung cancer risk. *Cancer Research*, Vol. 66, No. 12, pp 6449-6456, 00085472

Ergene, S.; Çelik, A.; Çavaş, T. & Kaya, F. (2007). Genotoxic biomonitoring study of population residing in pesticide contaminated regions in Göksu Delta: Micronucleus, chromosomal aberrations and sister chromatid exchanges. *Environment International*, Vol. 33, pp 877–885, 01604120

Fenech, M. (2006). Cytokinesis-block micronucleus assay evolves into a "cytome" assay of chromosomal instability, mitotic dysfunction and cell death. *Mutation Research*, Vol. 600, pp 58–66, 00275107

Fenech, M. (2007). Cytokinesis-block micronucleus cytome assay. *Nature Protocols*, Vol. 2, pp 10084-1104, 17542189

Fenech, M.; Chang, W.P.; Kirsch-Volders, M.; Holland, N.; Bonassi, S. & Zeiger, E. (2003). HUMN project: detailed description of the scoring criteria for the cytokinesis-block micronucleus assay using isolated human lymphocyte cultures. *Mutation Research*, Vol. 534, pp 65–75, 00275107

Garry, V.F.; Tarone, R.E.; Kirsch, I.R.; Abdallah, J.M.; Lombardi, D.P.; Long, L.K.; Burroughs, B.L.; Barr, D.B.; & Kesner J.S. (2001). Biomarker correlations of urinary 2,4-D levels in foresters: genomic instability and endocrine disruption. *Environmental Health Perspectives*, Vol. 109, No. 5, pp 495-500, 00916765

Garry, V.F.; Tarone, R.E.; Long, L.; Griffith, J.; Kelly, J.T. & Burroughs, B. (1996). Pesticide appliers with mixed pesticide exposure: G-banded analysis and possible relationship to non-Hodgkin's lymphoma. *Cancer Epidemiology, Biomarkers and Prevention*, Vol. 5, pp 11-16, 10559965

Green, R.E. (1949). Poisoning by the organic phosphate insecticides. *Wisconsin Medical Journal*, Vol. 48, No. 11 p 1007, 00436542

Hadnagy, W.; Seemayer, N.H.; Kühn, K.H.; Leng, G. & Idel, H. (1999). Induction of mitotic cell division disturbances and mitotic arrest by pyrethroids in V79 cell cultures. *Toxicology Letters*, Vol. 107, No. 1-3, pp 81-87, 03784274

Hagmar, L.; Tinnerberg, H.; Mikoczy, Z.; Strömberg, U.; Bonassi, S.; Huici Montagud, A.; Hansten, I.-L.; Knudsen, L.E. & Norppa, H. (2000). Do cytogenetic biomarkers, used for occupational health surveillance predict cancer?, In: *Human Monitoring after Environmental and Occupational Exposure to Chemical and Physical Agents*, Anderson, D.; Karakaya, A.E. & Šrám, R.J. (Ed.), pp 1-6, IOS Press, 9051995958, Amsterdam

Hill, M.K. (2010). *Understanding Environmental Pollution*, pp 89-116, Cambridge University Press, Cambridge

Hoshizaki, H.; Niki, Y.; Tajima, H.; Terada, Y. & Kasahara, A. (1969). A case of leukemia following exposure to insecticide. *Nippon Ketsueki Gakkai Zasshi*, Vol. 32, No. 4, pp 672-677, 00015806

Hoyos, L.S.; Carvajal, S.; Solano, L.; Rodriguez, J.; Orozco, L.; Lopez, Y. & Au W.W. (1996). Cytogenetic monitoring of farmers exposed to pesticides in Colombia. *Environmental Health Perspectives*, Vol. 104, Supp. 3, pp 535-538, 00916765

IAEA (2001). *Cytogenetic analysis for radiation dose assessment: a manual*, IAEA, 9201021011, Vienna

Joksic, G.; Vidakovic, A. & Spasojevic-Tisma, V. (1997). Cytogenetic monitoring of pesticide sprayers. *Environmental Research*, Vol. 75, pp 113–118, 00139351

Jungmann, G. (1966). Arsenic cancer in vintagers. Landarzt, Vol. 42, pp 1244-1247, 00237728

Kang, D.; Park, S.K.; Beane-Freeman, L.E.; Lynch, C.F.; Knott, C.E.; Sandler, D.P.; Hoppin, J.A.; Dosemeci, M.; Coble, J.; Lubin, J.; Blair, A. & Alavanja, M. (2008). Cancer incidence among pesticide applicators exposed to trifluralin in the Agricultural Health Study. *Environmental Research*, Vol. 107, pp 271–276, 00139351

Kaszubiak, H. (1968). The effect of herbicides on Rhizobium. 3. Influence of herbicides on mutation. *Acta Microbiologica Polonica*, Vol. 17, No. 1, pp 51-57, 01371320

Khan, F.; Sherwani, A.F. & Afzal, M. (2009). Chromosomal aberration and micronucleus studies of two topoisomerase (II) targeting anthracyclines. *Journal of Environmental Biology*, Vol. 30, No. 3, pp 409-412, 02548704

Klayman M.B., Exposure to Insecticides, Arch Otolaryngol. 1968;88(1):116-117

Kourakis, A.; Mouratidou, M.; Barbouti A. & Dimikiotou M. (1996). Cytogenetic effects of occupational exposure in the peripheral blood lymphocytes of pesticide sprayers. *Carcinogenesis*, Vol. 17, No. 1, pp 99-101, 01433334

Legator, M. & Zimmering, S. (1975). Genetic Toxicology. *Annual Review of Pharmacology*, Vol. 15, pp 387-408, 00664251

Lindberg, H.K.; Wang, X.; Jarventaus, H.; Falck, GC.-M.; Norppa, H. & Fenech, M. (2007) Origin of nuclear buds and micronuclei in normal and folate-deprived human lymphocytes. *Mutation Research*, Vol. 617, pp 33-45, 00275107

Lucas, J.N. & Sachs, R.K. (1993). Using three-color chromosome painting to test chromosome aberration models. *Proceedings of National Academy of Science USA*, Vol. 90, pp 1484–1487, 00278424

Lynch, S.M.; Mahajan, R.; Beane Freeman, L.E.; Hoppin, J.A. & Alavanja, M.C.R. (2009). Cancer incidence among pesticide applicators exposed to butylate in the Agricultural Health Study. *Environmental Research*, Vol. 109, pp 860-868, 00139351

Matter, B. & Schmid, W. (1971). Trenimon-induced chromosomal damage in bone-marrow cells of six mammalian species, evaluated by the micronucleus test. *Mutation Research,* Vol. 12, No. 4, pp 417-425, 00275107

Maynard, S.; Schurman, S.H.; Harboe, C.; de Souza-Pinto, N.C. & Bohr, V.A. (2009). Base excision repair of oxidative DNA damage and association with cancer and aging. *Carcinogenesis*, Vol. 30, pp 2–10, 01433334

Mitelman, F.; Mertens, F. & Johansson, B. (1997). A breakpoint map of recurrent chromosomal rearrangements in human neoplasia. Nature Genetics, Vol. 15, pp 417–474, 10614036

Muller, J.; Decordier, I.; Hoet, P.H.; Lombaert, N.; Thomassen, L.; Huaux, F.; Linson, D. & Kirsh-Volders, M. (2008). Clastogenic and aneugenic effects of multi-wall carbon nanotubes in epitheliail cells. *Carcinogenesis*, Vol. 29, No. 2, pp 427-433, 01433334

Norppa, H. & Falck, G.C-M. (2003). What do human micronuclei contain? *Mutagenesis*, Vol. 18, No. 3, pp. 221–233, 02678357

Obe, G.; Pfeiffer, P.; Savage, J.R.K.; Johannes, C.; Gödecke, W.; Jeppesen, P.; Natarajan, A.T.; Martínez-López, W.; Folle, G.A. & Drets M.E. (2002). Chromosomal aberrations: formation, identification and distribution. *Mutation Research*, Vol. 504, pp 17–36, 00275107

Orsi, L.; Delabre, L.; Monnereau, A.; Delval, P.; Berthou, C.; Fenaux, P.; Marit, G.; Soubeyran, P.; Huguet, F.; Milpied, N.; Leporrier, M.; Hemon, D.; Troussard, X. & Clavel, J. (2009). Occupational exposure to pesticides and lymphoid neoplasms among men: results of a French case-control study. *Occupational and Environmental Medicine*, Vol. 66, No. 5, pp 291-298, 13510711

Prasad, I. (1970). Mutagenic effects of 3',4'-dichloropropionanilide and its degradation products. *Canadian Journal of Microbiology*, Vol. 16, No. 5, pp 369-372, 00084166

Preston, R.J. (2000). Mechanisms of Introduction of Chromosomal alterations and sister chromatid exchange, In: *Genetic Toxicology*. Li, A.P. & Heflich, R.H. (Ed.), pp 41-66, CRC Press, 0849388153, Boca Raton, FL

Price, J.D. (1973). Agricultural Entomology, In: *History of Entomology*, Smith, R.F.; Mittler, T.E. & Smith, C.N., (Ed.), pp 307-372, Annual Reviews, 0686092988, Palo Alto, CA

Raap, A.K. (1998). Advances in fluorescence in situ hybridization. *Mutation Research*, Vol. 400, pp 287-298, 00275107

Richardson, J.C. (1974). A preliminary assessment of cytogenetic effects of metepa on mose bone marrow using the micronucleus test. *Mutation Research*, Vol. 26, No. 5, pp 391-394, 00275107

Rossi, A.M.; Hansteen, I-L.; Skjelbred C.F.; Ballardin, M.; Maggini, V.; Murgia, E.; Tomei, A.; Viarengo, P.; Knudsen, L.E.; Barale, R.; Norppa, H. & Bonassi, S. (2009). Association between frequency of chromosomal aberrations and cancer risk is not influenced by genetic polymorphisms in GSTM1 and GSTT1. *Environmental Health Perspectives*, Vol. 117, No. 2, pp 203-208, 00916765

Rossner, P.; Boffeta, P.; Ceppi, M.; Bonassi, S.; Smerhovsky, Z.; Landa, K.;, Juzova, D. & Šrám, R.J. (2005). Chromosomal aberrations in lymphocytes of healthy subjects and risk of cancer. *Environmental Health Perspectives*, Vol. 113, No. 5, pp 517-520, 00916765

Rull, R.P.; Gunier, R.; von Behren, J.; Hertz, A.; Crouse, V.; Buffler, P.A. & Reynolds, P. (2009). Residential proximity to agricultural pesticide applications and childhood acute lymphoblastic leukemia. *Environmental Research*, Vol. 109, pp 891-899, 00139351

Rusiecki, J.A.; Hou, L.; Lee, W.J.; Blair, A.; Dosemeci, M.; Lubin, J.H.; Bonner, M.; Samanic, C.; Hoppin, J.A.; Sandler, D.P. & Alavanja, M.C.R. (2009). Cancer incidence among pesticide applicators exposed to metolachlor in the Agricultural Health Study. *International Journal of Cancer*, Vol., 118, pp 3118–3123, 0020-7136

Smith, A.E. & Secoy, D.M. (1975). Forerunners of Pesticides in Classical Greece and Rome. *Agricultural and Food Chemistry*, Vol. 23, No. 6, pp 1050, 00218561

Smith, E.H. & Kennedy, G.G. (2002). History of Pesticides, In: *Encyclopedia of Pest Management*, Pimentel, D. (Ed.), 0824706326, pp 376-372, Taylor & Francis, London

Steenland, K.; Cedillo, L.; Tucker, J.; Hines, C.; Sorensen, K.; Deddens, J. & Cruz, V. (1997). Thyroid hormones and cytogenetic outcomes in backpack sprayers using ethylenebis(dithiocarbamate) (ebdc) fungicides in Mexico. *Environmental Health Perspectives*, Vol. 105, No. 10, pp 1126-1130, 00916765

Stehrer-Schmid, P. & Wolf, H.U. (1995). Genotoxic evaluation of three heterocyclic N-methylcarbamate pesticides using the mouse bone marrow micronucleus assay and the Saccharomyces cerevisiae strains D7 and D61. *Mutation Research*, Vol. 345, pp 111–125, 00275107

Titenko-Holland, N.; Moore, L.E. & Smith, M.T. (1994). Measurement and characterization of micronuclei in exfoliated human cells by fluorescence in situ hybridization with a centromeric probe. *Mutation Research*. Vol. 312, No. 1, pp 39-50, 00275107

Tucker, J.D.; Ramsey, M.J.; Lee, D.A. & Minkler, J.L. (1993). Validation of chromosome painting as a biodosimeter in human peripheral lymphocytes following acute exposure to ionizing radiation in vitro. *International Journal of Radiation Biology*, Vol. 64, pp 27–37, 09553002

Usmani, K.A.; Hodgson, E. & Rose, R.L. (2004). In vitro metabolism of carbofuran by human, mouse, and rat cytochrome P450 and interactions with chlorpyrifos, testosterone, and estradiol. *Chemico-biological Interactions*, Vol. 150, pp 221–232, 00092797

van Bemmel, D.M.; Visvanathan, K.; Beane Freeman, L.E.; Coble, J.; Hoppin, J.A. & Alavanja, M.C.R. (2008). S-ethyl-N,N-dipropylthiocarbamate exposure and cancer incidence among male pesticide applicators in the agricultural health study. *Environmental Health Perspectives,* Vol. 116, No. 11, pp 1541-1556, 00916765

van Maele-Fabry, G.; Duhayon, S.; Mertens, C. & Lison, D. (2008). Risk of leukaemia among pesticide manufacturing workers: A review and meta-analysis of cohort studies. *Environmental Research*, Vol. 106, pp 121–137, 00139351

Vidal, C.J. (2005). Expression of cholinesterases in brain and non-brain tumours. Chemico-biological Interactions, Vol. 157–158, pp 227–232, 00092797

Wandahwa, P.; van Ranst, E. & van Damme, P. (1996). Pyrethrum (*Chrysanthemum cinerariaefolium* Vis.) cultivation in West Kenya: origin, ecological conditions and management. *Industrial Crops and Products,* Vol. 5, No. 4, pp. 307-322, 09266690

Ware, G. & Whitacre, D. (2004). *The pesticide book,* pp 128-132, Meister Media Worldwide, 1892829118, Willoughby, OH

Weinstock, D.M.; Richardson, C.A.; Elliott, B. & Jasin, M. (2006). Modeling oncogenic translocations: distinct roles for double strand break repair pathways in translocation formation in mammalian cells. *DNA Repair*, Vol. 5, pp 1065-1074, 15687864

Zeljezic, D. & Garaj Vrhovac, V. (2001). Chromosomal aberration and single-cell gel electrophoresis (comet) assay in longitudinal risk assessment of occupational exposure to pesticides. *Mutagenesis*, Vol. 16, No. 4, pp 359-363, 02678357

Zeljezic, D.; Lucic Vrdoljak, A.; Lucas, J.N.; Lasan, R.; Fucic, A.; Kopjar, N.; Katic, J.; Mladinic, M. & Radic, B. (2009). Effect of occupational exposure to multiple pesticides on translocation yield and chromosomal aberrations in lymphocytes of plant workers. *Environmental Science & Technology*, Vol. 43, pp 6370–6377, 0013936X

Zeljezic, D.; Lucic Vrdoljak, A.; Radic, B.; Fuchs, N.; Berend, S.; Orescanin, V. & Kopjar, N. (2007). Comparative evaluation of acetylcholinesterase status and genome damage in blood cells of industrial workers exposed to carbofuran. *Food and Chemical Toxicology*, Vol. 45, pp 2488–2498, 02786915

Zhang, L.J.; Min, S.G.; Li, G.X.; Xiong, Y.M. & Sun, Y. (2005). The mechanism of carbofuran interacts with calf thymus DNA. *Guang Pu Xue Yu Guang Pu Fen Xi*, Vol. 25, No. 5, pp 739-742, 10000593

Zheng, T.; Zahm, S.H.; Cantor, K.P.; Weisenburger, D.D.; Zhang, Y. & Blair, A. (2001). Agricultural exposure to carbamate pesticides and risk of non-Hodgkin lymphoma. Journal of Occupational and Environmental Medicine, Vol. 43, pp 641–649, 10762752

15

Pesticides: Genotoxic Risk of Occupational Exposure

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1. Introduction

Exposure to pesticides remains a major environmental health problem. Pesticides are one of the most extensively used chemical products to control agricultural pests. The progress of agrochemical industries in the 20th century originated a great number of highly aggressive compounds against humans and altered the equilibrium in the ecosystems. To a high or low degree, human populations are unavoidably exposed to environmental pollution in physical, chemical or biological forms through products degraded in the air, water, soil or food and their inclusion in the alimentary chain.

With the objective of increasing and preserving crops, chemicals have been used to control and eliminate a wide variety of insects and other noxious organisms in agricultural production causing illnesses that affect plants and decrease the amount of food products obtained; therefore, it is important to protect the crops during the periods of sowing, harvest, storage and distribution of the products. The increase in the world population and the need to produce more food are major factors that stimulate the industry to produce new and more effective pesticides. DDT used to be effective for controlling insects, but later the synthesis of new substances became the main source to fight pests, and the world production of crops duplicated between 1970 and 1985.

It has been estimated that 2.5 million tons of pesticides are applied worldwide each year and the amount continues increasing with the passage of time. At the end of the 20th century, sales reached 40 thousand million dollars annually in the world, which corresponds to 2,800 million kilograms of active ingredients and more than 50 thousand commercial formulations. Developing countries use around 40% of this total. More than one thousand formulations or commercial names are used throughout the world as insecticides, fungicides, herbicides, rodenticides and antimicrobians (WHO/UNEP, 1990; PNUMA/OMS, 1992; OPS/OMS, 1993; OPS, 2002).

As to pesticide categories, herbicides represent 49% of the world sales, followed by insecticides 27%, fungicides 20% and other uses 4%. Hundreds of active ingredients and thousands of formulations are available in an uncontrolled fashion and they are promoted by both manufacturer and distributor as being essential for crop production (Eddleston et al., 2002).

The leading producers and exporters of pesticides in the world are Germany, the USA, England, Switzerland, France, Japan and Italy, countries that export the major part of their production to the Third World; regulation agencies consider that around 30% of such production are pesticides designated to agriculture and public health with a value of 900 million dollars, substances that do not meet the quality norms accepted internationally. These pesticides frequently contain compounds or impurities that are restricted in many countries because they constitute a risk for human health and the environment (OMS, 1990).

Occupational exposure may occur through the pesticide formulation, manufacture and application phases which involve the exposure to complex mixtures of different types of chemicals, active ingredients and other substances included in the technical formulations such as impurities, solvents and other compounds produced during the storage procedure. Moreover, although inert ingredients do not have pesticidal activity, they may be biologically active and sometimes the most toxic component of a pesticide formulation (Bolognesi, 2003).

Pesticides are substances with very different characteristics and they are designed to kill a great variety of undesirable organisms for humans. They are toxic substances whose application should be controlled because their indiscriminate use and abuse constitute a risk to human health. Due to the large amount and variety of pesticides used at present to protect crops, great controversy has been generated about their use because of the adverse effects for humans, the environment and other organisms, although there is clear evidence of the benefits obtained by humans with the application of control vectors that have transmitted endemic diseases mainly in tropical countries, for example in Pakistan to control the dengue virus with sprayed deltamethrine or the malaria control program with malathion (Tariq et al., 2007), or to increase the daily production of food for the world population.

In several countries, the cotton plant still represents the most important crop and the main element for the national economy, as in the case of Egypt. Here the pests infesting cotton affect the quality and quantity of the yield, and thus pesticides are considered essential to protect this crop, with a high number of workers spraying three to five times each season (Mansour, 2004). The same problem occurred in Pakistan where about 80% of total pesticides used in this country are applied to the cotton plants (Tariq et al., 2007). The continuous shifting from one compound to another has been mainly attributed to the development of resistance of the cotton leaf worm (Mansour, 2004).

One of the main risks of pesticides for humans is occupational exposure, which occurs with agricultural workers in open fields, greenhouse workers, with individuals involved in the production of pesticides and exterminators of house pests, as well as with sanitation workers, workers packing pesticides, and other similar cases.

The World Health Organization estimates that every year between 500,000 and 1 million individuals suffer pesticide intoxications, and between 5,000 and 20,000 die. At least half of those intoxicated and 75% of those who die are agricultural workers, while the others die because of poisoning from contaminated food. Totally mortality for both groups was 220,000 annual deaths (OMS, 1990; Eddleston et al., 2002).

In accordance with the International Agency for Cancer Research (IARC) 56 pesticides have been classified as carcinogens in laboratory animals. The pesticide association for cancer in humans reported the use of 2,4,5-trichlorophenoxyacetic acid, lindane, methoxychlor, toxaphene and several organophosphates (IARC, 2002).
The International Network Against the Use of Pesticides has informed that developing countries account for the 5th part of the world consumption of these compounds, that the number of intoxications with such substances increased to 25 million cases and that 99% of the deaths are attributable to pesticides (PAN International, 1990).
The wide spectrum of effects on health produced by pesticides includes acute and persistent damage in the nervous system (Ecobichon et al., 1990; Kamel et al., 2005, 2007), lung and respiratory disorders (Barthel, 1981; Blair et al., 1983; Hoppin et al., 2008), alterations in the reproductive organs (Hileman, 1994) as well as in the immunological (Turner, 1994) and endocrine systems, in addition to birth defects (Gray, 1992; Rojas et al., 2000). Other causes of worry are the carcinogenic and genotoxic effects, considered as being among the most important of the effects, are possibly side effects associated with agricultural chemicals (Anwar, 1997).
In a review, Mansour (2004) concludes that there is strong scientific evidence that pesticides, as a whole, can induce severe effects to human health ranging from myelotoxicity to cytogenetic damage and carcinogenicity. The developed countries have already addressed the pesticide problem, but are still facing some problems in certain locations, whereas in the Third World countries pesticides should be used carefully since toxic outbreaks are often attributed to misuse of these substances.

2. Pesticides in Mexico

Pesticides are one of major sources of pollution derived from synthetic products and generated as a result of agricultural activity. Some are forbidden or restricted in many countries because they are toxic for human health and they affect natural resources, yet in Mexico there is an indiscriminate use which increases the risk of exposure to them on account of their genotoxic action.
Although there is wide-spread usage of pesticides in Mexico to control pests, as in other countries, this has caused environmental and human health problems. In accordance with the Mexican Association of Pesticide and Fertilizer Industrials (Asociación Mexicana de la Industria de Pesticidas y Fertilizantes), the pesticide volume used in Mexico in 1995 was 54,678.96 tons, 47% of which corresponded to insecticides, 29% to herbicides, 17% to fungicides and 7% to other uses. According to Cofepris (2010), the use of pesticides has increased and herbicides are the most utilized chemicals, followed by insecticides and fungicides.
In Mexico, the land available for agriculture is around 23 million hectares, which is 12% of the total surface of the country. The most important crops and the ones that are sprayed with the greatest volume of these chemicals are corn, bean, sorghum, wheat, potato, cotton, chilli, tomato, avocado, coffee, tobacco, pot-herb in amounts ranging from 395 to 13,163 pesticide tons per year (AMIPFAC, 1995). The official data for 2001 show that the population working in agriculture was around 7 million persons; however, this number does not include the rural population that was also exposed to pesticides and was calculated to be 25.4% of the total population in Mexico (AMIPFAC, 2001; Martínez Guerrero, 2001). Sixty percent of the 22 pesticides classified as dangerous to health and the environment are

commonly used in the Mexican Republic, and 42% are made in the country; 30 to 90% have been restricted or cancelled (INEGI, 1998). Pesticide handling and use in Mexico is regulated by different federal agencies: their transport by the Ministry of Communications and Transport (Secretaría de Comunicaciones y Transportes), their environmental impact by the Ministry of Environment and Natural Resources (Secretaria del Medio Ambiente y Recursos Naturales), their biological effectiveness for agricultural application by the Ministry of Agriculture, Livestock and Fisheries (Secretaría de Agricultura, Ganadería y Pesca), and the sanitary aspects by the Ministry of Health (Secretaría de Salud) (SEMARNAP, 1996; Rosales Castillo, 2001).

Besides the large amounts of pesticides imported by Mexico, there are industrial plants located in several states of the republic as Coahuila, Chihuahua, Guanajuato, Estado de México, Querétaro, Tlaxcala and Veracruz. The products for marketing are classified according to their toxicity: 57% slightly toxic, 25% moderately toxic, 9% highly toxic and 9% extremely toxic (Perea, 2006).

The Health Ministry considered that intoxications from pesticides registered every year in the world had occurred in developing countries. In Mexico 260 pesticide trademarks are used, 24 of these are prohibited and 3 restricted; in summary, the main cases of intoxication are due to lack of control and to deficient prevention. In agreement with the epidemiological norms of this Ministry, the number of intoxications caused by the use of pesticides decreased significantly from 8,000 to 2,532, between the years 1995 to 2001. In 2002 the number increased slightly to 2,802, in 2003 it increased again to 3,849 and in 2005 it was 3,898. However, the authority itself recognized the presence of a sub-register or "black data" as to the number of intoxications caused by the use of agrochemicals. The indiscriminate and exhaustive use of pesticides has originated very serious problems for the environment as well as for non-target organisms and humans (CICOPLAFEST, 1998). The states with highest use of pesticide are Sinaloa, Veracruz, Jalisco, Nayarit, Colima, Sonora, Baja California, Tamaulipas, Michoacán, Tabasco, Estado de México, Puebla and Oaxaca. Approximately 80% of all pesticides are applied in these regions (Grammont & Lara Flores, 2004; Albert, 2005).

3. Biomarkers used in cytogenetic biomonotoring studies in populations exposed to pesticides

Biomarkers are the measure of biochemical, physiological or morphological changes produced in a biological system and they are interpreted as a reflex or marker of a toxic agent (Garte & Bonassi, 2005). The studies of cytogenetic biomonitoring in human populations exposed to pesticides show different results because diverse biomarkers have been widely utilized in heterogeneous populations (Paldy et al., 1987; Rupa et al., 1989a,b; De Ferrari et al., 1991; Carbonell et al., 1993; Bolognesi et al., 2002, 2004). In studies on the exposure to pesticides the genotoxic effects of such biomarkers should be considered; the research should also take into account the damage resulting from the exposure, the robustness of the studies, the similarity of the control groups and the protocols used to determine the genotoxicity (Bull et al., 2006). Most of the adverse effects to health are the result of the genetic damage induced by genotoxic agents in somatic as well as in germinal cells. If this damage occurs, the condition can, among other effects, derive into cancer and contribute to premature aging, cause vascular illness and other similar ailments (Norppa, 2004). A large percentage of the chemical agents delivered to the environment has not been

assessed adequately in relation with genotoxic activity and it is essential to identify the subjects so as to determine the genetic risk to live organisms, including humans (Jamil et al., 2005). The word biomarker has been used very frequently in the last decade. Currently a great amount of research is being done with the objective of finding toxicological biomarkers that will detect different substances because persons are more exposed now than in past decades (Ríos & Solaris, 2010). Cytogenetic damage has been evaluated through biomarkers as chromosomal aberrations (AC), micronuclei (MN), sister chromatid exchange (SCE) and recently the unicellular alkaline electroforesis or comet assay (CoA).

3.1 Chromosomal aberrations

This assay can be used as a reliable biomarker of cellular damage whose increment in lymphocytes can predict cancer risk in humans (Hagmar et al., 1998; Bonassi et al., 2008). Several studies on the chromosomal effect of pesticides have been made using CA; the positive results obtained showed a correlation with the exposure time (Dulout et al., 1985; Paldy et al., 1987; Rupa et al., 1989a; Carbonell et al., 1993; Joksić et al., 1997; Kaioumova & Khabutdinova, 1998; Cuenca & Ramírez, 2004; Zeljezic et al., 2009). Other positive results did not find correlation between the exposure time and the CA induction (Rita et al., 1987; Rupa et al., 1988, 1989b, 1991a, El-Ghazali et al., 1990; Kourakis et al., 1992; Scarpato et al., 1996; Amr, 1999; Au, 1999; Antonucci & de Syllos Colus, 2000; Lander et al., 2000; Paz-y-Miño et al., 2002; Sailaja et al., 2006); meanwhile others authors although also found positive frequency of CA but did not determined this correlation (Nehéz et al., 1988; Jabloniká et al., 1989; De Ferrari et al., 1991; Carbonell et al., 1995; Mohammad et al., 1995; Kourakis et al., 1996; Lander et al., 2000; Garaj-Vrhovac & Zeljezic, 2001, 2002; Ascarrunz et al., 2006; Ergene et al., 2007; Mañas et al., 2009). As well negative results have been obtained (Mustonen et al., 1986; Steenland et al., 1986; Hoyos et al., 1996; D'Arce & de Syllos Colus, 2000; Costa et al., 2006).

3.2 Micronucleus

This assay is a genotoxic biomonitoring method widely used for evaluating exposure risk to pesticides. The micronuclei originate from acentric fragments or whole chromosomes that were not included in either of the daughter nuclei remaining in the cytoplasm, and in the interphase they are observed as small nuclei. The MN showed signs of chromosomal damage and afforded a marker of an early-stage of chronic diseases as cancer; they also revealed an increase in micronuclei frequency predicting cancer risk in humans (Bonassi et al., 2005, 2007). The use of the micronucleus assay in peripheral blood lymphocytes is useful for detecting clastogenic and aneuploidogenic effects together. Bolognesi et al. (1993a) suggested that micronuclei analysis in peripheral blood lymphocytes could be considered a good biomarker of genotoxic exposure to detect early biological effects in individuals having occupational contact with pesticides. The cytokinesis-block micronucleus technique supplied a robust methodology for monitoring human populations (Fenech & Morley, 1985); the studies in populations exposed to pesticides showed positive results with a correlation between the micronuclei frequencies and the years of exposure (Bolognesi et al., 1993a,b, 2002; Pasquini et al., 1996; Joksić et al., 1997; Falck et al., 1999; Bhalli et al. 2006; Costa et al., 2006); positive results without relation to the exposure time (Márquez et al., 2005; Kehdy et al., 2007; Da Silva et al., 2008); positive but not determining this correlation (Garaj-Vrhovac & Zeljezic, 2002; Vlastos et al., 2004; Ascarrunz et al., 2006; Tope et al., 2006; Bolognesi et al.,

2009; Rohr et al., 2010); and negative (Barbosa & Bonin, 1994; Scarpato et al., 1996; Titenko-Holland et al., 1997; Calvert et al., 1998; Venegas et al., 1998; Windham et al., 1998; Holland et al., 2002; Pastor et al., 2002a; Bolognesi et al., 2004; Vlastos et al., 2006).

The MN assay has also been performed in exfoliated buccal cells, which constitutes a minimally invasive method for monitoring populations exposed to pesticides. The assay in exfoliated cells was initially applied at the beginning of the 1980s, using cells of the buccal mucosa to evaluate the genotoxic effect of tobacco (Stich et al., 1982; Stich & Rosin, 1983). Micronuclei are formed by chromosomal damage in the basal cells of the epithelium; when these cells divide themselves, chromosomal fragments or entire chromosomes that lack an attachment to the spindle apparatus are excluded from the main nuclei in the daughter cells and they appear as Feulgen-specific bodies called micronuclei in the cytoplasm. Later, these cells mature and then exfoliate (Rosin, 1992). Other potential sites for MN studies included nasal cavity, bronchi, esophagus, cervix, bladder and urinary tract (Stich et al., 1983; Reali et al., 1987). The analysis of MN in exfoliated buccal cells is relevant because about 92% of cancer cases have an epithelial origin (Rosin & Gilbert, 1990) and recently has been considered as a tool for biomonitoring DNA damage (Holland et al., 2008). Likewise, other nuclear anomalies have been observed: for example, binucleate cells (presence of two nuclei within a cell), condensed chromatin (aggregated chromatin), broken eggs (cinched nuclei with a Feulgen-negative band), pycnosis (shrunken nuclei), karyorrhexis (disintegrated nuclei) and karyolysis (nuclear dissolution, with a Feulgen-negative ghost-like image of the nucleus remaining); all were classified according to Tolbert et al. (1992). This MN assay has been recently used to estimate exposure risk to pesticides. The studies realized have reveled positive results (Gómez-Arroyo et al., 2000; Sailaja et al., 2006; Ergene et al., 2007; Bortoli et al., 2009; Martínez-Valenzuela et al., 2009; Remor et al., 2009). In not any case, correlation between the micronuclei frequency and the exposure time was observed or determined. Other authors have described negative results for the analysis of MN in both peripheral blood lymphocytes and exfoliated buccal cells realized at the same time (Lucero et al., 2000; Pastor et al., 2001a,b, 2002b, 2003). Negative results were also found in peripheral blood lymphocytes, in oropharyngeal cells (Calvert et al., 1998) and in umbilical cord blood cells (Levario-Carrillo et al., 2005).

3.3 SCE

The SCE assay events produced in S-phase (Wolff et al., 1974), is a sensitive biomarker to detect DNA damage (Alptekin et al., 2006). It represents the symmetric interchange between homologous loci of replication products (Wolff, 1982). SCE occur without loss of either DNA or of changes in the chromosomal morphology, and it is possible detect them in metaphase. The assay was based on the incorporation of the thymidine DNA base analog 5-bromodeoxyuridine (BrdU) inside the DNA cells that replicated twice (Latt, 1979; Latt et al., 1981). In addition to SCE analysis, the BrdU differential staining technique can be used to assess the effects of pesticides in cell replication through the cell proliferation kinetics (CPK) (Gómez-Arroyo et al., 2000).

In studies in which SCE has been used to detect exposure risk to pesticides, results have varied: they were positive with correlation between the frequency and the exposure time (Rupa et al., 1991b; Padmavathi et al., 2000; Shaham et al., 2001; Martínez-Valenzuela et al., 2009); they were positive without correlation (Rupa et al., 1988, 1991a; Lander & Rønne, 1995; Scarpato et al., 1996); they were not determined (Jabloniká et al., 1989; De Ferrari et al., 1991; Dulout et al., 1992; Zeljezic & Garaj-Vrhovac, 2002; Ascarrunz et al., 2006), and they

were negative (Steenland et al., 1986; Carbonell et al., 1990; 1993; El-Ghazali et al., 1990; Gómez-Arroyo et al., 1992; Hoyos et al., 1996; Kourakis et al., 1996; Pasquini et al., 1996; Joksić et al., 1997).

3.4 Comet assay

The alkaline single cell gel electrophoresis assay or comet assay has constituted a useful tool for human biomonitoring studies in the detection of DNA single-strand breaks, alkali-labile sites, and incomplete excision repair events. It is a rapid and sensitive assay to demonstrate the damaging effect of several agents on DNA at the individual cell level. Cells in which DNA is damaged display an increased migration of DNA fragments from the nucleus originating a comet shape, given that during alkali gel electrophoresis the broken DNA strands move towards the anode forming a comet (Singh et al., 1988; Fairbairn et al., 1995).
The capacity of DNA to migrate depends upon the size as well as the number of breaks produced by the agent (Garaj-Vrhovac y Zeljezic, 2001). Each damaged cell has the appearance of a comet with head and tail bright; the undamaged cells appear intact or with complete nuclei and no tail (Möller, 2006).
The application of the comet assay to evaluate the DNA damage, biomontoring in populations occupationally exposed to pesticides has demonstrated positive results (Lebailly et al., 1998a,b; Garaj-Vrhovac & Zeljezic, 2000, 2001; Zeljezic & Garaj-Vrhovac, 2001; Ündeğer & Başaran, 2002; Grover et al., 2003; Ascarrunz et al., 2006; Castillo-Cadena et al., 2006; Remor et al., 2009; Rohr et al., 2010). Besides, correlation between the CoA and exposure time was not observed or determined in any of these cases. However, in other studies negative results have been found (Lebailly et al., 2003; Piperakis et al., 2003).

4. Cytogenetic biomonitoring studies

Several groups of workers are exposed to pesticides and the genotoxic effect has focused on evaluating the cytogenetic damage in those who work in open fields and greenhouses, with pesticide sprayers and applicators, as well as in industrial workers and individuals working in sanitation and pest eradication.
The cytogenetic biomonitoring in human populations is a useful tool to estimate the genetic risk from the exposure to complex mixtures of pesticides. Our analysis was based on the review of 88 cytogenetic biomonitoring studies done in the past 25 years (1985 to 2010). Table 1 shows that 64 results were positive and 34 negative; the total is primarily due to the fact that several of those studies included two or more biomarkers that indicated positive results in some and negative ones in others. In creating this table no exclusion criteria was applied, as suggested by Bull et al. (2006). In our case all the studies related with occupational exposure to pesticides were included. Biomonitoring was done using chromosomal aberrations (CA), micronucleus (MN), sister chromatid exchange (SCE) and comet assay (CoA). The studies mentioned above were carried out in different continents of the world as America (Argentina, Brazil, Bolivia, Chile, Colombia, Costa Rica, Ecuador, Mexico and the USA); Europe (Croatia, ex Czechoslavakia, ex Yugoslavia, Denmark, Finland, France, Greece, Hungary, Italy, Poland, Portugal, Spain, Russia and Turkey); Africa (Egypt and Syria); Asia (India, Pakistan and Turkey); the Middle East (Israel) and Australia.
Of all the studies shown in Table 1, 62 were made using only one biomarker: twenty with CA, twenty-five with MN (seventeen of these in peripheral blood lymphocytes, one in exfoliated buccal cells, five using both, one in peripheral blood lymphocytes and

oropharyngeal cells and one in peripheral blood lymphocytes of mothers exposed to pesticides and in the umbilical cord of her newborns); eight with SCE and five with CoA. In 18 studies two biomarkers were used: two with CA and MN (one in peripheral blood lymphocytes and one in exfoliated buccal cells); three with SCE and MN (one in peripheral blood lymphocytes and two in exfoliated buccal cells); two with CoA and MN (one in peripheral blood lymphocytes and one in exfoliated buccal cells); nine with CA and SCE and one with CA and CoA. In 5 studies that were carried out, the three biomarkers used were: CA, SCE and MN (two in peripheral blood lymphocytes; two with exfoliated buccal cells) and 2 with four biomarkers: CA, SCE, CoA and MN (in peripheral blood lymphocytes).

Duration of time exposure has been used as a substitute of exposure in a great number of studies due to the difficulty in making a quantitative evaluation of the exposure. The incidence of CA, MN, SCE and CoA correlated with duration exposure in many of these investigations has been included. The data in Table 1 show that of the 61 positive results only 17 of them established correlation between the time of exposure to pesticides and the cytogenetic effect, since in 20 there was no such correlation. In some cases the authors did not include evidence of exposure as they were considered not determined in this review; in 24 cases these data were not mentioned.

In the same table one observes that of the 85 cases of occupational exposure to pesticides, 67 of them correspond to agricultural workers (mainly greenhouse and open field workers, sprayers, pesticide applicators, mixture preparing workers, etc.), 12 to pesticide production (industrial plant workers), 2 to packing, 2 to agricultural pest eradication, 1 to sanitation programs, and 1 to mothers exposed to pesticides with their newborns. It is important to mention that the occupational groups with highest risk are the sprayers and the greenhouse workers as has been mentioned previously by other authors (Bolognesi, 2003; Bull et al., 2006).

In about 88% of the studies shown in Table 1 the workers had been exposed to pesticide mixtures, and therefore it is very difficult to know what to attribute their effect to. This constitutes a complicated factor for comparing the different studies due to the high number and variety of chemicals used. In several cases the pesticides applied are classified as carcinogenic by the U.S. Environmental Protection Agency (2005) and hazardous by the World Health Organization (WHO, 2004), or they are mentioned as carcinogens by the International Agency for Research on Cancer (IARC, 1991, 2002).

For validation of the studies, the robustness of the biomarkers employed must be known. The data given in Table 1 allow one to calculate that in 84% of the studies analyzing CA, the results are positive and that in 21% the correlation with the time of exposure was established. In relation with micronuclei, 56% of the studies done revealed positive results and 31% showed a correlation between their frequency and the exposure time. The SCE in 66% of the studies was positive and in 50% there was correlation with the exposure time. As to the CoA, 87% of the studies presented positive results, but correlation with the pesticide time exposure was not established. Several studies that have examined biomarkers have found that the micronuclei frequency is less sensitive than CA and SCE (Tates et al., 1994; Van Hummelen et al., 1994). The results obtained of the studies done in human populations exposed to pesticides CA, MN and SCE show these are adequate assays with a good percentage of positive results; besides, the studies carried out on CA and MN have been correlated as predictors of future cancer risk (Bonassi et al., 1995; Smerhovsky et al., 2001). Since in the case of the SCE their biological significance is unknown, their use has progressively disappeared from the scientific literature while new methods have become

available, as is the case of MN (Bonassi et al., 2005). In a review of evidence for the genotoxicity of pesticides Bull et al. (2006) excluded the SCE as endpoint, because the true biological relevance for mutagenecity or carcinogenicity risk were questionable and therefore not useful (Tucker et al., 1993). With respect to CoA , this is an assay that has been used recently to biomonitor populations exposed to pesticides and has had a good percentage of positive results, although correlation was not found with the time of exposure to pesticides.

5. Methods used in the studies on occupational exposure to pesticides in Mexican populations

5.1 Sister chromatid exchanges (SCE) (Fig. 1)

Venous samples were taken with heparinized syringes and transferred to the laboratory within a few hours. Eight drops of blood were added to 3 ml of RPMI medium 1640 with L-glutamine (Gibco) plus 0.2 ml of phytohemagglutinin (Gibco). The cultures were incubated at 37 °C for 72 h. Twenty-four hours later, 5-bromodeoxyuridine (BrdU, Sigma) was added to the culture medium to obtain a final concentration of 5 µg/ml. Afterwards, colchicine (100 µl) was added 2 h prior to the harvest.

Metaphase cells were harvested by centrifugation, treated with 0.075 M KCl and fixed in methanol-acetic acid (3:1). Slides were stained by the fluorescence-plus-Giemsa technique (FPG) (Perry & Wolff, 1974). Fifty second-division metaphases were scored for each sample. Besides SCE examination, the BrdU differential staining technique can be used to assess the effects of pesticides on cell replication. The cell proliferation kinetics (CPK), which is the proportion of first, second and third metaphases, was scored through the analysis of 100 consecutive mitoses for each individual (Fig. 2). The RI is the average number of replications completed by metaphase cells; it was obtained considering the CPK proportion and was

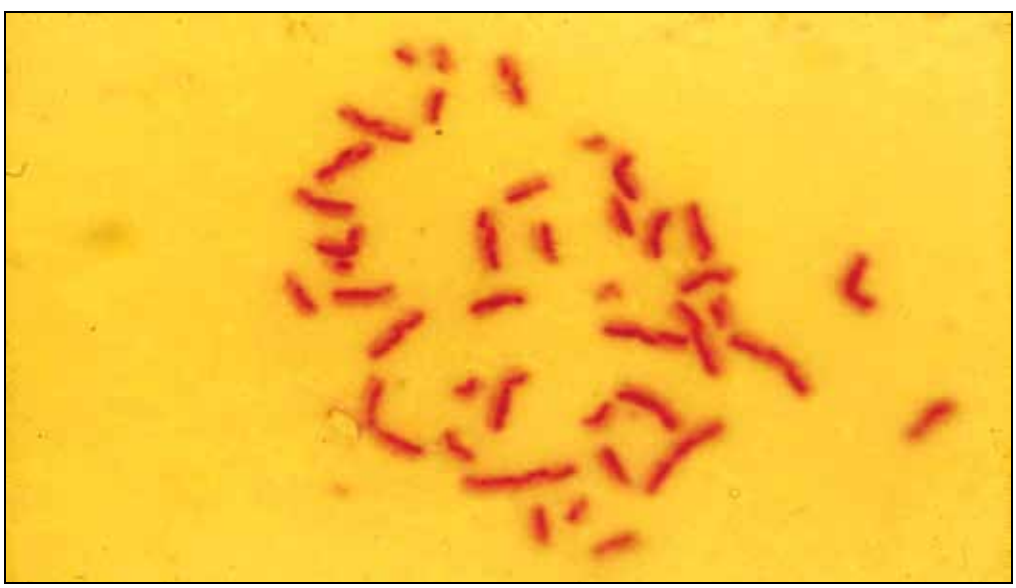

Fig. 1. Metaphase of human peripheral blood lymphocyte after differential staining of sister chromatid with FPG technique

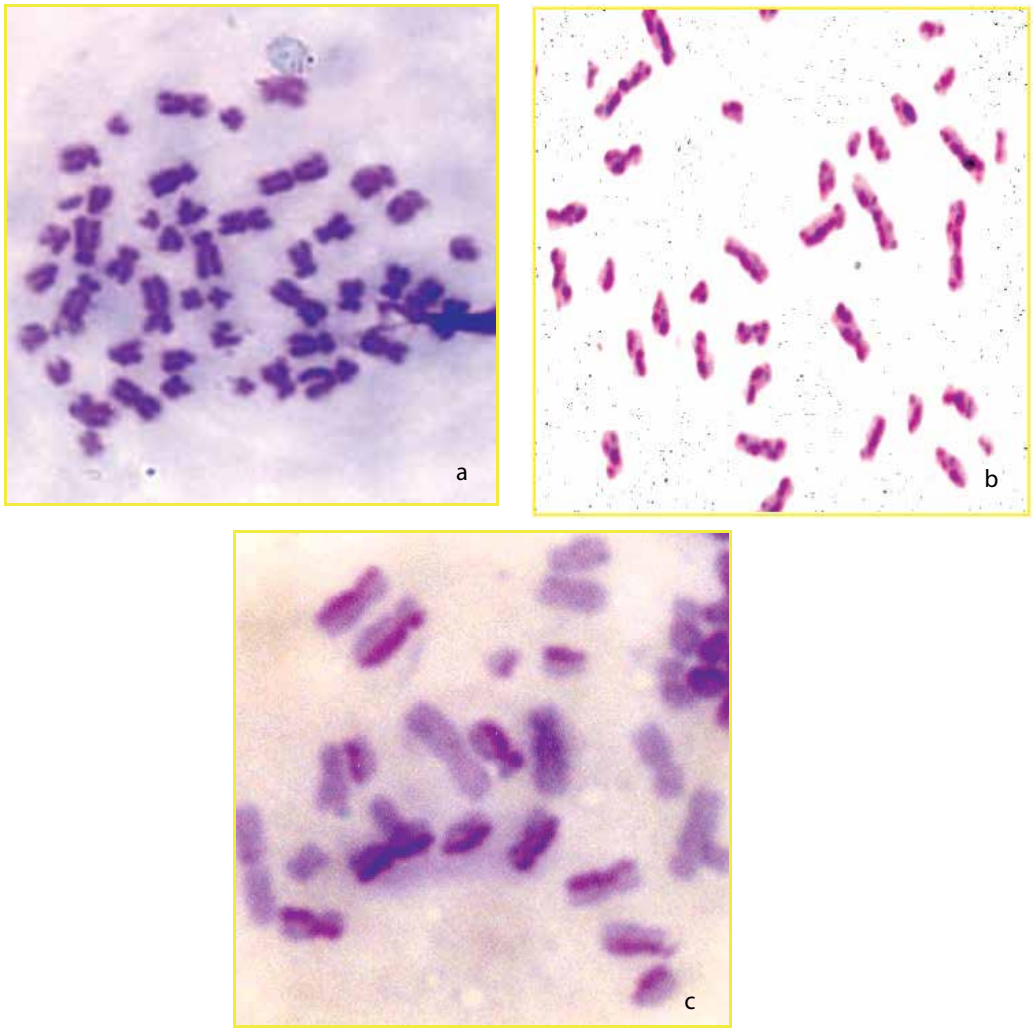

Fig. 2. Metaphases in first (a), second (b) and third (c) division in presence of BrdU

calculated following the formula RI = $1M_1 + 2M_2 + 3M_3/100$. The mitotic index (MI), considered as a measure of the proliferation status of a cell population, was determined in 3000 cells of each donor in order to ascertain the cytotoxic action of pesticides. The slides were handled by code in order to keep their origin unknown and avoid bias.

5.2 Micronucleus test in buccal exfoliated cells (Fig. 3)

The subjects were asked to rinse their mouth with water, and a wooden spatula was used to obtain the sample cells from the buccal mucosa. The sample was then applied to a clean microscope slide. Smears were air dried and fixed in methanol-acetic acid (3:1). The cell smears were stained using the Feulgen reaction technique described by Stich & Rosin (1984) and Stich (1987); it was modified as follows: smears were pretreated with 1 N HCl for 10 min at room temperature, placed for 10 min into 1 N HCl at 60 °C, rinsed in distilled water, put into Schiff's reagent for 90 min and washed with running tap water. The criteria

followed for estimating the frequency of micronucleated cells were according to Stich & Rosin (1984). Three thousand epithelial cells were screened for each individual to determine the micronucleus (MN) frequency, and other nuclear anomalies as broken eggs (BE), karyolysis (KL), karyorrhexis (KR), and binucleate cells (BN), which were classified according to Tolbert et al. (1992). All the slides were also coded before scoring so as to avoid bias.

Fig. 3. Micronucleus of exfoliated buccal cells

5.3 Comet assay in buccal exfoliated cells (Fig. 4)

The comet assay was carried out in the buccal epithelial cells. Alkaline comet assay was performed according to the procedure described previously (Singh et al., 1988; Tice et al., 2000; Speit & Hartmann, 2006) with some modifications. The buccal cells were collected with a small sterile spoon, rinsed three times and resuspended in 50 µl of physiological solution at 37 °C, then added to 50 µl agarose with low melting point (0.75% in phosphate buffer). The sample was carefully stirred, dropped on a coverslide and put on a microscope slide, precoated with normal agarose (1% in phosphate buffer) and kept on ice during the polymerization of each gel-layer. Two slides were made per donor. Slides were then immersed in a tank filled with a freshly made lysis solution (2.5 M NaCl, 100 mM EDTA, 1 mM Tris, 10% DMSO, and 1% Triton X-100, adjusted at pH 10) for 24 h. All the process was done under minimal illumination at room temperature.

To allow DNA unwinding, we incubated slides in a freshly made electrophoresis buffer (300 mM NaOH and 1 mM EDTA, pH 13) for 20 min. The slides were then placed in a horizontal electrophoresis chamber, immersed in fresh electrophoresis buffer, and exposed to 25 volts for 20 min at 300 mA. After electrophoresis, slides were washed twice in freshly prepared

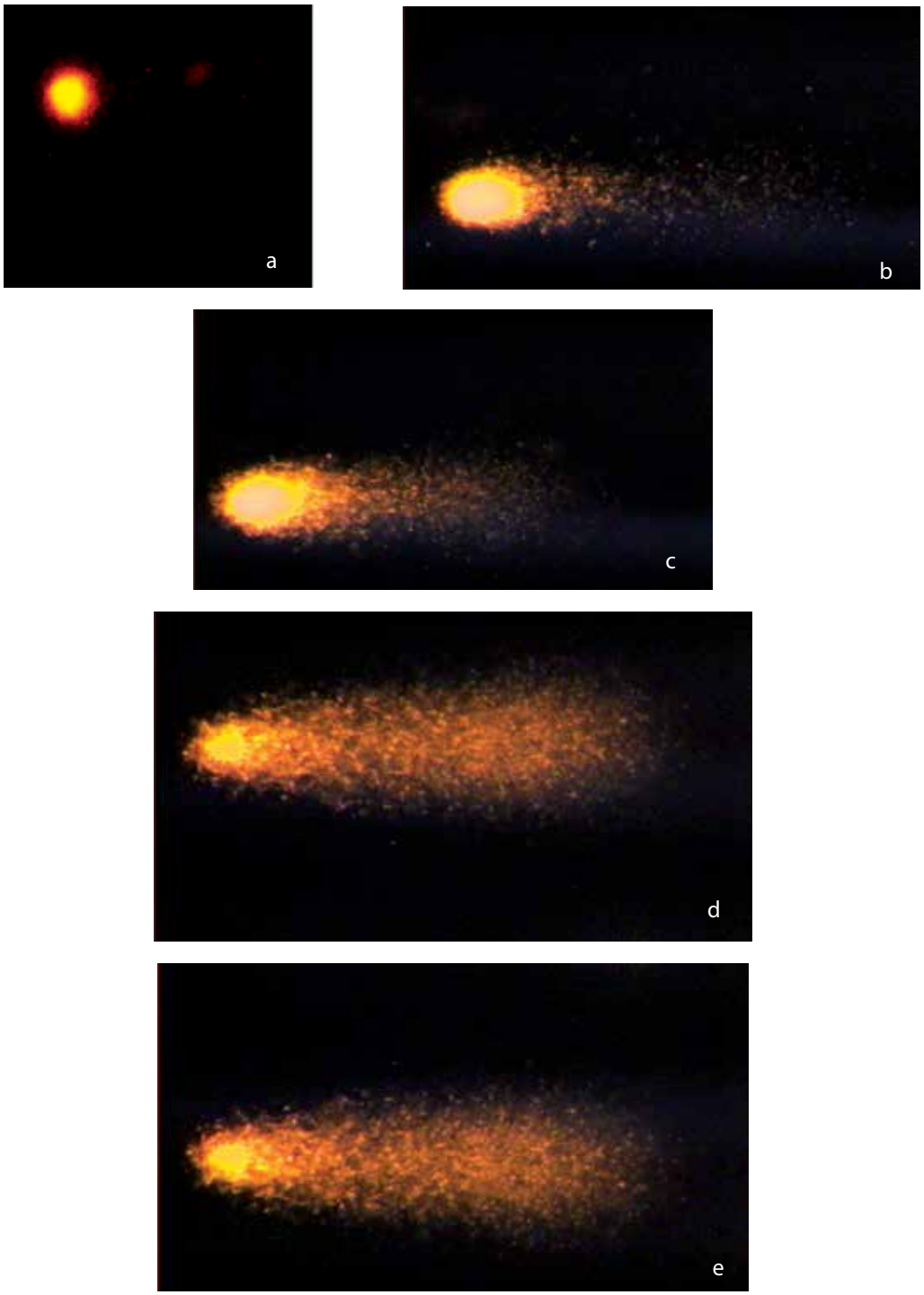

Fig. 4. Exfoliated buccal cells without (a) and with (b, c, d and e) comets having differential length

neutralization buffer (0.4 M Tris, pH 7.5) and fixed in absolute methanol for 5 min and stained with 10% ethidium bromide for 10 min. For each slide 50 cells were analyzed in an epifluorescence Axiostar Plus Zeiss microscope. All the slides were coded before scoring so as to avoid bias.

The statistical analysis was carried out through the Student's t-test, applied to the results of SCE and RI, and the Mann-Whitney U-test was used for MI, MN and other nuclear anomalies. The analysis of variance (ANOVA) was used to determine the effect on cell kinetics (M1, M2 and M3 cells) and the influence of smoking habits, alcohol consumption, age and gender; when significant values were found ($p < 0.001$), the Tukey-Kramer multiple comparison test was used to identify groups showing significant differences at $p < 0.001$. The analysis of correlation was applied between exposure time to pesticides and the frequencies of both SCE and micronuclei and comet assay.

6. Studies of occupational exposure carried out in Mexico

In Mexico human pesticide exposure occurs in workers in open fields and greenhouses, when the mixtures are prepared, and during the spraying; in all cases the individuals are in constant risk of suffering accidents related with these substances. Exposure is increased in tropical regions due the high level of humidity and environmental temperatures which cause these substances to remain in the air movement associated with water molecules; the winds then allow them to reach urban zones, as occurred in the states of Morelos, Sinaloa, Guerrero and other agricultural areas in Mexico. This is why we evaluated the genotoxic effect produced by pesticide mixtures, using SCE in peripheral blood lymphocytes as well as the micronucleus test and the comet assay in exfoliated buccal cells of workers occupationally exposed in four states of the Mexican Republic: Tlaxcala, Morelos, Sinaloa and Guerrero.

A study done in Tlaxcala, Mexico on a rural population exposed to pesticides (Gómez-Arroyo et al., 1992) produced the following results: of 170 men 94 were exposed, with age range under sixteen and older than sixty-five, and duration of pesticide range from one to thirty-five years; 76 were non-exposed showing SCE negative results. This lack of effect could possibly be due to the fact that people were exposed to the pesticides chronically, but for short periods each year and they work on very small parcels of land where the level of exposure was not enough to produce SCE.

The study in Morelos state (Gómez-Arroyo et al., 2000) was made in 30 floriculturists -22 women and 8 men- who worked in greenhouses, and who had ten and one and a half years of pesticide exposure, respectively. The data obtained of the questionnaire filled out by the exposed individuals showed that they did not have smoking or drinking habits. The medical examination revealed that the pesticide exposed workers did not show health problems as cancer or respiratory and digestive disturbances. However, the 22 female floriculturists presented acute intoxication, occasional cephalea, skin and nasal mucosa irritations, and nausea when they were in contact with the pesticides.

The 30 non-exposed individuals with an age average of forty years showed SCE mean $\pm$ S.E. of 4.0 $\pm$ 0.1 in a range of 3 to 5 SCE per cell; in the exposed group the age average was 35.5 $\pm$ 2.22 and the SCE mean $\pm$ S.E. 7.1 $\pm$ 0.17 in a range of 5.5 to 10.7. A Student's t-test showed a significant difference of $p < 0.001$ when these data were compared. When SCE frequencies

were compared between males (7.28 ± 0.27) and females (7.02 ± 0.27) no significant differences were observed, probably due to the different exposure duration among men and woman. Although men had been in contact with the pesticides for only one and a half years and women for ten years, men had to work 10 to 12 h a day, while woman were only exposed for 6 h. The exposure condition for both was in plastic greenhouses with poor ventilation, but men were in charge of spraying the pesticides once or twice every day while the women went out of the greenhouses and returned after the pesticide application; then both continued working in the greenhouses. The lack of correlation between exposure time to pesticides and SCE frequencies might be related to the fact that the group with lower time of exposure has the greatest pesticide exposure.

The cell proliferation kinetics (CPK) was also determined. The controls were 28.78 M1, 41.70 M2 and 29.21 M3, while for the exposed group they were 25.25 M1, 36.46 M2 and 39.23 M3 in which the M2 cells decreased and M3 cells increased significantly meaning that the pesticide exposure induced acceleration of the cell cycle, and the mitotic index also increased.

The micronucleus frequency in epithelial cells of the buccal mucosa of the workers exposed to pesticides was 1.01 ± 0.03 and in the non-exposed individuals it was 0.038 ± 0.021 ($p < 0.001$), a result which allowed concluding that pesticide exposure significantly increases cytogenetic damage in this population exposed to pesticide mixtures. According to Tolbert et al. (1992) the analysis of exfoliated cells of buccal mucosa also provides evidence of other nuclear anomalies as binuclated cells, condensed chromatin, broken egg, karyolysis, pycnosis and karyorrhexis; only in the last three, the results were significant between the individuals exposed to pesticides and the non-exposed.

The occupational exposure of the floriculture workers is intense and acute in closed plastic greenhouses without ventilation. Such workers are considered to have high risk exposure, which is worsened because not only do they not use protective clothing when working in the greenhouses but wear clothes impregnated with the pesticide outside of the work area.

In the study carried out in Sinaloa state (Martínez-Valenzuela et al., 2009) genotoxic damage was evaluated in 70 agricultural workers, 25 women and 45 men, exposed to pesticides in Las Grullas, Ahome, Sinaloa, Mexico, with an average of 7 years of exposure. The effect was detected through the sister chromatid exchanges (SCE) in lymphocytes of peripheral blood and micronuclei (MN) and other nuclear anomalies (NA) in buccal exfoliated cells. Also, the influence on (CPK) was studied by means of the replication index (RI) and the cytotoxic effect was examined with the mitotic index (MI). The non-exposed group consisted of 70 individuals, 21 women and 49 men from the city of Los Mochis, Sinaloa, Mexico. The SCE mean ± S.E. in the exposed group was 6.36 ± 0.22 and in the non-exposed 3.71 ± 0.11, significant differences were obtained between them $p < 0.001$. The analysis of correlation between the average values of SCE and exposure time to pesticides evidenced a significant correlation ($p < 0.001$). In the non-exposed group CPK had a parametric distribution with 30.7 M1, 45.9 M2 and 23.2 M3 cells, while in the exposed group the M1 was 24.3, M2 decreased significantly to 33.8 and M3 increased significantly to 41.9 when we applied the ANOVA and the Tukey-Kramer multiple comparisons test. These results mainly showed that in the pesticide exposed group there were induced alterations in the CPK due to the fact that M2 cells decreased but M3 cells increased significantly, suggesting that the pesticide exposure had induced an acceleration of the cell cycle; the same behavior was observed in the floriculture workers of Morelos (Gómez-Arroyo et al., 2000). No significant differences

were found regarding CPK between alcohol consumers and smokers in either the exposed group or the non-exposed group. Age, gender and time of exposure did not correlate with CPK.

MN frequencies in the exposed group were 2.83 ‰ and 0.37 ‰ in the non-exposed group; the Mann-Whitney U-test was significant ($p < 0.0001$). The MN frequency was not correlated with age, gender and exposure time to pesticides. When we compared MN frequency between exposed smokers and alcohol consumers, and between exposed non-smokers and non-alcohol consumers, no statistical differences were found. We did not find correlation between exposure time and MN frequency.

The agricultural workers referred to in the present study were exposed to complex mixtures of pesticides that had different active ingredients, mainly organophosphorus and carbamates. Some of those active ingredients include two compounds which according to WHO (2004) are "extremely hazardous" (parathion-methyl and aldicarb) and five that are "highly hazardous" (azinphos-methyl, monocrotophos, gusathion, lannate and vydate).

In three locations of Guerrero state (Carbajal-López et al., unpublished data), the study was made in 111 agricultural workers exposed to pesticide mixtures; the individuals were from the towns of Arcelia (62), Ajuchitlan (13) and Tlapehuala (36). Their exposure ranged from 1 to 57 years, and ages extended from 13 to 83 years; the non-exposed group constituted 50 individuals whose ages went from 15 to 66 years. All the participants were males working in open fields; they used no protective measures, but they mentioned that they did use clean cloths after handling the pesticides and washed their hands before eating. Cells of the buccal ephitelium were sampled and the comet assay was used as biomarker to know the DNA damage. The average of the comet tail was screened in 100 cells of each individual: in the exposed group, the mean $\pm$ S.D. of cells with comet was 81.11 $\pm$ 12.75 and the tail length was 190.33 $\pm$ 43.26 μm; in the non-exposed group it was 8.72 $\pm$ 3.85 and 106.08 $\pm$ 20.04 μm, respectively. The micronuclei test was carried out in 3000 epithelial cells for each participant: the mean $\pm$ S.D. in the exposed group was 2.33 $\pm$ 1.16 and in the non-exposed 0.88 $\pm$ 0.56; other nuclear anomalies as broken eggs, karyolysis, karyorrhexis and binucleate cells were also evaluated. The results revealed that in the exposed group of the three areas studied the frequency of cells with comet increased significantly in relation with the non-exposed group. The same behavior was observed in the tail migration of DNA. Micronuclei exhibited significant differences between the exposed and the non-exposed groups, and they showed nuclear anomalies associated with a cytotoxic or genotoxic effect. No positive correlation was noted between exposure time and comet tail length, nor between cells with comet and with micronuclei frequency. No significant effect on genetic damage was observed as a result of smoking and alcohol consumption. This study afforded valuable data for establishing the possible risk to human health associated with pesticide exposure.

Due to the fact that the smoking and alcohol drinking habits have been considered confounding factors that could influence the frequency of genetic damage, we evaluated these risk factors. In none of the four studies carried out we did find statistical differences between exposed smokers and alcohol consumers in relation with non-smoker and non-alcohol consumer (Gómez-Arroyo et al., 1992, 2000; Martínez-Valenzuela et al., 2009; Carbajal-López et al., unpublished). No significant difference was observed in SCE and micronuclei frequency in either gender or age (Gómez-Arroyo et al., 2000; Martínez-Valenzuela et al., 2009).

7. Relevance of our studies

Mexico can be considered as a "mega-diverse" country belonging to the group of countries that have a great diversity of animals and plants, and which have almost 70% of the world diversity of species. This group of twelve countries comprises Mexico, Colombia, Ecuador, Peru, Zaire, Madagascar, China, India, Malacca, Indonesia and Australia. However, Mexico is characterized with having regions for agriculture development mainly in the Northwest of the country, in addition to using mixtures of pesticides that affect not only the health of the persons involved but also the environment.

The population in the rural environment suffers various cultural conditions such as a high level of illiteracy and low education. Such factors prevent the agricultural workers from knowing and developing an awareness of the risk involved in working in direct and indirect contact with these compounds; for example, in most cases they handle pesticides without any type of protection.

Rural workers lack social support from the landowners who make labor arrangements verbally or in most cases through intermediaries. Besides, the workers do not have medical insurance (Gómez & García, 2002). In the Northwest of the Mexican Republic, the agricultural activity is outstanding but climatic conditions have favored the development of pests and plant diseases, creating a culture related with the use of pesticides. In the fields, children between the ages of 6 and 14 years very often collaborate in agricultural activities and are exposed to pesticides, as are scarcely newly born infants being carried by their mothers during the long work day in the crops. Likewise, persons living on or near treated croplands can be exposed through agricultural application, as in most parts of Mexico where huge amounts of pesticides are sprayed in the crop fields. The problem is more critical when the pesticide mixtures are applied aerially with the use of small planes, which is a method that contaminates more since only part of the pesticide mixtures reach the crops and the rest are distributed on other places.

In 2008 the Ministry of Health reported death in females older than 40 years produced by cancer in the breast (15%), in the uterus (14%), as well as in liver and bile ducts (9.2%). In men, deaths resulting from tumors were due to prostate (17.1%), and to lung, trachea and bronchi (16%). The register of the Ministry of Health showed that agricultural areas of Mexico present a high incidence of cancer. This is why we must mention the enormous lack of information related with scientific investigation, which evidences the effects generated by the use of pesticides and their mixtures, not only in the occupationally exposed workers but also in families that live on or near the crop lands and in the general population. Therefore, we carried out our studies in several states of the Mexican Republic which have been pioneers in the use of biomarkers as SCE, MN and CoA. They constitute evidence that supports the fact that pesticide exposure caused genotoxic damage, and they afford the scientific bases for the authorities to take the corresponding decisions.

Considering the abovementioned information, the introduction of agricultural practices to reduce the use of pesticides is important; furthermore, the utilization of measurements for biological control as well as the integration of pest management is relevant. Also important are efforts to intensify and permanently train the workers in agricultural practices so as to increase prevention activities and improve education in the community.

Cytogenetic biomonitoring is very important because it is the basis for integrating correct medical watchfulness; it allows evaluating the potential risk of occupational exposure and helps take the right steps to identify genetic risks earlier.

No. of individuals and exposure type	Biomarker	Country	Exposure time (years) [1]range [2]average N.D. not determined	Result [a]with or [b]without time exposure correlation [c]not determined	Reference
36 floriculturist (21 with chronic intoxication symptoms, 9 female and 11 male and 15 without intoxication symptoms 7 female and 9 male) exposed to mixtures of organophosphorus, carbamate and organochlorine pesticides; and 15 healthy donors	CA, SCE	Argentina	At least 10	Positive[a]	Dulout et al., 1985
19 male pesticide sprayers exposed to phenoxyacetic acids, and 15 male controls	CA	Finland	N.D.	Negative	Mustonen et al., 1986
60 male working in papaya-packing plants exposed to ethylene dibromide, and 42 male controls	CA, SCE	USA (Hawaii)	5[2]	Negative	Steenland et al., 1986
80 male workers exposed to complex mixtures of pesticides organophosphates, dithiocarbamates, nitro compounds, triazines, ureas, phtalamides, organochlorines, phenoxy-acetic acids, pyrethroids, cabamates, heterocyclic compounds, among others, and 24 male controls	CA	Hungary	1 to >15[1]	Positive[a]	Paldy et al., 1987
15 workers of grape gardens exposed to pesticides DDT, lindane, quinalphos, diethane M_{45}, metasystox, parathion, cooper sulfate, dichlorvos and dieldrin, and 10 controls	CA	India	5 to 15[1]	Positive[b]	Rita et al., 1987
55 male working with pesticides in open fields (14) or in closed space (41) exposed to mixtures of organophosphates, carbamates, pyrethroids, fungicides and acaricides, and 60 male controls	CA	Hungary	1 to 15[1]	Positive (in open fields) [c] Negative (in closed space)	Nehéz et al., 1988
25 male workers exposed to DDT, BHC, malathion, parathion, dimethoate, fenitrothion, urea and gromor, and 30 male controls	CA, SCE	India	5 to 38[1]	Positive[b]	Rupa et al., 1988
44 workers (30 male and 14 female) exposed to mancozeb during the production of the pesticide novozir Mn80 and 30 control (18 male and 12 female)	CA, SCE	Ex Czech oslavakia	Up to 2	Positive[c]	Jabloniká et al., 1989
50 smokers exposed to insecticides DDT, BHC, endosulfan, malathion, methyl parathion, monocrotophos, quinolphos, dimethoate, phosphamidon, cypermethrin, and fenvelrate, and 47 controls (30 non-smokers and 27 smokers)	CA	India	5 to 25[1]	Positive[a]	Rupa et al., 1989a
52 male pesticide sprayers exposed mainly to DDT, BHC, endosulfan, malathion, methyl parathion, monocrotophos, quinolphos,	CA	India	1 to 25[1]	Positive[b]	Rupa et al., 1989b

dimethoate, phosphamidon, cypermethrin, and fenvelrate, and 25 male controls					
50 smoking pesticide sprayers exposed to DDT, BHC, endosulfan, malathion, methyl parathion, dimethoate, monocrotophos, phosphamidon, quinolphos, fenvelrate and cypermethrin, and 47 controls (20 non-smokers and 27 smokers)	SCE	India	1 to 25[1]	Positive[a]	Rupa et al., 1989c
27 workers exposed to pesticide mixture mainly benomyl, captan, deltamethrin, fenvelrate, methomyl and paraquat, and 28 controls	SCE	Spain	10[2]	Negative	Carbonell et al., 1990
28 workers packing pesticides and 20 controls	CA, SCE	Egypt	12.9±6.2[2]	Positive (CA)[b] Negative SCE	El-Ghazali et al., 1990
32 healthy floricultors and 32 individuals hospitalized for bladder cancer (without radio- or chemo-therapy before blood sampling) exposed to pesticide mixtures as nitro-organic herbicides and fungicides, nitrothiorganics, organophosphates, organothiophosphates, organochlorines, pyrethroids, among others, and 31 controls	CA, SCE	Italy	N.D.	Positive[c]	De Ferrari et al., 1991
26 male pesticide applicators exposed to endosulfan, malathion, methyl parathion, dimethoate, phosphamidon, monocrotophos, quinalphos, cypermethrin and fenvelrate, and 26 male controls	CA	India	2 to 18[1]	Positive[b]	Rupa et al., 1991a
61 male pesticide applicators who sprayed DDT, BHC, endosulfan, malathion, methyl parathion, phosphamidon, dimethoate, monocrotophos, quinalphos, fenvelrate and cypermethrin, and 45 male controls	SCE	India	1 to 20[1]	Positive[a]	Rupa et al., 1991b
27 floriculturist exposed to pesticide mixture 14 with chronic intoxication symptoms and 13 without chronic intoxication symptoms, and 32 non-exposed	SCE	Argentina	About 10	Positive[c] in both groups (the median value is higher in floriculturist with chronic intoxication symptoms)	Dulout et al., 1992
94 male rural workers exposed to mixtures of insecticides organophosphates, organochlorines and carbamates, fungicides as manzate, mancozeb, benomyl and carbendazin, herbicides mainly triazines, hormones, thiocarbamics, and ureics, and 70 male controls	SCE	Mexico	1 to 35[1]	Negative	Gomez–Arroyo et al., 1992

29 pesticide greenhouses sprayers exposed at the same mixtures of organophosphates, carbamates, dithiocarbamates, and organochlorines, and 14 controls	CA	Greece	4 to 30 [1]	Positive[b]	Kourakis et al., 1992
71 floriculturists (57 male and 14 female) in open fields or in greenhouses exposed to pesticide mixtures as dithiocarbamates, organophosphates, and organochlorines, and 75 control (66 male and 9 female)	MN (peripheral blood lymphocytes)	Italy	2 to 55[1]	Positive[a]	Bolognesi et al., 1993a,b
70 male working in flower and fruit cultivation exposed to pesticides organochlorines, organophosphorus, carbamates, and pyretroids, fungicides as cooper compounds, thiocarbamates, heterocycles, and antibiotics, and 69 male controls	CA, SCE	Spain	5 to 29[1]	Positive (CA)[a] Negative (SCE)	Carbonell et al., 1993
31 fumigators exposed to phosphine during the high fumigation season, and 21 controls	MN (peripheral blood lymphocytes)	Australia	1.5 to 32[1] 11.6[2]	Negative	Barbosa & Bonin, 1994
9 male sprayers exposed to deltamethrin and cypermethrin and 7 agricultural workers exposed to pesticide mixture, and 6 male controls	CA	Syria	3 to 38[1]	Positive[c]	Mohammad et al., 1995
134 greenhouse sprayers (118 male and 16 female) exposed to pesticide complex mixture of almost 50 insecticides, fungicides and growth regulators, and 157 referents (137 male and 20 female)	SCE	Denmark	1 to 50[1] 17[2]	Positive[b]	Lander & Rønne 1995
29 male agricultural workers exposed to pesticide mixtures manly carbamates, heterocycles, organochlorines, organophosphorus, and pyrethroids, among others, and 29 and 24 male controls	CA	Spain	N.D.	Positive (in the period of major exposure) [c] Negative (in the period of minor exposure)	Carbonell et al., 1995
30 workers (26 male and 4 female) exposed to mixtures of insecticides as carbamates and orgaphosphates, fungicides as dithiocarbamates and carbamates, and 30 controls (26 male and 4 female)	CA, SCE	Colombia	16.5±8.8[1]	Negative	Hoyos et al., 1996
56 (29 indoor and 27 outdoor) agricultural workers exposed to mixture of orgaonophosphorus, carbamates, dithiocarbamates, and organochlorine, and 30 controls	CA, SCE	Greece	At least 6	Positive (CA)[c] Negative (SCE)	Kourakis et al., 1996
48 male agricultural workers exposed to pesticide mixtures of carbaryl, delthametrin, benomyl, dinocap, oxadixyl, propineb, mancozeb, triadimenol, alachlor, atrazine, linuron, MCPA, metobromuron, metalachlor and oxyfluorfen, and 50 male controls	MN (peripheral blood lymphocytes), SCE	Italy	4 to 50[1]	Positive (MN) [a] Negative (SCE)	Pasquini et al., 1996

43 greenhouse floriculturist (24 male and 19 female) exposed to pesticide mixtures of more than 100 of active ingredients, and 42 controls (22 male and 20 female)	CA, MN (peripheral blood lymphocytes), SCE	Italy	N.D.	Positive (SCE and CA in smokers) [b] Negative (MN)	Scarpato et al., 1996
27 male vineyard growers exposed to pesticide mixtures of the insecticide diazinon and fungicides dithiocarbamates being the most commonly used, and 35 male controls	CA, MN (peripheral blood lymphocytes), SCE	Ex Yugoslavia	12.1±6.02[2]	Positive (CA and MN)[a] Negative (SCE)	Joksić et al., 1997
38 malathion exposed workers (29 male and 9 female) involved in the Mediterranean Fruit Fly Eradication Program, and 16 unexposed (9 male and 7 female)	MN (peripheral blood lymphocytes)	USA	At least 6 months	Negative	Titenko-Holland et al., 1997
32 male methyl bromide-exposed fumigation workers, and 28 male referents	MN (peripheral blood lymphocytes and oropharyngeal cells)	USA	0.3 to 22[1] 3[2]	Negative	Calvert et al., 1998
19 herbicide plant workers (17 male and 2 female) exposed to 2,4,5-trichlorphenol (2,4,5-T) and 2,4-dichlorophenoxiacetic acid (2,4-D) (dioxin-containing products), and 36 controls	CA	Russia	10 to 30 [1]	Positive[a]	Kaioumova & Khabutdinova, 1998
29 male farmers (14 non-smokers, 7 ex-smokers and 8 smokers) exposed to pesticides	CoA	France	N.D.	Positive[c]	Lebailly et al., 1998a
29 male farmers (8 smokers) exposed to mixtures of dimethoate, ethephon, omethoate, oxydemeton-methyl, thiometon, befenthrin, b-cyfluthrin, deltamethrin, mancozeb, carbendazin, endosufan, chlorothalanil, iprodine, diflufenicanil, l-cyhalothrin, pymethanil, fluroxypyr, cyproconazole, epoxyconazole, flutriafol, tebucanazole, atrazine, MCPA, isoproturon, 2,4-D, amidosulfuron, bentazon, bifenox, bromoxynil, clopyralid, fenopropadin, imazamethabenz-methyl, ioxynil, mecoprob and sethoxydim a longitudinal study on the same individuals (each farmer was his own control)	CoA	France	N.D.	Positive[c]	Lebailly et al., 1998b
22 pesticide sprayers exposed to mixture of the insecticides deltamethrin, dichlorvos, diazinon, methamidophos, cyfluthrin, propoxur, cypermethrin, endosulfan, parathion, among others, herbicides and fungicides as linuron, captan pentachlorophenol, methyl bromide,	MN (peripheral blood lymphocytes)	Chile	About 7	Negative	Venegas et al., 1998

among others, and the raticides bromadialone, brodifacoum, coumatetralyl, and diphacinone, and 16 controls					
53 workers exposed to malathion in Medfly eradication (40 male and 13 female), and 4 male controls	MN (peripheral blood lymphocytes)	USA	N.D.	Negative	Windham et al., 1998
39 male formulators and 32 male applicators exposed to insecticides organochlorine, carbamates as propoxur, organophosphates as dichlorvos, dimethoate and malathion and pyrethroids as cypermethrin, D-allethrin, deltamethrin, and sumithrin, and 40 male controls (20 for each group)	CA	Egypt	5 to 15 [1] 5 to 25 [1]	Positive[c]	Amr, 1999
20 male workers exposed to pesticide mixture of chlorpyriphos, dibromochloropropene, fenamiphos, gramoxone, imalzabile, terbufos, and thiabendazole, and 20 male controls	CA	Costa Rica	N.D.	Positive[c]	Au et al., 1999
34 greenhouse workers (20 male and 14 female) exposed mainly to acephate, azocyclotin, benfuracarb, captan, chlorothalonil, dichlorvos, dicofol, dimethoate, endosulfan, fenopropathrin, iprodione, mancozeb, mathalaxyl, methiocarb, metiram, methomyl, procymidone, propineb, toclofos, methyl trichlorfon, and vinclozin, and 33 controls (17 male and 16 female)	MN (peripheral blood lymphocytes)	Italy	7 to 41[1] in sprayers with extensive contact, 8 to 27[1] and 2 to 26[1] in sprayers and others with less contact	Positive [a]	Falck et al., 1999
23 workers exposed to pesticide mixtures of carbamates and organophosphates, and 23 controls	CA	Brazil	0 to 16[1]	Positive[b]	Antonucci & de Syllos Colus, 2000
20 male workers exposed to the insecticides tamaron, orthene, nuvacron, folidol, endosulfan, lannate and vertimec, the bactericides agrimicin, primycin, microshield and recop; the fungicides manzate, benlate, dacosar, cercobin, folicur and curzate and the herbicides roundup, and sencor, and 16 male controls	CA	Brazil	10 to 40[1]	Negative	D'Arce & de Syllos Colus, 2000
10 workers (7 male and 3 female) employed in pesticide production simultaneously exposed to atrazine, alachlor, cyanazine, 2,4-dichlorophenoxyacetic acid, and malathion, and 10 controls (7 male and 3 female)	CoA	Croatia	4 to 30[1] 22.2[2]	Positive (after period of high pesticide exposure) [c] Positive (but significantly decreased 6 months out of the pesticide exposure) [c]	Garaj-Vrhovac & Zeljezic, 2000

30 greenhouse floriculturist (22 female and 8 male) exposed to pesticide mixtures meanly organochlorine, organophosphates, and carbamates, and 30 controls (28 female and 2 male)	MN (exfoliated buccal cells), SCE	Mexico	1.5 to 10[1]	Positive[b]	Gómez-Arroyo et al., 2000
116 greenhouse workers exposed to a complex mixture of almost 50 insecticides, fungicides and growth regulators, and 29 non-pesticide exposed	CA	Denmark	N.D.	Negative (in preseason) Positive (after summer season)[c]	Lander et al., 2000
64 male greenhouse workers exposed to insecticides as abamectine, acrinathrin, buprofesin cyromazine, dichlorvos, endosufan, formetanate, midacloprid, malathion, methamidophos, methomyl, oxamyl, permethrin, pyriproxyfen, tebufenozide and tralomethrin, bactericides as kasugamycin, fungicides as carbendazim, cymoxanil, diethofencarb, mancozeb, nuarimol, fosetyl-aluminium, procymidone, propamocarb, and propineb, and 50 male controls	MN (peripheral blood lymphocytes and exfoliated buccal cells)	Spain	9.82±1.01[2]	Negative	Lucero et al., 2000
135 workers (83 non-smokers and 52 smokers) from organophosphorus pesticide industry and 111 control (65 non-smokers and 46 smokers)	SCE	India	1 to 24[1]	Positive[a]	Padmavathi et al., 2000
20 workers (17 male and 3 female) of pesticide production of atrazine, alachlor, cyanazine, 2,4-dichlorophenoxyacetic acid, and malathion, and 20 controls (12 male and 8 female)	CA, MN (peripheral blood lymphocytes), SCE, CoA	Croatia	4 to 30[1]	Positive (after period of high pesticide exposure and 8 months out of the pesticide exposure)[c]	Garaj-Vrhovac & Zeljezic, 2001
49 male workers exposed to pesticides as the insecticides deltamethrin, dimethoate, methomyl, carbosulfan, lamba-cyhalothrin, cafenvalerate, pirimicarb, acetamiprid, diazinon, and the fungicides chlorothalonil, propamocarb, vinclozolin, iprodione, triforine, thiophanate, bupirimate, captan, among others, and 50 male controls	MN (peripheral blood lymphocytes and exfoliated buccal cells)	Poland	16.28±1.1[2]	Negative	Pastor et al., 2001a
50 agricultural workers (30 male and 20 female) exposed to complex mixtures, and 66 non-exposed (41 male and 25 female)	MN (peripheral blood lymphocytes and exfoliated buccal cells)	Greece	8.62±1.13[2]	Negative	Pastor et al., 2001b
104 greenhouse farmers exposed to pesticide mixture mainly organophosphates, carbamates, glyphosate, pyrethroids, triazoles, phthalamides, organochlonines, and phenoxyacetic acid, and 44 unexposed individuals	SCE	Israel	2.5 to 55.5[1]	Positive[a]	Shaham et al., 2001

20 workers (17 male and 3 female) of pesticide production simultaneously exposed to complex mixture of atrazine, alachor, cyanazine, 2,4-dichlorophenoxyacetic acid, and malathion, and 20 controls (12 male and 8 female)	CA, CoA	Croatia	4 to 30[1] 22.5[2]	Positive (after period of high pesticide exposure and 8 months out of the pesticide exposure)[c]	Zeljezic & Garaj-Vrhovac, 2001
107 floriculturist (92 male and 15 female) of greenhouses and open field exposed to mixture mainly of organophosphates and carbamates, and 61 control subjects (42 male and 19 female)	MN (peripheral blood lymphocytes)	Italy	2 to 70[1] 27.8±15.5[2]	Positive[a]	Bolognesi et al., 2002
10 workers (7 male and 3 female) of pesticide production simultaneously exposed to complex mixture of atrazine, alachlor, cyanazine, 2,4-dichlorophenoxyacetic acid, and malathion, and 20 controls (12 male and 8 female).	CA, MN, (peripheral blood lymphocytes), CoA	Croatia	4 to 30[1] 22.5[2]	Positive[c]	Garaj-Vrhovac & Zeljezic, 2002
12 male applicators exposed to the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D), and 9 male controls	MN (peripheral blood lymphocytes)	USA	N.D.	Negative	Holland et al., 2002
39 male greenhouse workers exposed to pesticide mixture mainly carbamates, organophosphorus, and pyrethroids, and 22 male non-exposed	MN (peripheral blood lymphocytes)	Spain	8.31±1.12[2]	Negative	Pastor et al., 2002a
84 workers (58 male and 26 female) exposed in greenhouses and open fields to mixtures of insecticides, fungicides, and herbicides, and 65 controls (53 male and 12 female)	MN (peripheral blood lymphocytes and exfoliated buccal cells)	Hungary	18.75±0.89[2]	Negative	Pastor et al., 2002b
41 workers (28 male and 13 female) exposed to pesticides as aldicarb, fenamiphos, benomyl, captan, carbofuran, cypermethrin, deltamethrin, endosulfan, methyl bromide, among others, and 41 non-exposed (28 male and 13 female)	CA	Ecuador	6 to 66[1] 39.49[2]	Positive[b]	Paz-y-Miño et al., 2002
33 male workers exposed to pesticide mixtures such as pyrethroids, carbamates, heterocycles, and organophosphates, and 33 male controls	CoA	Turkey	1 to 23[1] 10[2]	Positive [b]	Ündeğer & Başaran, 2002
20 workers (17 male and 3 female) of pesticide production simultaneously exposed to complex mixture of atrazine, alachlor, cyanazine, 2,4-dichlorophenoxyacetic acid, and malathion, and 20 (12 male and 8 female) controls	SCE	Croatia	4 to 30[1]	Positive (after period of high pesticide exposure and 8 months out of the pesticide exposure)[c]	Zeljezic & Garaj-Vrhovac, 2002
54 pesticide workers (42 male and 12 female) employed in a pesticide manufacturing mainly acephate, chlorpyriphos, phorate, fenvalerate, cypermethrin, monocrotophos, and dimethoate, and 54 controls (43 male and 11 female)	CoA	India	3 to 13[1] 8.57[2]	Positive[b]	Grover et al., 2003

19 male fruit growers exposed to captan mixed with other pesticides as fungicides, insecticides, and herbicides, and 1 male control	CoA	France	N.D.	Negative	Lebailly et al., 2003
247 agricultural workers (201 male and 46 female) exposed to complex mixtures of pesticides mainly carbamates, organophosphates, and pyrethroids, and 231 unexposed controls (194 male and 37 female)	MN (peripheral blood lymphocytes and exfoliated buccal cells)	Greece Spain Poland Hungary	8.62±1.3[2] 9.82±1.03 16.28±1.1 18.75±0.89	Negative	Pastor et al., 2003
50 workers of greenhouse (30 male and 20 female) exposed to pesticide mixture mainly insecticides as buprofezin, cyromazine, dichlorvos, endosulfan, imidacloprid, malathion, mathamidophos, methomyl, oxamyl, permetryn, pyriproxyfen and tralometrin; bacetericides as kasugamycin and fungicides as carbendazin, cymoxalin, diethofencard, mancozeb, fosetyl-aluminium, procymidone, propamocard, and propined, and 66 controls (41 male and 25 female)	CoA	Greece	8.62±1.13[2]	Negative	Piperakis et al., 2003
52 floriculturists in greenhouses (86.2% male and 7% female) exposed to pesticide mixture mainly organophosphates, carbamates benzimidazoles, pyrethroids, tiophtalamides, pyrimidinol compounds, organochlorines, bypiridylics, amides, and morpholinics, and 24 controls (62.50% male and 9% female)	MN (peripheral blood lymphocytes)	Italy	26.35±14.46[2]	Negative	Bolognesi et al., 2004
10 female workers directly exposed to the fungicides imazalil and tiabendazol, and the insecticide chlorpyriphos, and 10 female controls	AC	Costa Rica	14[2]	Positive[a]	Cuenca & Ramírez, 2004
11 farmers exposed to a mixture of metalaxyl and imidacloprid before and after spaying, and 11 controls	MN (peripheral blood lymphocytes)	Greece	23.64. ±4.13[2]	Positive[c] (before and after spraying)	Vlastos et al., 2004
21 healthy newborns who preganacies developed without complications of urban areas; 12 healthy newborns from Mexico City whose pregnancies were without complications and the mothers no reported occupational exposure to toxic compounds, 16 whose mothers lived in agricultural areas, and 15 with mothers with high risk pregnancies	MN (umbilical cord blood and peripheral blood from the mothers)	Mexico	N.D.	Negative	Levario-Carrillo et al., 2005
64 female agricultural workers exposed to pesticide mixtures in thinning and pruning fruit trees in harvesting and packing fruits the pesticides most often used were carbamates, organophosphates, and pyrethroids, and 30 female controls	MN (peripheral blood lymphocytes)	Chile	8.0±4.8[2]	Positive[b]	Márquez et al., 2005

259 individuals were studied: 131 agricultural workers exposed to pesticides, 77 controls and 51 pesticide manager (30% female and 70% male)	CA, SCE, MN (peripheral blood lymphocytes), CoA	Bolivia	At least 5	Positive[c]	Ascarrunz et al., 2006
29 male workers involved in the pesticide manufacturing industry exposed to mixtures specifically organophosphates and pyrethroids, and 35 male controls	MN (peripheral blood lymphocytes), SCE	Pakistan	13.48±3.48[2]	Positive[a]	Bhalli et al., 2006
52 floriculturist (37 male and 15 female) in greenhouse exposed to pesticide mixture mainly organophosphates, organochlorines, carbamates, and pyrethroids, and 38 controls (22 male and 16 female)	CoA	Mexico	2 to 48[1]	Positive[b]	Castillo-Cadena et al., 2006
33 farmers (17 male and 16 female) exposed to fungicides as benzimidazoles, azoles, pyrimidines, dithiocarbamates, triazines, insecticides mainly organophosphates, pyrerthroids, carbamates and organochlorine, the rodenticide acrinathrin, acaricides as N-methyl carbamates and herbicides as phosphoglycines and ureas, among others, and 33 controls (17 male and 16 female)	CA, MN (peripheral blood lymphocytes), SCE	Portugal	0.5 to 48[1]	Positive (MN and SCE)[a] Negative (CA)	Costa et al., 2006
54 pesticide workers (12 female and 42 male) employed in a pesticide-manufacturing exposed simultaneously to a complex mixture of organophosphates, carbamates, and pyrethroids, and 54 controls (11 female and 43 male)	CA, MN (bucal epithelial cells)	India	3 to 13[1] 8.57[2]	Positive[b]	Sailaja et al., 2006
15 farm workers (3 female and 12 male) exposed to pesticides as endosulfan, chlorpyriphos, dimethoate, diazinon, and maleic hydrazide, and 10 controls (4 female and 6 male)	MN (peripheral blood lymphocytes)	USA	18.2±1.3[2]	Positive[c]	Tope et al., 2006
11 farmers exposed to pesticide mixtures as abamectin, cypermethrin deltamethrin, dimethoate, fenthion, methamidophos, mathidathion, parathion, nenomyl, among others, and 11 controls	MN (peripheral blood lymphocytes)	Greece	25 to 60[1] 26.45±3.38[2]	Negative	Vlastos et al., 2006
32 male exposed to pesticide mixture mainly to organoclhlorine, organophosphates, carbamates, pyrethroids, benzol ureas, among others, and 32 male controls	CA, MN (buccal epithelial cells), SCE	Turkey	34.5±10.47[2]	Positive[c]	Ergene et al., 2007
29 male sanitation workers exposed to the insecticides a-cypermethrin, cypermethrin, deltamethin, temephos, malathion, fenitrothion, and the rodenticides brodifacum, coumachlor, coumafuryl, coumatetralyl, difethialone,	MN (peripheral blood lymphocytes)	Brazil	1.5 to 18[1] 5.28±0.60[2]	Positive [b]	Kehdy et al., 2007

flocoumafen, difenacoum, bromadiolone, diphacinone, and pindone, and 30 male controls					
108 male vineyard workers exposed to pesticides mainly carbamates and organophosphates, and 65 male controls	MN (peripheral blood lymphocytes), CoA	Brazil	29.8±14.2[2]	Positive[b]	Da Silva et al., 2008
14 rural workers (12 male and 2 female) exposed mainly to glyphosate, cypermetrhin, and atrazine, and 12 controls (10 male and 2 female)	CA	Argentina	8 to 35[1]	Positive[c]	Mañas et al., 2009
29 male exposed to a complex mixture mainly organophosphates and pyrethroids, and 37 male non-exposed	MN (buccal epithelial cells)	Brazil	16.3±10.0[2]	Positive[b]	Bortoli et al., 2009
137 female and 137 male exposed to glyphosate	MN (peripheral blood lymphocytes)	Colombia	N.D.	Positive[c]	Bolognesi et al., 2009
70 agricultural workers (45 male and 25 female) exposed to pesticide mixtures mainly organophosphopus, carbamates and pyretroids and 70 non exposed (49 male and 21 female)	MN (buccal epithelial cells), SCE	Mexico	7[2]	Positive (SCE)[a] (MN)[b]	Martínez-Valenzuela et al., 2009
37 male pesticide appliers exposed to insecticides organophosphorus, carbamates, and pyrethroids, fungicides as cooper compounds, dithiocarbamates and azoles, and herbicides triazine, ureas, phosphanoglycine, bypyridilium, imidazolidones, chloronicotinyls, and 20 male controls	MN (buccal epithelial cells), CoA	Brazil	25.29±10.1 4[2]	Positive[b] (CoA) Negative (MN)	Remor et al., 2009
32 pesticide plant workers (18 male and 14 female) exposed to carbofuran, matalaxyl, and dodine, and 32 controls (18 male and 14 female)	CA	Croatia	1 to 36[1] 16.2±10.9[2]	Positive[a]	Zeljezic et al., 2009
108 vineyard male workers exposed to pesticides mainly bipyridyls, organophosphates, cooper sufates, carbamates, among others, and 65 male non-exposed	MN (peripheral blood lymphocytes), CoA	Brazil	More than 10	Positive[c]	Rohr et al., 2010
111 male workers exposed to methamidophos, malathion, methyl parathion, methomyl, propoxur, cypermethrin, atrazine, compounds bypiridylics, 2,4-dichlorophenoxyacetic acid, paraquat, glyphosate, among others, and 50 male controls	MN, CoA (buccal epithelial cells)	Mexico	1 to 57[1]	Positive[b]	Carbajal et al., Unpublished

Table 1. Cytogenetic biomonitoring studies by the use of chromosomal aberrations (CA), micronucleus (MN), sister chromatid exchanges (SCE) and comet assay (CoA) in human populations exposed to pesticides in different countries

8. References

Albert, L. (2005). Panorama de los plaguicidas en México. *Revista de Toxicología* in Line (retel) No. 8, October, ISSN: 1668-091X

Alptekin, D., Lüleyap, H.U., Demirhindi, H., Gökel, Y., Pazarbaşi, A., Dokur, M., Kasap, M. & Kasap, H. (2006). The sister-chromatid exchange and acetylcholine esterase enzyme levels among patients with insecticide intoxication in the Cukurova region, Turkey. *Acta Medica Okayama* 60 (2): 121-126, ISSN: 0386-300X

AMIPFAC. (1995). Asociación Mexicana de la Industria de Plaguicidas y Fertilizantes. México.

AMIPFAC. (2001). Asociación Mexicana de la Industria de Plaguicidas y Fertilizantes. México.

Amr, M.M. (1999). Pesticide monitoring and its health problems in Egypt, a Third World country. *Toxicology Letters* 107 (1-3): 1-13, ISSN**:** 0378-4274

Antonucci, G.A. & de Syllos Colus, I.M. (2000). Chromosomal aberrations analysis in a Brazilian population exposed to pesticides. *Teratogenesis, Carcinogenesis and Mutagenesis* 20 (5): 265-272, ISSN: 1520-6866

Anwar, W.A. (1997). Biomarkers of human exposure to pesticides. *Environmental Health Perspectives* 105 (4): 801-806, ISSN: 0091-6765, 1552-9924

Ascarrunz, M.E., Tirado, N., González, A.R., Cuti, M., Cervantes, R., Huichi, O. & Jors, E. (2006). Evaluación de riesgo genotóxico: biomonitorización de trabajadores agrícolas de Caranavi, Guanay, Palca y Mecapaca, expuestos a plaguicidas. *Cuadernos Hospital de Clínicas* 51 (1): 1-15

Au, W.W., Sierra-Torres, C.H., Cajas-Salazar, N., Bryan, K.S. & Legator, M.S. (1999). Cytogenetic effects from exposure to mixed pesticides and influence from genetic susceptibility. *Environmental Health Perspectives* 107 (6): 501-505, ISSN: 0091-6765

Barbosa, A. & Bonin, A. (1994). Evaluation of phosphine genotoxicity at occupational levels of exposure in New South Wales, Australia. *Occupational and Environmental Medicine* 51 (10): 700-705, *ISSN*: *1351-0711*

Barthel, E. (1981). Increased risk of lung cancer in pesticide-exposed male agricultural workers. *Journal of Toxicology and Environmental Health* 8 (5-6): 1027-1040, ISSN: 1528-7394

Bhalli, J.A., Khan, Q.M., Haq, M.A., Khalid, A.M. & Nasim, A. (2006). Cytogenetic analysis of Pakistani individuals occupationally exposed to pesticides in a pesticide production industry. *Mutagenesis* 21 (2): 143–148, ISSN 0267-8357

Blair, A., Grauman, D.J., Lubin, H.J. & Fraumeni, J.F. (1983). Lung cancer and other causes of death among licensed pesticide applicators. *Journal National Cancer Institute* 71 (1): 31-37, *ISSN: 0027-8874*

Bolognesi, C., Parrini, M., Bonassi, S., Lanello, G. & Salanito, A. (1993a). Cytogenetic analysis of a human population occupationally exposed to pesticides. *Mutation Research* 285 (2): 239-249, ISSN: 1383-5718

Bolognesi, C., Parrini, M., Merlo, F. & Bonassi, S. (1993b). Frequency of micronuclei in lymphocytes from a group of floriculturists exposed to pesticides. *Journal of Toxicology and Environmental Health* 40 (2-3): 405-411, ISSN: 1528-7394

Bolognesi, C.; Perrone, E. & Landini, E. (2002). Micronucleus monitoring of a floriculturist population from western Liguria, Italy. *Mutagenesis* 17 (5): 391-397, ISSN 0267-8357

Bolognesi, C. (2003). Genotoxicity of pesticides: a review of human biomonitoring studies. *Mutation Research* 543 (3): 251-272, ISSN: 1383-5718

Bolognesi, C., Landini, E., Perrone, E. & Roggieri, P. (2004). Cytogenetic biomonitoring of a floriculturist population in Italy: micronucleus analysis by fluorescence in situ hybridization (FISH) with an all-chromosome centromeric probe. *Mutation Research* 557 (2): 109-107, ISSN: 1383-5718

Bolognesi, C., Carrasquilla, G., Volpi, S., Solomon, K.R. & Marshall, E.J.P. (2009). Biomonitoring of genotoxic risk in agricultural workers from five Colombian regions: association to occupational exposure to glyphosate. *Journal of Toxicolgy and Environmental Health, Part A* 72 (15-16): 986-997, ISSN: 1528-7394

Bonassi, S., Abbondandolo, A., Camurri, L., Dal Prá, A., De Ferrari, M., Degrassi, F., Forni, A., Lamberti, L., Lando, C., Padovani, P., Sbrana, I., Vecchio, D. & Puntoni, R. (1995). Are chromosome aberrations in circulating lymphocytes predictive of future cancer onset in humans? Preliminary results of an Italian cohort study. *Cancer Genetic and Cytogenetic* 79 (2): 133-135, ISSN:0165-4608

Bonassi, S., Ugolini, D., Kirsch-Volders, M., Stromberg, U., Vermeulen, R. & Tucker, J.D. (2005). Human population studies with cytogenetic biomarkers: review of the literature and future prospectives. *Environmental and Molecular Mutagenesis* 45 (2-3): 258-270, ISSN: 0267

Bonassi, S., Znaor, A., Ceppi, M., Lando, W.P., Chang, W.P., Holland, N., Kirsch-Volders, M., Zieger, E., Ban, S., Barale, R., Bigatti, M.P., Bolognesi, C., Cebulska-Wasilewska, A., Fabianova, E., Fucic, A., Hagmar, L., Joksić, G., Martell, A., Migliore, L., Mirkova, E., Scarfi, A., Zijno, A., Norppa, H. & Fenech, M. (2007). An increased micronucleus frequency in peripheral blood lymphocytes predicts the risk of cancer in humans. *Carcinogenesis* 28 (3): 625-631, ISSN: 0143-3334

Bonassi, S., Norppa, H., Ceppi, M., Strongberg, U., Vermeulen, R., Znaor, A., Cebulska-Wasilewska, A., Fabianova, E., Fucic, A., Gundy, S., Hansteen, I.L., Knudsen, L.E., Latzuka, J., Rossner, P. Sram, R.J. & Boffeta, P. (2008). Chromosomal aberrations frequency in lymphocytes predicts the risk of cancer: results from a pooled cohort study of 22,358 subjects in 11 countries. *Carcinogenesis* 29 (6): 1178-1183, ISSN: 0143-3334

Bortoli de Moura, G.M., Barbieri de Azevedo, M. & Basso da Silva, L. (2009). Cytogenetic biomonitoring of Brazilian workers exposed to pesticides: micronucleus analysis in buccal cells of soybean growers. *Mutation Research 675:* 1-4, ISSN: 1383-5718

Bull, S., Fletcher, K., Boobis, A.R. & Battershill, J.M. (2006). Evidence for genotoxicity of pesticides in pesticide applicators: a review. *Mutagenesis* 21 (2): 93-103, ISSN: 0267

Calvert, G.M., Talaska, G., Mueller, C.A., Ammenheuser, M., Fleming, L.E., Briggle, T. & Ward, E. (1998). Genotoxicity in workers exposed to methyl bromide. *Mutation Research* 417 (2-3): 115-128, ISSN: 1383-5718

Carbajal-López, Y., Gómez-Arroyo, S., Villalobos-Pietrini, R. & Calderón-Segura, M.E. (2010). Evaluation of genetic damage in workers exposed to pesticides in Guerrero state, Mexico, utilizing comet assay and micronucleus test in exfoliated buccal cells. (Unpublished).

Carbonell, E., Puig, M., Xamena, N., Creus, A. & Marcos, R. (1990). Sister chromatid exchange in lymphocytes of agricultural workers exposed to pesticides. *Mutagenesis* 5 (4): 403-405, ISSN: 0267-8357

Carbonell, E., Xamena, N., Creus, A. & Marcos, R. (1993). Cytogenetic biomonitoring in a Spanish group of agricultural workers exposed to pesticides. *Mutagenesis* 8 (6): 511-517, ISSN: 0267-8357

Carbonell, E., Valbuena, A., Xamena, N., Creus, A. & Marcos, R. (1995). Temporary variations in chromosomal aberrations in a group of agricultural workers exposed to pesticides. *Mutation Research* 344 (3-4): 127-134, ISSN: 1383-5718

Castillo-Cadena, J., Tenorio-Vieyra, L.E., Quintana-Carabia, A.I., García-Fabila, M.M., Ramírez-San Juan, E. & Madrigal-Bujaidar, E. (2006). Determination of DNA damage in floriculturists exposed to mixtures of pesticides. *Journal of Biomedicine and Biotechnology* 2: 1-12, ISSN: 11107243

CICOPLAFEST. (1998). Comisión Intersecretarial para el Control del Proceso y Uso de Plaguicidas, Fertilizantes y Sustancias Tóxicas 1998. Catalogo Oficial de Plaguicidas. SEMARNAP, SECOFI, SAGAR y SSA, México D.F.

Cofepris (2010). Comisión Federal para la Protección contra Riesgos Sanitarios. http://201.147.97.103/wb/cfp/uso de plaguicidas/rid/882? page=5. Last visit August 13, 2010.

Costa, C., Teixeira, J.P., Silva, S., Roma-Torres, J., Coehlo, P., Gaspar, J., Alves, M., Laffon, B., Rueff, J. & Mayan, O. (2006). Cytogenetic and molecular biomonitoring of a Portuguese population exposed to pesticides. *Mutagenesis* 21 (5): 343-350, ISSN: 0267-8357

Cuenca, P. & Ramírez, V. (2004). Aberraciones cromosómicas en trabajadoras expuestas a plaguicidas. *Revista de Biología Tropical* 52 (2): 623-628, ISSN: 0034-7744

Da Silva, J., Moraes, R., Heuser, V.D., Andrade, V.M., Silva, F.R., Kvitko, K., Emmel, V., Rohr, P., Bordin, D.L., Andreazza, C., Salvador, M., Henriques, J.A. & Erdtmann, B. (2008). Evaluation of genetic damage in a Brazilian population exposed to pesticides and its correlation with polymorphisms in metabolizing genes. *Mutagenesis* 23 (5): 415-422., ISSN: 0267-8357

D'Arce, L.P.G. & de Syllos Colus, I.M. (2000). Cytogenetic and molecular biomonitoring of agricultural workers exposed to pesticides in Brazil. *Teratogenenesis, Carcinogenenesis and Mutagenenesis* 20 (3): 161-170, ISSN: 1520-6866

De Ferrari, M., Artuso, M., Bonassi, S., Cavalieri, Z., Pescatore, D., Marchini, E., Pisano, V. & Abbondandolo, A. (1991). Cytogenetic biomonitoring of an Italian population exposed to pesticides: chromosome aberration and sister-chromatid exchange analysis in peripheral blood lymphocytes. *Mutation Research* 260 (1): 105-113, ISSN: 1383-5718

Dulout, F.N., Pastori, M.C., Olivero, O.A., González Cid, M., Loria, D., Matos, E., Sobel, N., de Bujan, E.C. & Albiano, N. (1985). Sister-chomatid exchanges and chromosomal aberrations in a population exposed to pesticides. *Mutation Research* 143 (4): 237-244, ISSN: 1383-5718

Dulout, F.N., López Camelo, J.S. & Guradze, H.N. (1992). Analysis of sister chromatid exchanges (SCE) in human populations studies. *Revista Brasileña de Genetica* 15 (1): 169-182, ISSN: 0100-8455

Ecobichon, D.J., Davis, J.E., Doull, J., Ehrich, M., Joy, R., McMillan, D., McPhail, R., Reiter, I.W., Slikker, W. & Tilson, H. (1990). Neurotoxic effects of pesticides. In: *The Effects of Pesticides on Human Health*. Baker, S.R. & Wilkinson, C.F (Eds). 131-199. Princenton Scientific Publishers. New Jersey.

Eddleston, M., Karalliedde, L., Buckley, N., Fernando, R., Hutchinson, G., Isbister, G., Konradsen, F., Murray, D., Piola, J.C., Senanayake, N., Sheriff, R., Singh, S., Siwach, S.B. & Smit, L. (2002). Pesticide poisoning in the developing world-a minimum pesticide list. *The Lancet* 360 (9340): 1163-1167, ISSN: 01440-6736

El-Ghazali, S., Au, W.W., Anwar, W., Legator, M. & Massoud, A. (1990). Cytogenetic study among workers paking pesticides. *Environmental and Molecular Mutagenesis* 15 (1-2) suppl. 17 (Abstract): 18, ISSN: 0893-6692

Ergene, S., Çelik, A., Çavaş, T. & Kaya, F. (2007). Genotoxic biomonitoring study of population residing in pesticide contaminated regions in Gösku delta: micronucleus, chromosomal aberrations and sister chromatid exchanges. *Environmental International* 33: 877-885, ISSN: 0160-4120

Falck, G.C.-M., Hirvonen, A., Scarpato, R., Sirkku, T., Saarikoski, S., Migliore, L. & Norppa, H. (1999). Micronuclei in blood lymphocytes and genetic polymorphism GSTM1, GSTT1 and NAT2 in pesticide exposed greenhouse workers. *Mutation Research* 441 (2): 225-237, ISSN: 1383-5718

Fairbairn, D.W., Olive, P.L. & O'Neill, K.L. (1995). The comet assay: a comprehensive review. *Mutation Research* 339 (1): 37-59, ISSN: 1383-5718

Fenech, M. & Morley, A.A. (1985). Measurement of micronuclei in human lymphocytes. *Mutation Research* 148 (1-2): 29-36, ISSN: 1383-5718

Garaj-Vrhovac, V. & Zeljezic, D. (2000). Evaluation of DNA damage in workers occupationally exposed to pesticides using single-cell gel electrophoresis (SCGE) assay pesticide genotoxicity reveled by comet assay. *Mutation Research* 469 (2): 279-285, ISSN: 1383-5718

Garaj-Vrhovac, V. & Zeljezic, D. (2001). Cytogenetic monitoring of Croatian population occupationally exposed to a complex mixture of pesticides. *Toxicology* 165 (2-3): 153-162, ISSN: 0300-483X

Garaj-Vrhovac, V. & Zeljezic, D. (2002). Assessment of genome damage in a population of Croatian workers employed in pesticide production by chromosomal aberration analysis, micronucleus assay and comet assay. *Journal of Applied Toxicol*ogy 22 (4): 249-255, ISSN: 1099-1263

Garte, S. & Bonassi, S. (2005). Linking toxicology to epidemiology: biomarkers and new technologies-special issue overview. *Mutation Research* 592 (1-2): 3-5: ISSN: 1383-5718

Gómez, E. & García, G. (2002). La problemática de la población jornalera agrícola migrante ante las transformaciones de la agricultura mexicana. *Nueva Época-UNAM* 612, 60-61.

Gómez-Arroyo, S., Noriega-Aldana, N., Osorio, A., Galicia, F., Ling, S. & Villalobos-Pietrini, R. (1992). Sister-chromatid exchange analysis in a rural population of Mexico exposed to pesticides. *Mutation Research* 281 (3): 173-179, ISSN: 1383-5718

Gómez-Arroyo, S., Díaz-Sánchez, Y., Meneses-Pérez, M.A., Villalobos-Pietrini, R. & De León-Rodríguez, J. (2000). Cytogenetic biomonitoring in a Mexican floriculture

worker group exposed to pesticides. *Mutation Research* 466 (1): 117-124, ISSN: 1383-5718

Grammont, H. & Lara Flores, S. (2004). Encuesta a hogares de jornaleros migrantes en regiones hortícolas de México: Sinaloa, Sonora, Baja California Sur y Jalisco. Instituto de Investigaciones Sociales-UNAM. Cuadernos de Investigación. 30 p.

Gray, L.E. (1992). Chemical-induced alterations of sexual differentiation: a review of effects in humans and rodents. In: *Chemically-Induced Alterations in Sexual and Functional Development: The Wild/Human Connextion*. Colborn, T. & Clement, C. (Eds.) 203-230, New Jersey.

Grover, P., Danadevi, K., Mahboob, M., Rozati, R., Banu, B.S. & Rahman, M.F. (2003). Evaluation of genetic damage in workers employed in pesticide production utilizing the comet assay. *Mutagenesis* 18 (2): 201-205, ISSN: 0267-8357

Hagmar, L., Bonassi, S., Stromberg, U., Brogger, A., Knudsen, L.E., Norppa, H., Reuterwall, C. & European Study Group on Cytogenetic Biomarkers and Health. (1998). Chromosomal aberrations in lymphocytes predict human cancer: a report from European Study Group on Cytogenetic Biomarkers and Health (ESCH). *Cancer Research* 58 (18): 4117-4121, ISSN: 0008-5472

Hileman, B. (1994). Environmental estrogens linked to reproductive abnormalities and cancer. *Chemical and Engineering News* 72: 19-23, *ISSN: 0009-2347*

Holland, N., Duramad, P., Rothman, N., Figgs, L.W., Blair, A., Hubbard, A. & Smith, M.T. (2002). Micronucleus frequency and proliferation in human lymphocytes after exposure to herbicide 2,4-dichlorophenoxyacetic acid in vitro and in vivo. *Mutation Research* 521 (1-4): 165-178, ISSN: 1383-5718

Holland, N., Bolognesi, C., Kirsch-Volders, M., Bonassi, S., Zieger, E., Knasmueller, S. & Fenech, M. (2008). The micronucleus assay in human buccal cells as a tool for biomonitoring DNA damage: Te HUMN project perspective on current status and knowledge gaps. *Mutation Research* 659 (1-2): 93-108, ISSN: 1383-5718

Hoppin, J.A., Umbach, D.M., London, S.J., Henneberger, P-K., Kullman G.J. & Sandler, D.P. (2008). Pesticides and atopic and nonatopic asthma among farm women in the agricultural health study. *American Journal of Respiratory and Critical Care Medicine* 177 (1): 11-18, ISSN: 1073-449X

Hoyos, L S., Carvajal, S., Solano, L., Rodríguez, J., Orozco, L. & López, V. (1996). Cytogenetic monitoring of farmers exposed to pesticides in Colombia. *Environmental Health Perspectives* 104 (Suppl. 3): 535-538, ISSN: 0091-6765

IARC (International Agency for Research on Cancer). (1991). Occupational exposure in insecticide application and some pesticides. IARC Monographs on the Evaluation of Carcinogenic Risk to Humans, vol. 53, IARC, Lyon, pp. 179-250.

IARC (International Agency for Research on Cancer). (2002). Monographs on Evaluation of Carcinogenic Risk of Chemicals to Humans, vol. 82, Lyon, France.

INEGI (Instituto Nacional de Estadística, Geografía e Informática). (1998). Informe 1997. Estadística del Medio Ambiente. México

Jabloniká, A., Poáakova H., Karelová, J. & Vargova, M. (1989). Analysis of chomosome aberrations and sister-chromatid exchanges in peripheral blood lymphocytes of workers with occupational exposure to the mancozeb-containing fungicide Novozir Mn80. *Mutation Research* 224 (2): 143-146, ISSN: 1383-5718

Jamil, K., Shaik, A. & Lakshimi, J. (2005). Pesticide induced cytogenetic risk assessment in human lymphocyte culture in vitro. *Bulletin of Environmental and Contamination Toxicology* 75 (1): 7-14, ISSN: 0007-4861

Joksić, G., Vidaković, A. & Spasojević-Tišma, V. (1997). Cytogenetic monitoring of pesticide sprayers. *Environmental Research* 75 (2): 113-118, ISSN: 0013-9351

Kaioumova, D. & Khabutdinova, L. (1998). Cytogenetics characteristics of herbicide production workers in Ufa. *Chemosphere* 37 (9-12): 1755-1759, ISSN: 0045-6535

Kamel, F., Engel, L.S., Gladen, B.C., Hoppin, J.A., Alavanja, M.C.R. & Sandler, D.P. (2005). Neurologic symptoms in licensed private pesticide applicators in the agricultural health study. *Environmental Health Perspectives* 113 (7): 877-882, ISSN: 0091-6765

Kamel, F., Engel, L.S., Gladen, B.C., Hoppin, J.A., Alavanja, M.C.R. & Sandler, D.P. (2007). Neurologic symptoms in licensed pesticide applicators in the agricultural health study. *Human and Experimental Toxicology* 26 (3): 243-250, ISSN: 0960-3271

Kehdy, F.S.G., Cerqueira, E.M.M., Bonjardim, M.B., Camelo, R.M. & Castro, M.C.L. (2007). Study of the cytogenetic effects of occupational exposure of pesticides on sanitation workers in Belo Horizonte, Brazil. *Genetics and Molecular Research* 6 (3): 581-593, ISSN: 1676-5680

Kourakis, A., Mouratidou, M., Kokkinos, G., Barbouti, A., Kotsis, A., Mourelatos, D. & Dozi-Vassiliades, J. (1992). Frequencies of chromosomal aberrations in pesticide sprayers working in plastic green houses. *Mutation Research* 279 (2): 145-148, ISSN: 1383-5718

Kourakis, A., Mouratidou, M., Barbouti, A. & Dimikiotou, M. (1996). Cytogenetic effects of occupational exposure in the peripheral blood lymphocytes of pesticide sprayers. *Carcinogenesis* 17 (1): 99-101, ISSN: 0143-3334

Lander, F. & Rønne, M. (1995). Frequency of sister chromatid exchange and hematological effects in pesticide-exposed greenhouse sprayers. *Scandinavian Journal Work Environmental Health* 21: 283-288, ISSN: 0355-3140

Lander, F., Knudsen, L.E. Gamborg, M.O., Jarventaus, H. & Norppa, H. (2000). Chromosome aberrations in pesticide-exposed greenhouse workers. *Scandinavian Journal Work Environmental Health* 26 (5): 436-442, ISSN: 0355-3140

Latt, S. (1979). Microflurometric detection of deoxyribonucleic acid replication in human metaphase chromosomes. *Proceedings of the National Academy of Science* 70 (12): 3395-3399, ISSN: 0027-8424

Latt, S., Allen, J., Blom, S., Carrano, A., Falke, E., Kram, D., Schneider, E., Schreck, R., Tice, R., Whitfield, B. & Wolff, S. (1981). Sister chromatid exchanges in human lymphocytes after exposure to diagnostic ultrasound. *Science* 205 (4412): 1273-1275, ISSN: 0036-8075

Lebailly, P., Vigreux, C., Lechevrel, D., Ledemeney, D., Godard, T., Sichel, F., LeTalaër, J.Y., Henry-Amar, M. & Gauduchon, P. (1998a). DNA damage in mononuclear leukocytes of farmers measured using the alkaline comet assay: discussion of critical parameters and evaluation of seasonal variations in relation to pesticide exposure. *Cancer Epidemiology Biomarkers & Prevention* 7 (10): 917-927, ISSN: 1055-9965

Lebailly, P., Vigreux, C., Lechevrel, D., Ledemeney, D., Godard, T., Sichel, F., LeTalaër, J.Y., Henry-Amar, M. & Gauduchon, P. (1998b). DNA damage in mononuclear

leukocytes of farmers measured using the alkaline comet assay: modifications of DNA damage levels after a one-day field spraying period with selected pesticides. *Cancer Epidemiology Biomarkers & Prevention* 7 (10): 929-940, ISSN: 1055-9965

Lebailly, P., Devaux, A., Pottier, D., De Meo, M., Andre, V., Baldi, I., Severin, F., Bernaud, J., Durand, B., Henry-Amar, M. & Gauduchon, P. (2003). Urine mutagenicity and lymphocyte DNA damage in fruit growers occupationally exposed to the fungicide captan. *Occupational Environmental Medicine* 60 (12): 910-917, ISSN: 1351-0711

Levario-Carrillo, M., Sordo, M., Rocha, F., González-Horta, C., Amato, D. & Ostrosky-Wegman, P. (2005). Micronucleus frequency in human umbilical cord lymphocytes. *Mutation Research* 586 (1): 68-75, ISSN: 1383-5718

Lucero, L., Pastor, S., Suárez, S., Durban, R., Gómez, C., Parrón, T., Creus, A. & Marcos, R. (2000). Cytogenetic biomonitoring of Spanish greenhouse workers exposed to pesticides: micronuclei analysis in peripheral blood lymphocytes and buccal epithelial cells. *Mutation Research* 464 (2): 255-262, ISSN: 1383-5718

Mansour, S. (2004). Pesticide exposure-Egyptian scene. Toxicology 198 (1-3): 91-115, ISSN: 0300-483X

Mañas, F., Peralta, L., Gorla, N., Bosh, B. & Aissa, D. (2009). Aberraciones cromosómicas en trabajadores rurales de la provincia de Córdoba expuestos a plaguicidas. *Journal of Basic and Applied Genetics* 20 (1): version on line ISSN: 1852-6233

Márquez, C., Villalobos, C., Poblete, S., Villalobos, E., García M. A. & Duk, S. (2005). Cytogenetic damage in female Chilean agricultural workers exposed to mixtures of pesticides. *Environmental and Molecular Mutagenesis* 45 (1): 1-7, ISSN: 1098-2280

Martínez Guerrero, M. (2001). Retrospectiva y perspectiva de la cuestión agraria mexicana. *Tribunal Superior Agrario*, México. 382 p.

Martínez-Valenzuela, C., Gómez-Arroyo, S., Villalobos-Pietrini, R., Waliszewski, S., Calderón-Segura, M.E., Félix-Gastélum, R. & Álvarez-Torres, A. (2009). Genotoxic biomonitoring of agricultural workers exposed to pesticides in the north of Sinaloa state, Mexico. *Environment International* 35 (8): 1155-1159, ISSN: 0160-4120

Mohammad, O., Walid, A.A. & Ghada, K. (1995). Chromosomal aberrations in human lymphocytes from two groups of workers occupationally exposed to pesticides in Syria. *Environmental Research* 70 (1): 24-29, ISSN: 0013-9351

Möller, P. (2006). The alkaline comet assay: towards validation in biomonitoring of DNA damaging exposures. *Basic & Clinical Pharmacology & Toxicology* 98 (4): 336-345, ISSN: 0300-483X

Mustonen, R., Kangas, J., Vuojolahti, P. & Linnainmaa, K. (1986). Effects of phenoxyacetic acids on the induction of chromosome aberrations in vitro and in vivo. *Mutagenesis* 1 (4): 241-246, ISSN: 0267-8357

Nehéz, M., Boros P., Ferke, A., Mohos, J., Palotás, M., Vetró, G., Zimányi, M. & Desi, I. (1988). Cytogenetic examination of people working with agrochemicals in the southern region of Hungary. *Regulatory Toxicology and Pharmacology* 8 (1): 37-44, ISSN: 0273-2300

Norppa, H. (2004). Cytogenetic biomarkers and genetic polymorphisms. *Toxicology Letters* 149 (1-3): 309-334, ISSN: 0378-4274

OMS (1990). Organización Mundial de la Salud. Plaguicidas. *Informe Técnico* No. 12.

OPS/OMS (1993). Organización Panamericana de la Salud. Plaguicidas y Salud en las Américas. Washington, D.C. OPS, pp. 25-27.

OPS (2002). Organización Panamericana de la Salud. Situación epidemiológica de las intoxicaciones agudas por plaguicidas en el istmo centroamericano 1992-2000. *Boletín Epidemiológico* 23: 7-22.

Padmavathi, P., Prabhavathi, A.P. & Reddy, P. (2000). Frequencies of SCEs in peripheral blood lymphocytes of pesticide workers. *Bulletin of Environmental Contamination and Toxicology* 64 (2): 155-160, ISSN: 0007-4861

Paldy, A., Puskás, N., Vincze, N. & Hadházi, M. (1987). Cytogenetic studies on rural populations exposed to pesticides. *Mutation Research* 187 (3): 127-132, ISSN: 1383-5718

PAN International (Pesticide Action Network International) (1990). Consult Manual.

Pasquini, R., Scassellati-Sforzolini, G., Angeli, G., Fatigoni, C., Monarca, S., Beneventi, L., DiGiulio, A.M. & Bauleo, F. (1996). Cytogenetic biomonitoring of pesticide-exposed farmers in central Italy. *Journal of Environmental Pathology, Toxicology and Oncology* 15 (1): 29-39, ISSN: 0731-8898

Pastor, S., Gutiérrez, S., Creus, A., Cebulska-Wasilewska, A. & Marcos, R. (2001a). Micronuclei in peripheral blood lymphocytes and buccal epithelial cells of Polish farmers exposed to pesticides. *Mutation Research* 495 (1-2): 147-156, ISSN: 1383-5718

Pastor, S., Gutiérrez, S., Creus, A., Xamena, N., Piperakis, S. & Marcos R. (2001b). Cytogenetic analysis of Greek farmers using the micronucleus assay in peripheral lymphocytes and buccal cells. *Mutagenesis* 16 (6): 539-545, ISSN: 0267-8357

Pastor, S., Lucero, L., Gutiérrez, S., Durbán, R., Gómez, C., Parrón, T., Creus, A. & Marcos, R. (2002a). A follow up study on micronucleus frequency in Spanish agricultural workers exposed to pesticides. *Mutagenesis* 17 (1): 79-82, ISSN: 0267-8357

Pastor, S., Creus, A., Xamena, N., Siffel, C. & Marcos, R. (2002b). Occupational exposure to pesticides and cytogenetic damage: results of a Hungarian population study using the micronucleus assay in lymphocytes and buccal cells. *Environmental and Molecular Mutagenesis* 40 (2): 101-109, ISSN: 1098-2280

Pastor, S., Creus, A., Parrón, T., Cebulska-Wasilewska, A., Siffel, C., Piperakis, S. & Marcos, R. (2003). Biomonitoring of four European populations occupationally exposed to pesticides: use of micronuclei as biomarkers. *Mutagenesis* 18 (3): 249-258, ISSN: 0267-8357

Paz-y-Miño, C., Bustamante, G., Sánchez, M.E. & Leone, P.E. (2002). Cytogenetic monitoring in a population occupationally exposed to pesticidas in Ecuador. *Environmental Health Perspectives* 110 (11): 1077-1080, ISSN: 0091-6765

Perea, E. (2006). Plaguicidas la peste de la ignorancia. *Teorema Ambiental*. Editorial 3w, México.

Perry, P. & Wolff, S. (1974). New Giemsa method for differential staining of sister chromatids. *Nature* 261 (5471): 156-158, ISSN: 0028-0836

Piperakis, S., Petrakou, E., Tsilimigaki, S., Sagnou, M., Monogiudis, E., Haniotakis, G., Karkaseli, H. & Sarikaki, E. (2003). Biomonitoring with the comet assay of Greek greenhouse workers exposed to pesticides. *Environmental and Molecular Mutagenenesis* 41 (2): 104-110, ISSN: 1098-2280

PNUMA/OMS (1992). Programa de las Naciones Unidas para el Medio Ambiente. Consecuencias Sanitarias del Empleo de Plaguicidas en la Agricultura. Geneva.

Reali, D., Di Marino, F., Bahramandpour, S., Carducci, A., Barale, R. & Loprieno, N. (1987). Micronuclei in exfoliated urothelial cells and urine mutagenicity in smokers. *Mutation Research* 192 (2): 145-149, ISSN: 1383-5718

Remor, A.L., Caprini Totti, C., Alves Moreira, D., Pimentel Dutra, G., Dahlström Heuser, V. & Boeira J.M. (2009). Occupational exposure of farm workers to pesticides: biochemical parameters and evaluation of genotoxicity. *Environment International* 35 (2): 273-278, ISSN: 0160-4120

Ríos, B. & Solari, G. (2010). Biomonitoring pesticides: a national need? *Revista Médica Chilena* 138: *515-518,* ISSN: 0034-9887

Rosin, M.P. & Gilbert, A. (1990). Modulation of genotoxic effects in humans. *Environmental Mutagenesis* 347 Part E: 351-359, ISSN: 0192-2521

Rosin, M. (1992). The use of the micronucleus test on exfoliated cells to identify anticlastogenic action in humans: a biological marker of the efficacy of chemopreventive agents. *Mutation Research* 267 (2): 265-267, ISSN: 1383-5718

Rita, P., Reddy, P.P. & Reddy, S.V. (1987). Monitoring of workers occupationally exposed to pesticides in grape gardens of Andhra Pradesh. *Environmental Research* 44 (1): 1-5, ISSN: 0013-9351

Rohr, P., da Silva, J., Erdtmann, B., Saffi, J., Nikolova Guecheva, T., Pegas Heriques, J.A. & Kvitko, K. (2010). BER gene polymorphisms (OGG1 Ser326Cys and XRCC1 Arg194Trp) and modulation of DNA damage due to pesticide exposure. *Environmental and Molecular Mutagenesis* (in press), ISSN: 1098-2280

Rojas, A., Ojeda, M. & Barraza, X. (2000). Congenital malformations and pesticide exposure. *Revista Médica Chilena* 128 (4): 399-404, ISSN: 0034-9887

Rosales Castillo, J.A. (2001). La toxicología y la regulación de plaguicidas en México. Comisión Federal para la Protección Contra Riesgos Sanitarios, 24

Rupa, D.S., Rita, P., Reddy, P.P. & Reddi, O.S. (1988). Screening of chromosomal aberrations and sister chromatid exchanges in peripheral lymphocytes of vegetable garden workers. *Human Toxicology* 7 (4): 333-336, ISSN: 0144-5952

Rupa, D.S., Reddy, P.P. & Reddi, O.S. (1989a). Frequencies of chromosomal aberrations in smokers exposed to pesticides in cotton field. *Mutation Research* 222 (1): 37-41, ISSN: 1383-5718

Rupa, D.S., Reddy, P.P. & Reddi, O.S. (1989b). Chromosomal aberrations in peripheral lymphocytes of cotton field workers exposed to pesticides. *Environmental Research* 49 (1): 1-6, ISSN: 0013-9351

Rupa, S., Reddy, P.P. & Reddi, O.S. (1989c). Analysis of sister-chromatid exchanges, cell kinetics and mitotic index in lymphocytes of smoking pesticide sprayers. *Mutation Research* 223 (2): 253-258, ISSN: 1383-5718

Rupa, D.S., Reddy, P.P. & Reddi, O.S. (1991a). Clastogenic effect of pesticides in peripheral lymphocytes of cotton-field workers. *Mutation Research* 261 (3): 177-180, ISSN: 1383-5718

Rupa, D.S., Reddy, P.P., Sreemannarayana, K. & Reddi, O.S. (1991b). Frequency of sister chromatid exchange in peripheral lymphocytes of male pesticide applicators. *Environmental and Molecular Mutagenesis* 18 (2): 136-138, ISSN: 1098-2280

Sailaja, N., Chandrasekhar, M., Rekhadevi, P., Mahboob, M., Rahman, M., Saleha, B., Vuyyuri, B., Danadevi, K., Hussain, S. & Grover, P. (2006). Genotoxic evaluation of workers employed in pesticide production. *Mutation Research* 609 (1): 74–80, ISSN: 1383-5718

Scarpato, R., Migliore, L., Angotzi, G., Fedi, A., Miligi, L. & Loprieno, N. (1996). Cytogenetic monitoring of a group of Italian floriculturists: no evidence of DNA damage related to pesticides exposure. *Mutation Research* 367 (2): 73-82, ISSN: 1383-5718

Shaham, J., Kaufman, Z., Gurvich, R. & Levi, Z. (2001). Frequency of sister chromatid exchange among greenhouse farmers exposed to pesticides. *Mutation Research* 491 (1): 71-80, ISSN: 1383-5718

Singh, N.P., McCoy, M.T., Tice, R.R. & Schneider, E.L. (1988). A simple technique for quantitation of low levels of DNA damage in individual cells. *Experimental Cell Research* 175 (1): 184-191, ISSN: 0014-4827

SEMARNAP (1996). Lo que usted debe saber sobre la gestión de los plaguicidas en México. Serie Plaguicidas no. 4.

Smerhovsky, Z., Landa, K., Rossner, P., Brabec, M., Zudova, Z., Hola, N., Pokorna, Z., Mareckova, J. & Hurychova, D. (2001). Risk of cancer in an occupationally exposed cohort with increased level of chromosomal aberrations. *Environmental Health Perspectives* 109 (1): 41-45, ISSN: 0091-6765

Speit, G. & Hartmann, A. (2006). The comet assay: a sensitive genotoxicity test for the detection of DNA damage and repair. *Methods in Molecular Biology* 314: 275-286, ISSN: 1064-3745

Steenland, K., Carrano, A., Ratcliffe, J., Clapp, D., Ashworth, L. & Meinhardt, T. (1986). A cytogenetic study of papaya workers exposed to ethylene dibromide. *Mutation Research* 170 (3): 151-160, ISSN: 1383-5718

Stich, H.F., Stich, W. & Parida, B.B. (1982). Elevated frequency of micronucleated cells in the buccal mucosa of individuals at high risk for oral cancer: betel quid chewers. *Cancer Letters* 17 (2): 125-134, ISSN: 0304-3835

Stich, H. F. & Rosin, M.P. (1983). Quantitating the synergistic effect of smoking and alcohol consumption with the micronucleus test on human buccal mucosa cells. *International Journal of Cancer* 31 (3): 305-308, ISSN: 0020-7136

Stich, H., San, R. & Rosin, M.P. (1983). Adaptation of the DNA repair and micronucleus test to human cell suspensions and exfoliated cells. *Annals of the N.Y. Academy of Sciences* 407: 93-105, ISSN: 0077- 8923

Stich, H. F. & Rosin, M.P. (1984). Micronuclei in exfoliated human cells as a tool for studies in cancer risk and cancer intervention. *Cancer Letter 22 (3):* 241-253, ISSN: 0304-3835

Stich, H.F. (1987). Micronucleated exfoliated cells as indicators for genotoxic damage and as markers in chemo-prevention trials. *Journal of Nutrition, Growth and Cancer* 4: 9-18, ISSN: 0736-8283

Tarik, M.I., Afzal, S., Hussain, I. & Sultana, N. (2007). Pesticide exposure in Pakistan: a review. *Environment International* 33 (8): 1107-1122, ISSN: 0160-4120

Tates, A.D., Grummt, T., van Dam, F.J., de Zwart, F., Kasper, F.J., Rothe, S., Stirn, H., Zwinderman, A.H. & Natarajan, A.T. (1994). Measurement of frequencies of HPRT mutants, chromosomal aberrations, micronuclei, sister-chromatid exchanges and

cells with high frequencies of SCEs in styrene/dichloromethane-exposed workers. *Mutation Research* 313 (2-3): 249-262, ISSN: 1383-5718

Tice, R.R., Agurell, E., Anderson, D., Burlinson, B., Hartmann, A., Kobayashi, H., Miyamae, Y., Rojas, E., Ryu, J.C. & Sasaki, Y.F. (2000). Single cell/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. *Environmental and Molecular Mutagenesis* 35 (3): 206-221, ISSN: 1098-2280

Titenko-Holland, N., Windham, G., Kolachana, P., Reinisch, F., Parvatham, S., Osorio, A.M. & Smith, M. (1997). Genotoxicity of malathion in human lymphocytes assessed using the micronucleus assay in vitro and in vivo: a study of malathion-exposed workers. *Mutation Research* 388 (1): 85-95, ISSN: 1383-5718

Tolbert, P.E., Shy, C.M. & Allen, J.M. (1992). Micronuclei and other nuclear anomalies in buccal smears: methods development. *Mutation Research* 271 (1): 69-77, ISSN: 1383-5718

Tope, A., Bebe, F.N. & Panemangalore, M. (2006). Micronuclei frequency in lymphocytes and antioxidants in the blood of traditional limited-resource farm workers exposed to pesticides. *Journal of Environmental Science and Health* B 41 (6): 843-853, ISSN: 0360-1234

Tucker, J.D., Auletta, A., Cimino, M.C., Dearfield, K.L., Jacobson-Kram, D., Tice, R.R. & Carrano, A.V. (1993). Sister-chromatid exchange: second report of the Gene-Tox Program. *Mutation Research* 297 (2): 101-180, ISSN: 1383-5718

Turner, R.J. (Ed.). (1994). Immunolgy: *A Comparative Approach.* Wiley, J. & Sons Ltd., Chichester, U.K.

Ünderğer, Ü & Başaran, N. (2002). Assessment of DNA damage in workers occupationally exposed to mixtures by the alkaline comet assay. *Archieves of Toxicology* 76 (7): 430-436, ISSN: 0340-5761

U.S. Environmental Protection Agency Guidelines for Carcinogen Risk Assessment EPA/630/P-03001B;EPA: Washington, D.C. 2005.

Van Hummelen, P., Severi, M., Pauwels, A., Roosels, W., Veulemans, H. & Kirsch-Volders, M. (1994). Cytogenetic analysis of lymphocytes from fiberglass-reinforced plastic workers occupationally exposed to styrene. *Mutation Research* 310 (1); 133-150, ISSN: 1383-5718

Venegas, W., Zapata, I., Carbonell, E. & Marcos, R. (1998). Micronuclei analysis in lymphocytes of pesticide sprayers from Concepcion, Chile. *Teratogenesis Carcinogenesis and Mutagenesis* 18 (3): 123-129, ISSN: 1520-6866

Vlastos, D., Demsia, G. & Matthopoulos, D. (2004). Evaluation of genetic damage in tobacco-growing farmers occupationally exposed to a mixture of matalaxyl and imidacloprid. *International Journal of Environmental Analytical Chemistry* 84 (1-3): 183-191, ISSN: 0306-7319

Vlastos, D., Stivaktakis, P. & Matthopoulos, D.P. (2006). Pesticide exposure and genotoxicity correlations within a Greek farmers' Group. *International Journal of Environmental Analytical Chemistry* 86 (3-4): 215-223, ISSN: 0306-7319

WHO (World Health Organization) (2004). The WHO recommended classification of pesticides by hazard and guidelines to classification: 2004, Geneva WHO.

WHO/UNEP (1990). Public Health Impact of Pesticides used in agriculture. World Health Organization. Geneva.

Windham, C.G., Titenko-Holland, N., Osorio, A.M., Gettner, S., Reinisch, F., Haas, R. & Smith, M. (1998). Genetic monitoring of malathion-exposed agricultural workers. *American Journal of Industrial Medicine* 33 (2): 164-174 ISSN: 0271-3586

Wolff, S., Bodycote, J. & Painter, R.B. (1974). Sister chromatid exchanges induced in Chinese hamster cells by UV irradiation of different stages of the cell cycle: the necessity for cells to pass through S. *Mutation Research* 25: 73-81, ISSN: 1383-5718

Wolff, S. (1982). Chromosome aberrations, sister chromatid exchanges, and the lesions that produce them. In: *Sister Chromatid Exchanges*, Wolff, S. (Ed.) 59-86. Wiley & Sons, ISBN 0-471-05987-0, New York

Zeljezic, D. & Garaj-Vrhovac, V. (2001). Chromosomal aberration and single cell gel electrophoresis (comet) assay in the longitudinal risk assessment of occupational exposure to pesticides. *Mutagenesis* 16 (4): 359-363, ISSN: 0267-8357

Zeljezic, D. & Garaj-Vrhovac, V. (2002). Sister chromatid exchange and proliferative rate index in the longitudinal risk assessment of occupational exposure to pesticides. *Chemosphere* 46 (2): 295-303, ISSN: 0045-6535

Zeljezic, D., Vrdoljak, A.L., Lucas, J.N., Lasan, R., Fucic, A., Kopjar, N., Katic J., Mladinic, M. & Radic, B. (2009) Effect of occupational exposure to multiple pesticides on translocation yield and chromosomal aberrations in lymphocytes of plant workers. *Environmental Science & Technology* 40 (6): 6370-6377, ISSN: 0013-936X

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Effects of Pesticides on Neuronal and Glial Cell Differentiation and Maturation in Primary Cultures

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1. Introduction

Since many pesticides are developed to target the nervous system of different organisms their effects on the human brain are of great concern, in particular the effects on the immature brain. The acute neurotoxicity of pesticides is well-known from occupational exposure studies, poisoning events and suicide data (Kimbrough *et al.,* 1989). Furthermore, developmental neurotoxicity (DNT) effects, such as reduced short-term memory, hand-eye coordination, drawing ability and visuospatial deficits have been observed in several epidemiological studies (Grandjean *et al.,* 2006; Guillette *et al.,* 1998; Ruckart *et al.,* 2004).

It is widely accepted that the developing central nervous system (CNS), is much more vulnerable to injury induced by different classes of chemicals, including pesticides, than the adult CNS. This is partly due to the fact that the adult brain is well protected against chemicals by the blood brain barrier (BBB) while children's BBB is not fully differentiated until around 6 months after the birth (Adinolfi, 1985; Tilson, 2000) and at the same time they have increased absorption and a diminished ability to detoxify many exogenous compounds in comparison to that of adults (NRC, 2000).

Moreover, the development of the CNS is a very complex process involving several different important events, *e.g.* differentiation of progenitor cells, proliferation and cell migration, synaptogenesis, myelination, cell death, synthesis of neurotransmitters, formation of receptors, trimming of connections or electrical activity stimulation. These events are occurring within strictly controlled time frames and therefore each event creates different windows of vulnerability to xenobiotic exposure (Rice and Barone, 2000; Rodier, 1994; Rodier, 1995). Furthermore, the brain consists of many different cell types (*e.g.* neuronal, glial and endothelial cells) that have specific functions and different roles. Also each cell type is produced at a defined moment during the development and is therefore susceptible to environmental disturbances at different developmental time periods. Some events take place during a very short time period and interference by chemicals during these stages could have serious consequences for the individual.

Exposure to pesticides, industrial chemicals, or drugs, might contribute to the increasing incidences of neurodevelopmental disorders (Boyle et al., 1994; Grandjean and Landrigan, 2006; Lein et al., 2007; Schettler, 2001). There are some studies suggesting that one out of every six children has developmental disabilities and many of them are neurodevelopmental disorders such as learning disabilities, dyslexia, attention deficits, hyperactivity disorders and autism (Boyle et al., 1994; Grandjean and Landrigan, 2006; Lein et al., 2007; Schettler, 2001). Moreover, a report from the National Academia of Science (NAS) suggests that 28 % of all major developmental disorders in children are linked entirely or partly to environmental exposures (NRC, 2000).

A vast amount of papers in the peer-reviewed scientific literature recommends that the exposure to toxic substances during development should be considered as a non-negligible risk factor for triggering neurodevelopmental disorders in children. However, due to lack of studies only a very few substances have been identified as developmental neurotoxicants so far (Grandjean and Landrigan, 2006). Furthermore, in the case of pesticides there are no general requirements that they have to be tested for DNT effects before their registration in Europe or the US. The US Environmental Protection Agency (EPA) does only require developmental and reproductive toxicity studies for registration of pesticides used in food. Currently, DNT testing is only recommended on a case-by-case basis and at regulatory level discussions are ongoing to establish a number of criteria in the different regulatory frameworks (for chemicals, pesticides, food additives *etc.*) to be used as triggers for deciding when testing is needed (Coecke *et al.,* 2007). Moreover, the current DNT guidelines (Organisation for Economic Co-operation and Development (OECD) TG 426 and US EPA 712-C-98-239) (OECD, 2007; US EPA, 1998) are based entirely on *in vivo* studies that are time consuming, complex, costly and not suitable for the testing of large numbers of substances. In addition, the interpretation of the data from these studies can be difficult to predict human toxicity and often does not provide sufficient amount of information to facilitate regulatory decision-making. This puts pressure on all stakeholders involved (academia, industry and regulatory bodies) to look for alternative methods such as *in vitro* testing, *in silico* modeling and application of non-mammalian models that could accelerate the process of DNT testing for regulatory requirements. Both *in vitro* and non-mammalian test systems offer the possibility of providing early screening tools for a large number of substances, to identify the chemicals with DNT potential and additionally could be particularly useful in characterizing the compound-induced mechanisms of toxicity of various developmental processes.

We have evaluated an *in vitro* approach to detect DNT-induced effects after exposure to pesticides using primary cultures of rat cerebellar granule cells (CGCs). Pesticide toxicity was evaluated at the level of critical neuro-developmental processes, measured by gene expression and immunocytochemistry staining. Five pesticides with different mode of actions were selected to evaluate if our *in vitro* approach could be a useful tool for detection of DNT effects. Indeed the results allowed us to identify which cell type (neuronal or glial) and which state of development (proliferation, differentiation and maturation) was affected by pesticide exposure.

2. Material and methods

2.1 Chemicals and reagents

Reagents for cell cultures were purchased from Gibco Invitrogen (Milano, Italy); DMEM, fetal bovine serum, horse serum, L-glutamine, gentamicin, versene, HEPES and from Sigma-Aldrich (Milano, Italy); Poly-L-lysine, D (+) glucose and potassium chloride.

2.2 Primary cultures of rat cerebellar granule cells (CGCs)

The primary cultures of cerebellar granule cells (CGCs) were prepared from 7-day old Wistar rat pups as described previously (Hogberg *et al.,* 2009). The cerebella were dissociated in versene solution (1:5000) and plated at 0.25 x 10^6 cells/cm^2 in 12- or 96-well plates (Costar) coated with poly-L-lysine (0.01% diluted 1:10 (v/v) in sterile MilliQ water). Cultures were maintained in DMEM supplemented with 5% heat inactivated horse serum, 5% heat inactivated fetal bovine serum, 13 mM glucose, 0.5 mM HEPES buffer, 2 mM L-glutamine, 25 mM KCl and 10 μg/ml gentamicin. Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2. The medium of CGCs was not changed throughout the whole experimental period as these cells have to be cultured in self-conditioned medium. Cell samples from control (non-treated) and treated cultures were prepared at 1, 4 and 12 Days *In Vitro* (DIV) for real time PCR analysis of mRNA expression.

2.3 Pesticide treatments of CGCs

Five pesticides (parathion, dichlorvos, parquat, pentachlorophenol and cycloheximide) and one non-neurotoxic compound (aspirin) were studied. All compounds were purchased from Sigma-Aldrich (Milano, Italy). To prepare the stock solutions toxicants were dissolved in culture medium or dimethyl sulphoxide (DMSO). The concentrations of tested pesticides were chosen based on preliminary range-finding experiments, where wide ranges of concentrations have been tested using the Alamar Blue (AB) (resazurin, Sigma, Milano, Italy) cell viability assay (data not shown). In final experiments three non-cytotoxic concentrations (shown in Table 1-7) were selected based on the AB assay results. In the case of pesticides dissolved in DMSO, a non-cytotoxic concentration (0.5% (v/v)) of DMSO was used and it was constant in all wells of the test plates, independently from the studied chemical concentrations. Twenty-four hours after isolation, the neuronal cultures were exposed to the chemicals for up to 12 DIV, to cover critical developmental processes at various stages of cell maturation. To determine whether the presence of the pesticides influenced the selected gene expression, cell samples were prepared for real time PCR analysis after 4 DIV exposures (immature culture) and 12 DIV (mature culture).

2.4 Assessment of cell viability using Alamar Blue

Cell viability was determined after exposure to the selected pesticides at 4 and 12 DIV using the AB (resazurin) assay (O'Brien *et al.,* 2000) (data not shown). The blue coloured indicator dye resazurin is reduced into fluorescent resorufin by red-ox reactions in viable cells. Resazurin (10 μl of 100 μM stock) in Hank's Buffered Salt Solution was added directly to the 96-well plates, without removing the medium (100 μl). The plates were incubated for 2 h at 37°C, 5% CO_2. After incubation the fluorescence of the resazurin metabolite, resorufin was measured at 530 nm/ 590 nm (excitation/emission) in a multiwell fluorometric reader (Fluoroskan Ascent, Labsystem, Helsinki, Finland).

2.5 Selection of genes for DNT evaluation in CGCs

Seven different genes that could identify the presence of key processes during brain development such as neuronal differentiation (neurofilament (NF) 68 and 200) and functional maturation (N-methyl D-aspartate glutamate receptor (NMDA-R) and gamma-aminobutyric acid A receptor ($GABA_A$-R), proliferation and differentiation of astrocytes (S100β) as well as the presence of neural precursor cells (nestin) were studied.

2.6 RNA purification, reverse transcription and quantitative real-time PCR

Cell samples for analysis of mRNA expression were lysed and total RNA extraction was performed according to the manufacturer's protocol of RNeasy Mini Kit (Qiagen, Milan, Italy). Any contaminating DNA was removed by digestion using an RNase-free DNase set (Qiagen). RNA concentration and protein contamination were assessed spectrophotometrically (Biophotometer; Eppendorf, Milan, Italy). Reverse transcription was performed as follows: 500 ng RNA was incubated with 2.5 mM PCR Nucleotide Mix (Promega, Milan Andorra, Italy) and 12.5 µg/ml random primers (Promega) for 5 min at 65°C using a Perkin-Elmer Geneamp PCR system 9600. Subsequently 2 units/µl RNaseOut inhibitor (Invitrogen), 10 units/µl M-MLV reverse transcriptase (Promega) were added with the respective M-MLV buffer (Promega) and the samples were incubated for 10 min at 25°C for annealing, 60 min at 37°C for cDNA synthesis and 15 min at 70°C for inactivation of enzymes. An AbiPrism 7000 sequence detector system in conjunction with TaqMan® Universal PCR Master Mix and TaqMan® Real-Time PCR Assays-on-Demand (Applera Italia, Monza, Italy) was used for investigating the gene expression and the house keeping gene according to the manufacturer's protocol. The primers used were: 18S ribosomal RNA (18S rRNA, Hs99999901_s1) (TaqMan® Gene Expression Assays ID), nestin (Nes, Rn00564394_m1), neurofilament, light polypeptide 68kDa (Nfl, Rn00582365_m1), neurofilament, heavy polypeptide 200kDa (Nefh, Rn00709325_m1), ionotropic glutamate receptor N-methyl D-aspartate 1 (GRIN1, Rn00433800_m1), gamma-aminobutyric acid A receptor delta (Gabrd, Rn01517015_g1), and S100 protein, beta polypeptide (S100β, Rn00566139_m1). Relative RNA quantification was performed using the comparative C_T method, normalizing the data to a standard calibrator (a mixture of samples from the different time points of the cell proliferation and differentiation), and to the 18S rRNA content (Livak and Schmittgen, 2001).

2.7 Immunocytochemistry

CGCs cultures for immunocytochemistry were fixed for 20 minutes with 4 % paraformaldehyde in PBS at room temperature at 1, 4 and 12 DIV. The cells were permeabilised for 15 minutes with 0.1 % TritonX100 and were followed by a blocking step (10 % goat serum) for 2h at room temperature. Primary antibodies (all from Sigma, Milano, Italy) diluted in 1 % goat serum in PBS against GFAP (mouse monoclonal 1:800), nestin (rabbit, 1:200) and NF-200 (rabbit, 1:1000) were applied to the cells over night at 4°C. Subsequently, the secondary antibodies, goat anti-mouse IgG Alexa 546 (1:1000) and IgG Alexa 488 (1:1000) (Gibco Invitrogen, Milano, Italy) were applied. Cell nuclei were stained by Hoechst 33342 (10 µg/ml) purchased from Molecular Probes Europe (Leiden, The Netherlands). Controls for specific immunostaining were performed by omitting the primary antibodies from the procedure. All stained cultures were examined by fluorescent microscopy (Olympus IX70, Hamburg, Germany).

2.8 Statistical analysis

The GraphPad Prism 5.0 (GraphPad software, San Diego, USA) program was used for statistical analyses. All data given are the means of at least three independent experiments performed in duplicates ± standard error of the mean (S.E.M.). Two-way ANOVA was performed to assess differences between treated and non-treated cultures.

All data were log-transformed to achieve Gaussian distribution. Statistical significance was indicated as follows * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ treated vs. Control.

3. Results

3.1 Characterization of primary cultures of CGCs during development

Initially, control (non-treated) cultures were characterized over time using phase-contrast microscopy and immunocytochemistry. The morphology of the cell cultures (as seen by phase-contrast microscopy) was dramatically changing over the time starting with round cell bodies at 1 DIV (Fig. 1A). At 4 DIV (Fig. 1D) the cultures started to differentiate into a characteristic neuronal morphology with neurite outgrowths which by 12 DIV formed an intense network (Fig. 1G). Immunocytochmistry staining for NF-200 (green) increased over the time, being barely expressed at 1 and 4 DIV (Fig. 1B and E) while the staining showed rich neuronal connections at 12 DIV (Fig. 1H). Similarly, the immunocytochemistry staining for the astrocytic protein GFAP (red) was increasing over the time, not expressed at 1 DIV (Fig. 1C), increased expression at 4 DIV (Fig. 1F) and highly expressed at 12 DIV (Fig. 1I). Only a few cells stained positive for the neural precursor protein nestin (green) with the same intensity at 1 DIV (Fig. 1C), 4 DIV (Fig. 1F) and 12 DIV (Fig. 1I). The observed changes in protein staining for NF-200, GFAP and nestin correlated well with the observed changes in the mRNA expression over the time (data not shown).

3.2 Exposure to the selected pesticides decreased the gene expression of the neuronal markers (NF-68 and NF-200)

To evaluate if exposure to the selected pesticides was affecting the neuronal differentiation in primary cultures of CGCs, two neuronal cytoskeleton proteins, the earlier expressed NF-68 and the later expressed NF-200 were studied. Exposure to all pesticides significantly down-regulated the mRNA level of both NF-68 (Table 1 and Fig. 2A) and NF-200 (Table 2 and Fig. 2B), however, at different concentrations and time points. The effects on the gene expression of NF-68 were as follow, with increasing toxicity starting from pentachlorophenol (50μM), dichlorvos (50μM), parathion (25μM), paraquat (2.5μM) and cycloheximide (0.05μM) (Table 1 and Fig. 2A). Moreover, the significant decrease in gene expression of NF-68 was already observed at 4 DIV for parathion (25μM), dichlorvos (50μM) and cycloheximide (0.05μM) with persisting decrease after exposure to parathion at 12 DIV. In contrast the exposure to dichlorvos and pentachlorophenol reached the control level after the prolonged exposure of 12 DIV. Only the 12 days exposure to paraquat (2.5μM) and pentachlorophenol (50μM) induced significant decrease in the gene expression of NF-68. Increasing toxicity observed at the mRNA level of NF-200 was induced by pentachlorophenol (50μM), dichlorvos (50μM), parathion (10μM), paraquat (2.5μM) and cycloheximide (0.05μM) (Table 2 and Fig. 2B). The decrease was already observed at 4 DIV for all studied pesticides. However, after 12 days exposure to dichlorvos and cycloheximide the gene expression of NF-200 went up again. The observed effects suggest that the neuronal differentiation and morphology could be affected by exposure to pesticides and was confirmed by phase-contrast microscopy after the exposure to 2.5 μM paraquat for 12 DIV (Fig. 1J). Moreover, the decreased expression of NF-200 was confirmed at the protein level by immunocytochemistry after exposure to the same concentration of paraquat (2.5 μM) for 12 DIV (Fig. 1K). Exposure to aspirin (negative control) up to 500μM did not induce any changes in the gene expression of the studied neuronal cytoskeleton proteins.

3.3 The mRNA level of the NMDA receptor and the $GABA_A$ receptor was affected differently by exposure to the various pesticides

To evaluate the neuronal maturation, subunits of the NMDA and the $GABA_A$ receptors were studied. Paraquat (2.5μM), parathion (25μM) and pentachlorophenol (50μM) exposure significantly down-regulated the mRNA expression of the NMDA receptor subunit 1 after 12 DIV (Table 3 and Fig. 2C). No significant effect at studied concentrations could be observed after the exposure to dichlorvos and cycloheximide. The mRNA level of the $GABA_A$ receptor subunit delta was affected to a higher degree than the NMDA receptor, as it was down regulated by all pesticides except cycloheximide (Table 4 and Fig. 2D). The decrease was observed at 4 DIV after exposure to (given with increasing toxicity) dichlorvos (50μM), parathion (25μM) and paraquat (2.5μM) and at 12 DIV by pentachlorophenol (50μM), parathion (25μM) and paraquat (2.5μM) exposure (Table 4 and Fig. 2D). Exposure to aspirin up to 500μM did not alter the gene expression of the $GABA_A$ receptor subunit delta or the mRNA expression of the NMDA receptor subunit 1. The obtained results indicate that the neuronal maturation could be affected by exposure to selected pesticides at given concentrations as any disturbance in the expression of NMDA (excitatory receptor) and GABA (inhibitory receptor) could lead to the changes in neuronal function.

3.4 The gene expression of the astrocytic marker S100β was decreased after exposure to paraquat, pentachlorophenol and cycloheximide

Proliferation and differentiation of astrocytes are of great importance during the brain development as they play an important role in the neuronal-glial interaction. To evaluate whether astrocytes were affected by the exposure to pesticides the zinc and calcium binding protein S100β expressed in astrocytes was studied. Exposure to paraquat (2.5μM) at 4 DIV, pentachlorophenol (50μM) at 12 DIV and cycloheximide (0.05μM) at 4 and 12 DIV significantly down-regulated the gene expression of S100β (Table 5 and Fig 2E). These results indicate that the proliferation and differentiation of astrocytes were affected by the exposure to these pesticides that consequently could lead to neuronal and DNT effects. No effects on the mRNA level of S100β were observed after exposure to parathion, dichlorvos and aspirin (negative control) at the selected concentrations (Table 5 and Fig 2E).

3.5 Exposure to parathion and paraquat induced an up-regulation in the mRNA level of the neural precursor marker nestin

To identify the presence of neural progenitor cells, nestin, a neural precursor cytoskeleton protein was studied. Exposure to parathion (50μM) at 4 and 12 DIV and paraquat (2.5μM) at 12 DIV significantly up-regulated the gene expression of nestin (Table 6 and Fig. 2F). Nestin has also been reported to be re-expressed in activated astrocytes after brain injury or neuronal damage and has been recognized as a sensitive marker for reactive astrocytes in the CNS (Chen *et al.*, 2002; Clarke *et al.*, 1994; Rutka *et al.*, 1999). The observed increase in the mRNA expression of nestin could therefore be due to proliferation of precursor cells, higher expression of nestin per cell or and most likely because of re-expression of nestin in astrocytes that became activated in response to the possible neuronal damage. In contrast exposure to pentachlorphenol (50μM) significantly decreased the gene expression of nestin. The increased expression of nestin was confirmed at the protein level by immunocytochemistry after exposure to 2.5 μM paraquat for 12 DIV (Fig. 1L). Exposure to

dichlorvos and cycloheximide at the selected concentrations as well as exposure to aspirin (500μM) did not induce any changes in the mRNA level of nestin (Table 6 and Fig. 2F).

4. Tables and figures

NF-68	DIV 4	DIV12	DIV 4	DIV12	DIV 4	DIV12
Parathion	**10μM**		**25μM**		**50μM**	
% of control	70±8	57±14	38±10***	40±13***	13±9***	2±1***
Dichlorvos	**10μM**		**50μM**		**75μM**	
% of control	111±6	84±3	55±3*	95±5	60±10*	86±3
Paraquat	**0.16μM**		**0.63 μM**		**2.5 μM**	
% of control	89±11	86±13	91±15	67±10	68±11	23±6***
Pentachlorophenol	**10μM**		**25μM**		**50μM**	
% of control	116±17	87±7	95±4	84±11	84±6	22±5***
Cycloheximide	**0.01μM**		**0.05μM**		**0.075μM**	
% of control	71±7	97±12	52±5*	83±7	53±2**	86±2
Aspirin	**100μM**		**250μM**		**500μM**	
% of control	97±4	92±6	99±6	93±7	93±9	108±4

Table 1. Changes in mRNA levels (expressed as % of control) of the neuronal marker NF-68 in primary cultures of CGCs exposed to pesticides (parathion, dichlorvos, paraquat, pentachlorophenol or cycloheximide) or to aspirin (negative control). *P<0.05, **P<0.01 and ***P<0.001 comparing to untreated culture.

NF-200	DIV 4	DIV12	DIV 4	DIV12	DIV 4	DIV12
Parathion	**10μM**		**25μM**		**50μM**	
% of control	51±8***	39±12***	25±7***	26±8***	41±9***	2±1***
Dichlorvos	**10μM**		**50μM**		**75μM**	
% of control	111±4	75±4	42±3***	76±4	33±9***	65±9**
Paraquat	**0.16μM**		**0.63 μM**		**2.5 μM**	
% of control	77±6	97±16	74±13	71±10	49±5**	15±3***
Pentachlorophenol	**10μM**		**25μM**		**50μM**	
% of control	111±27	86±8	81±9	63±8	52±7**	9±5***
Cycloheximide	**0.01μM**		**0.05μM**		**0.075μM**	
% of control	71±9	75±10	47±5***	66±6	35±4***	72±5
Aspirin	**100μM**		**250μM**		**500μM**	
% of control	99±9	99±7	95±10	88±7	95±11	95±9

Table 2. Changes in mRNA levels (expressed as % of control) of the neuronal marker NF-200 in primary cultures of CGCs exposed to pesticides (parathion, dichlorvos, paraquat, pentachlorophenol or cycloheximide) or the non-neurotoxic chemical aspirin. **P<0.01 and ***P<0.001 comparing to untreated culture.

NMDA-R	DIV 4	DIV12	DIV 4	DIV12	DIV 4	DIV12
Parathion	**10µM**		**25µM**		**50µM**	
% of control	87±3	78±15	65±12	46±15***	43±7***	2±1***
Dichlorvos	**10µM**		**50µM**		**75µM**	
% of control	121±8	86±5	97±7	94±5	108±11	91±6
Paraquat	**0.16µM**		**0.63 µM**		**2.5 µM**	
% of control	92±9	89±16	95±17	62±7	72±10	24±7***
Pentachlorophenol	**10µM**		**25µM**		**50µM**	
% of control	105±6	107±15	99±9	94±15	94±5	21±5***
Cycloheximide	**0.01µM**		**0.05µM**		**0.075µM**	
% of control	99±8	111±9	107±8	113±9	107±7	113±4
Aspirin	**100µM**		**250µM**		**500µM**	
% of control	102±9	93±7	104±9	95±5	103±9	100±8

Table. 3 Changes in mRNA levels (expressed as % of control) of subunit 1 of the NMDA receptor in primary cultures of CGCs exposed to pesticides (parathion, dichlorvos, paraquat, pentachlorophenol or cycloheximide) or the non-neurotoxic chemical aspirin. ***P<0.001 comparing to untreated culture.

$GABA_A$-R	DIV 4	DIV12	DIV 4	DIV12	DIV 4	DIV12
Parathion	**10µM**		**25µM**		**50µM**	
% of control	90±9	63±14	47±10**	41±13***	24±13***	1±0.5***
Dichlorvos	**10µM**		**50µM**		**75µM**	
% of control	113±11	83±5	51±3*	85±3	47±14**	68±12*
Paraquat	**0.16µM**		**0.63 µM**		**2.5 µM**	
% of control	73±8	76±9	71±11	50±6	56±10	28±7***
Pentachlorophenol	**10µM**		**25µM**		**50µM**	
% of control	146±40	97±11	99±4	107±15	107±8	47±13**
Cycloheximide	**0.01µM**		**0.05µM**		**0.075µM**	
% of control	90±10	97±11	93±8	103±5	89±5	109±6
Aspirin	**100µM**		**250µM**		**500µM**	
% of control	96±8	84±6	93±10	87±5	84±9	100±8

Table 4. Changes in mRNA levels (expressed as % of control) of subunit delta of the $GABA_A$ receptor in primary cultures of CGCs exposed to pesticides (parathion, dichlorvos, paraquat, pentachlorophenol or cycloheximide) or the non-neurotoxic chemical aspirin. *P<0.05, **P<0.01 and ***P<0.001 comparing to untreated culture.

S100β	DIV 4	DIV12	DIV 4	DIV12	DIV 4	DIV12
Parathion	**10μM**		**25μM**		**50μM**	
% of control	110±17	116±23	112±19	123±32	101±14	80±15
Dichlorvos	**10μM**		**50μM**		**75μM**	
% of control	132±17	104±10	104±9	89±10	117±10	76±4
Paraquat	**0.16μM**		**0.63 μM**		**2.5 μM**	
% of control	62±6	116±18	63±9	96±16	61±9*	97±15
Pentachlorophenol	**10μM**		**25μM**		**50μM**	
% of control	135±26	129±11	110±32	144±11	88±17	47±5**
Cycloheximide	**0.01μM**		**0.05μM**		**0.075μM**	
% of control	104±17	99±21	61±9*	52±17***	61±13*	25±7***
Aspirin	**100μM**		**250μM**		**500μM**	
% of control	107±14	111±11	111±12	112±8	107±12	91±3

Table 5. Changes in mRNA levels (expressed as % of control) of the astrocytic marker S100β in primary cultures of CGCs exposed to pesticides (parathion, dichlorvos, paraquat, pentachlorophenol or cycloheximide) or the non-neurotoxic chemical aspirin. *P<0.05, **P<0.01 and ***P<0.001 comparing to untreated culture.

Nestin	DIV 4	DIV12	DIV 4	DIV12	DIV 4	DIV12
Parathion	**10μM**		**25μM**		**50μM**	
% of control	89±5	127±25	94±5	142±14	263±106**	338±86***
Dichlorvos	**10μM**		**50μM**		**75μM**	
% of control	123±8	103±4	110±5	104±11	128±7	93±10
Paraquat	**0.16μM**		**0.63 μM**		**2.5 μM**	
% of control	69±4	120±17	82±10	127±21	76±6	228±31***
Pentachlorophenol	**10μM**		**25μM**		**50μM**	
% of control	130±22	117±11	93±13	104±18	71±4	34±10***
Cycloheximide	**0.01μM**		**0.05μM**		**0.075μM**	
% of control	116±12	125±15	119±11	94±11	136±17	83±10
Aspirin	**100μM**		**250μM**		**500μM**	
% of control	105±12	121±16	97±9	124±13	97±8	92±10

Table 6. Changes in mRNA levels (expressed as % of control) of the neural precursor marker nestin in primary cultures of CGCs exposed to pesticides (parathion, dichlorvos, paraquat, pentachlorophenol or cycloheximide) or the non-neurotoxic chemical aspirin. **P<0.01 and ***P<0.001 comparing to untreated culture.

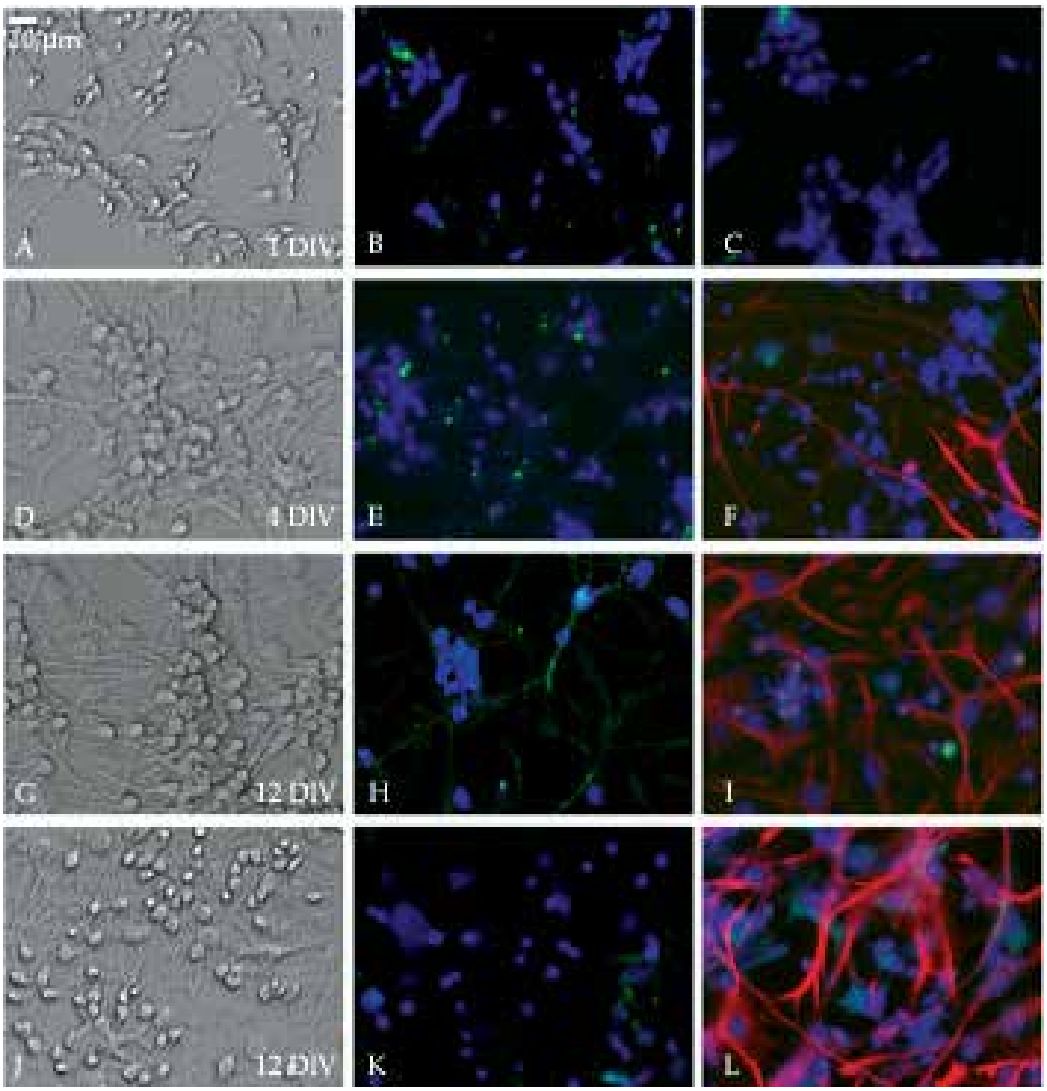

Fig. 1. Characterisation of CGCs by phase-contrast microscopy and immunocytochemistry in control (non-treated) and exposed to paraquat cultures. Neurons with round cell bodies (A) at 1 DIV progressively differentiated into neuronal phenotype showing outgrowth of neuritis at (D) 4 DIV and a dense neuronal network over the time (G) 12 DIV. In control cultures, protein expression of NF-200 (green) was very low at (B) 1 DIV and (E) 4 DIV but was higher expressed at (H) 12 DIV. The astrocytic protein GFAP (red) was not expressed at (C) 1 DIV but the expression increased over the time at (F) 4 DIV and (I) 12 DIV. The neural precursor protein nestin (green) was low expressed and at the same level over the time (C) 1 DIV, (F) 4 DIV and (I) 12 DIV. Exposure to paraquat (2.5 µM) at 12 DIV decreased the (J) neuronal network and (K) the protein expression of NF-200 (green) while the protein expression of (L) nestin (green) was increased. Nuclei were co-stained with Hoechst 33342 (blue).

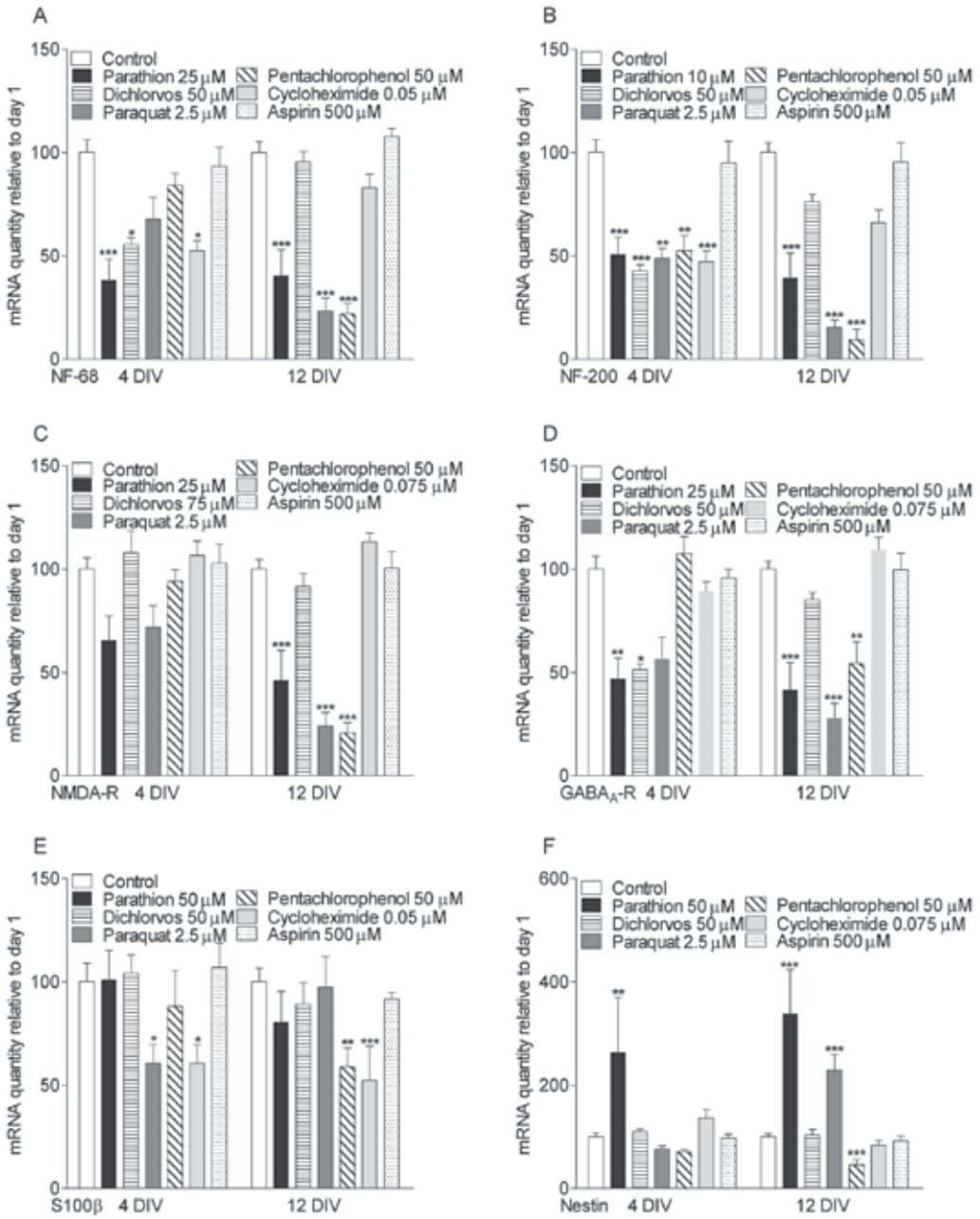

Fig. 2. The lowest concentration that induced the changes in mRNA levels (expressed as % of control) after exposure to pesticides (or aspirin). The neuronal markers: (A) NF-68 and (B) NF-200, (C) NMDA-R and (D) GABA$_A$-R. The astrocytic marker (E) S100β and the neural precursor marker (F) nestin. In the case of no observed changes the highest concentration tested is shown. *P<0.05, **P<0.01 and ***P<0.001 comparing to untreated culture.

5. Discussion

It is well known that a wide range of pesticides (~45%) can cause neurotoxicity. Taking into consideration the vulnerability of the developing brain it is likely that many of these substances could also cause developmental neurotoxicity. So to protect children's health it is important from a regulatory and public health prospective to have reliable tools in order to identify pesticides (and other chemicals) with DNT potential. In these studies we have demonstrated that gene expression is a relevant and promising tool to detect compounds with potential to induce DNT. Interestingly we could identify pesticides with different toxic mechanisms and at concentrations lower than those found in human plasma (Hogberg et al., 2009) suggesting that it is both a specific and sensitive endpoint.

To evaluate if we could identify toxic effects induced by various classes of pesticides we selected five pesticides with diverse mechanisms of toxicity. Since insecticides are especially known to induce neurotoxic effects, two insecticides (parathion and dichlorvos) from the most widely used class, organophosphates, were studied. Organophosphates were developed in the 1940's and 50's (Costa, 1988) and their main target is the acetyl cholinesterase enzyme. Inhibition of this enzyme causes accumulation of acetylcholine at the cholinergic synapses leading to overstimulation of cholinergic receptors that can give various effects in the CNS. Young animals and presumably children are more sensitive to the acute toxicity of these inhibitors than adults, possibly due to lower detoxification abilities (Costa, 2006; Mileson *et al.,* 1998; Pope, 1999; Thullbery *et al.,* 2005). In addition, the adverse effects on the developing brain could also be mediated by additional mechanisms, such as damage to DNA and RNA synthesis (Crumpton *et al.,* 2000a; Crumpton *et al.,* 2000b; Song *et al.,* 1997), deregulation of signal transduction pathways (Ehrich, 1995; Song *et al.,* 1997), oxidative stress (Crumpton *et al.,* 2000b) and astroglial cell proliferation (Garcia *et al.,* 2001; Guizzetti *et al.,* 2005). Indeed, in our study exposure to parathion and dichlorvos induced several changes in gene expression of both neuronal and glial cells indicating multiple mechanisms of toxicity (besides the inhibition of acetyl cholinesterase). Moreover, the neurotoxicity of these two organophosphates seems to differ in mechanism as they affected the gene expression differently, with parathion being the most toxic since it affected more genes at lower concentrations and at earlier time points.

Herbicides and fungicides are in general less toxic for the brain than insecticides, with the exceptions of paraquat (herbicide) which was studied here. Paraquat induces neurodegeneration of dopaminergic neurons (Castello *et al.,* 2007; Thiruchelvam *et al.,* 2002; Wu *et al.,* 2005) and can cause oxidative stress. The obtained results show that neurons are more affected by paraquat than the astrocytes as the gene expression of the neuronal markers (NF-68, NF-200, NMDA-R and $GABA_A$-R) were more affected than the astrocytic marker (S100β). This correlates well with the toxic mechanism, as neurons are known to be particularly vulnerable to oxidative stress. Interestingly, the mRNA level of nestin was up-regulated suggesting the presence of activated astrocytes possibly due to the neuronal damage. In fact, activated glial cells (astrocytes and microglia) can enhance the neurotoxcity as they release a variety of proinflammatory and neurotoxic factors such as cytokines and free radicals (Bal-Price and Brown, 2001).

In addition, two pesticides (pentachlorophenol and cycloheximide) with more general toxic mechanisms affecting any kind of cell type were studied. The mechanism of toxicity induced by pentachlorophenol is through the uncoupling of the mitochondrial phosphorylation leading to decreased levels of ATP production which is an essential source of energy for all

fundamental cell functions (Godfraind *et al.,* 1971). Indeed, in our study the expression of all genes were altered by the exposure to pentachlorophenol indicating that all cells (neurons, astrocytes and neural precursor) were affected. The fungicide cycloheximide is inhibiting the protein biosynthesis, which could lead to serious effects on the brain development (Harris *et al.,* 1968). Our results show that the mRNA expression of NF-68 and NF-200 was decreased by the cycloheximide exposure, however, only at the early time point (4 DIV). This indicates a delay in the synthesis of the neuro cytoskeleton proteins that might affect the neuronal morphological differentiation and final neuronal function. Moreover, the gene expression of S100β was down regulated at both 4 and 12 DIV suggesting that the proliferation and/or differentiation of the astrocytes were affected by cycloheximide exposure.

One non-neurotoxic chemical (aspirin) was selected as a negative control to evaluate if the gene expression could be a selective endpoint to detect specific neurotoxic effects. Indeed, aspirin exposure (up to 500μM) did not induce any changes suggesting that only neurotoxic compounds were detected by this endpoint. However, more non-neurotoxic chemicals should be tested to evaluate the robustness of this endpoint.

Since the development of the brain is based on precise events in space and time (such as proliferation, migration and differentiation) that are strictly controlled, *e.g.* by neurotransmitters, electrical activity and hormones, the pesticides-induced mechanisms of toxicity identified in the above studies are likely to have a potential to cause DNT. There are more than 600 pesticides registered on the market, including insecticides, fungicides and rodenticides, and several of these are produced in high volumes (Grandjean and Landrigan, 2006). Even though the uses of many pesticides are restricted and an increase in DNT testing is demanded, the general lack of DNT data for agricultural chemicals is of particular concern because of their widespread use and ubiquitous exposure (Whyatt *et al.,* 2004). A population based study has reported that over 90 % of the children in the US have detectable urinary remains of neurotoxic pesticides (Schettler, 2001). This exposure might be a risk to children's health, as these substances are suggested to contribute to developmental disorders such as attention deficits, hyperactivity disorders, autism and learning disabilities (Costa *et al.,* 2008; Jurewicz and Hanke, 2008; Shafer *et al.,* 2005). To protect children's health the reliable testing strategy has to be built up, as the current one that is based on *in vivo* approaches is too complex and not effective enough. Incorporation of alternative approaches such as *in vitro* studies, together with *in silico* modeling (Coecke et al., 2007) and non-mammalian animal models (zebra fish, medaka or *caenorhabditis elegance*) could significantly facilitate the decision making process for regulatory purposes.

6. Conclusion

The results from our study suggests that adverse effects induced by studied pesticides (parathion, dichlorvos, paraquat, pentachlorophenol and cycloheximide) were mediated by multiple toxicity mechanisms. Indeed, our *in vitro* approach allowed us to determine which cell type (neuronal or glial) and at which stage of development (proliferation, differentiation or maturation) was affected by the exposure to the different pesticides. Furthermore, the induced toxicity was observed at concentrations relevant to environmental exposure as compared to concentrations found in human plasma (Hogberg *et al.,* 2009). This suggests that gene expression could be used as a sensitive endpoint for testing DNT effects of pesticides. Incorporation of this endpoint together with other neuronal/glial specific assays

into an *in vitro* DNT testing strategy could be useful for an initial identification and further prioritization of compounds that have DNT potential. Such a testing strategy could speed up the process of DNT chemical assessment for regulatory purposes leading to their restricted use and a tighter control of children's exposure to potential DNT compounds.

7. References

Adinolfi, M. (1985). The development of the human blood-CSF-brain barrier. *Dev Med Child Neurol.* 27, 532-537.

Bal-Price, A. and Brown, G. C. (2001). Inflammatory neurodegeneration mediated by nitric oxide from activated glia-inhibiting neuronal respiration, causing glutamate release and excitotoxicity. *J Neurosci* 21, 6480-6491.

Boyle, C. A., Decoufle, P., and Yeargin-Allsopp, M. (1994). Prevalence and health impact of developmental disabilities in US children. *Pediatrics* 93, 399-403.

Castello, P. R., Drechsel, D. A., and Patel, M. (2007). Mitochondria are a major source of paraquat-induced reactive oxygen species production in the brain. *J Biol Chem* 282, 14186-14193.

Coecke, S., Goldberg, A. M., Allen, S., Buzanska, L., Calamandrei, G., Crofton, K., Hareng, L., Hartung, T., Knaut, H., Honegger, P., Jacobs, M., Lein, P., Li, A., Mundy, W., Owen, D., Schneider, S., Silbergeld, E., Reum, T., Trnovec, T., Monnet-Tschudi, F., and Bal-Price, A. (2007). Workgroup report: incorporating *in vitro* alternative methods for developmental neurotoxicity into international hazard and risk assessment strategies. *Environ Health Perspect* 115, 924-931.

Costa,L.G. (1988). Organophosphorus compounds. *In Recent Advances in Nervous System Toxicology* (C.L.Galli, L.Manzo, and P.S.Spencer, Eds.), pp. 203-246. Plening Publishing Corp, New York, US.

Costa, L. G. (2006). Current issues in organophosphate toxicology. *Clin Chim.Acta* 366, 1-13.

Costa, L. G., Giordano, G., Guizzetti, M., and Vitalone, A. (2008). Neurotoxicity of pesticides: a brief review. *Front Biosci.* 13, 1240-1249.

Crumpton, T. L., Seidler, F. J., and Slotkin, T. A. (2000a). Developmental neurotoxicity of chlorpyrifos *in vivo* and *in vitro*: effects on nuclear transcription factors involved in cell replication and differentiation. *Brain Res* 857, 87-98.

Crumpton, T. L., Seidler, F. J., and Slotkin, T. A. (2000b). Is oxidative stress involved in the developmental neurotoxicity of chlorpyrifos? *Brain Res Dev Brain Res* 121, 189-195.

Garcia, S. J., Seidler, F. J., Crumpton, T. L., and Slotkin, T. A. (2001). Does the developmental neurotoxicity of chlorpyrifos involve glial targets? Macromolecule synthesis, adenylyl cyclase signaling, nuclear transcription factors, and formation of reactive oxygen in C6 glioma cells. *Brain Res* 891, 54-68.

Grandjean, P. and Landrigan, P. J. (2006). Developmental neurotoxicity of industrial chemicals. *Lancet* 368, 2167-2178.

Godfraind, J.M., Kawamura, H., Krnjevic, K., Pumain, R. (1971). Actions of dinitrophenol and some other metabolic inhibitors on cortical neurones. *J. Physiol.* 215, 199–222.

Guillette, E. A., Meza, M. M., Aquilar, M. G., Soto, A. D., and Garcia, I. E. (1998). An anthropological approach to the evaluation of preschool children exposed to pesticides in Mexico. *Environ Health Perspect* 106, 347-353.

Guizzetti, M., Pathak, S., Giordano, G., and Costa, L. G. (2005). Effect of organophosphorus insecticides and their metabolites on astroglial cell proliferation. *Toxicology* 215, 182-190.

Harris, C., Grady, H., Svoboda, D. (1968). Alterations in pancreatic and hepatic ultrastructure following acute cycloheximide intoxication. *J. Ultrastruct. Res.* 22, 240–251.

Hogberg, H. T., Kinsner-Ovaskainen, A., Hartung, T., Coecke, S., and Bal-Price, A. K. (2009). Gene expression as a sensitive endpoint to evaluate cell differentiation and maturation of the developing central nervous system in primary cultures of rat cerebellar granule cells (CGCs) exposed to pesticides. *Toxicol Appl Pharmacol* 235, 268-286.

Jurewicz, J. and Hanke, W. (2008). Prenatal and Childhood Exposure to Pesticides and Neurobehavioral Development: Review of Epidemiological Studies. *Int J Occup.Med Environ Health* 1-12.

Kimbrough, R. D., Mahaffrey, K. R., Grandjean, P., Sandoe, S. H., and Ruttstein, D. D. (1989). Clinical effects of environmental chemicals: A software approach to etiologic diagnosis, Hemisphere, New York, US.

Lein, P., Locke, P., and Goldberg, A. (2007). Meeting report: alternatives for developmental neurotoxicity testing. *Environ Health Perspect* 115, 764-768.

Livak, K. J. and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25, 402-408.

Mileson, B. E., Chambers, J. E., Chen, W. L., Dettbarn, W., Ehrich, M., Eldefrawi, A. T., Gaylor, D. W., Hamernik, K., Hodgson, E., Karczmar, A. G., Padilla, S., Pope, C. N., Richardson, R. J., Saunders, D. R., Sheets, L. P., Sultatos, L. G., and Wallace, K. B. (1998). Common mechanism of toxicity: a case study of organophosphorus pesticides. *Toxicol Sci* 41, 8-20.

NRC (2000). NRC: Scientific frontiers in developmental toxicology and risk assessment., National Academy Press, Washington DC.

O'Brien, J., Wilson, I., Orton, T., and Pognan, F. (2000). Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. *Eur.J Biochem* 267, 5421-5426.

OECD. Test Guideline 426. OECD Guideline for Testing of Chemicals Developmental Neurotoxicity Study. http://www.oecdbookshop.org/oecd/display.asp?lang=en&sf1=DI&st1=5L4FG25MNKXS 2007 [accessed 30 April 2010]

Pope, C. N. (1999). Organophosphorus pesticides: do they all have the same mechanism of toxicity? *J Toxicol Environ Health B Crit Rev* 2, 161-181.

Rice, D. and Barone, S. Jr. (2000). Critical periods of vulnerability for the developing nervous system: evidence from humans and animal models. *Environ Health Perspect* 108 Suppl 3, 511-533.

Rodier, P. M. (1994). Vulnerable periods and processes during central nervous system development. *Environ Health Perspect* 102 Suppl 2, 121-124.

Rodier, P. M. (1995). Developing brain as a target of toxicity. *Environ Health Perspect* 103 Suppl 6, 73-76.

Ruckart, P. Z., Kakolewski, K., Bove, F. J., and Kaye, W. E. (2004). Long-term neurobehavioral health effects of methyl parathion exposure in children in Mississippi and Ohio. *Environ Health Perspect* 112, 46-51.

Schettler, T. (2001). Toxic threats to neurologic development of children. *Environ Health Perspect* 109 Suppl 6, 813-816.

Shafer, T. J., Meyer, D. A., and Crofton, K. M. (2005). Developmental neurotoxicity of pyrethroid insecticides: critical review and future research needs. *Environ Health Perspect* 113, 123-136.

Song, X., Seidler, F. J., Saleh, J. L., Zhang, J., Padilla, S., and Slotkin, T. A. (1997). Cellular mechanisms for developmental toxicity of chlorpyrifos: targeting the adenylyl cyclase signaling cascade. *Toxicol Appl Pharmacol* 145, 158-174.

Thiruchelvam, M., Richfield, E. K., Goodman, B. M., Baggs, R. B., and Cory-Slechta, D. A. (2002). Developmental exposure to the pesticides paraquat and maneb and the Parkinson's disease phenotype. *Neurotoxicology* 23, 621-633.

Thullbery, M. D., Cox, H. D., Schule, T., Thompson, C. M., and George, K. M. (2005). Differential localization of acetylcholinesterase in neuronal and non-neuronal cells. *J Cell Biochem* 96, 599-610.

Tilson, H. A. (2000). Neurotoxicology risk assessment guidelines: developmental neurotoxicology. *Neurotoxicology* 21, 189-194.

US EPA. Health effects guidelines OPPTS 870.6300 Developmental neurotoxicity study EPA 712-C-98-239. http://www.epa.gov/opptsfrs/publications/OPPTS_Harmonized/870_Health_Effects_Test_Guidelines/Series/870-6300.pdf 1998 [accessed 30 April 2010]

Whyatt, R. M., Rauh, V., Barr, D. B., Camann, D. E., Andrews, H. F., Garfinkel, R., Hoepner, L. A., Diaz, D., Dietrich, J., Reyes, A., Tang, D., Kinney, P. L., and Perera, F. P. (2004). Prenatal insecticide exposures and birth weight and length among an urban minority cohort. *Environ Health Perspect* 112, 1125-1132.

Wu, X. F., Block, M. L., Zhang, W., Qin, L., Wilson, B., Zhang, W. Q., Veronesi, B., and Hong, J. S. (2005). The role of microglia in paraquat-induced dopaminergic neurotoxicity. *Antioxid.Redox.Signal.* 7, 654-661.

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Agrochemicals: Horticulture Use Conditions Determine Genotoxic Effects and Oxidative Damage in Rural Populations in Santa Fe, Argentina

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1. Introduction

The horticultural productivity in the subtropical regions of the world is severely limited by the pests and diseases affecting crops. The losses in the field and the reduction of the commercial values of the products caused by pests and diseases make the horticultural business less profitable than expected. The fact that the quality of the products has become a priority worldwide has led to the generation of a group of quality standards in response to the demands of the consuming market. The main criterion used regarding this issue is related to the visual aspect related to the shape, the color and absence of damages. The use of agrochemicals is the most common method used for the control of pests and diseases but also one of the most important factors affecting natural resources as well as the health of the rural workers and potential consumers.

Horticultural activity in Santa Fe Province, Argentina, has low participation respect other crops: 4.9 % to field and 1.2 % in crops under cover, being leafy vegetables, cruciferous crops and other crops such as tomato and pepper some of the most relevant (Giunta et al., 2004; 2005).

The so-called "Cinturón hortícola Santafesino" (horticultural belt of Santa Fe) contributes 1.2% to the national market of products. This productive zone is in The Capital Department of Santa Fe Province (Argentina), an area constituted of approximately 3100 Hectares (ha), 1200 ha of which are cultivated in intensive form, and where 2000 people are employed as temporary workers, according to the season. Historically, the horticulture has been an agricultural activity of great economic importance for the region. Nevertheless, throughout time, and considering the low productivity, the technical lag and climatic phenomena such as floods and hail, the activity has modified its principal parameters.

The number of producers decreased from 350 in 1980 to about 150 in 2008. Since 2006, the establishments of productive activity (EAP) with larger surface (more than 50 ha) have

tended to replace the horticultural activity by the cultivation of soybean, maize, alfalfa and wheat.
At present, the horticultural production is focused on leaf-vegetable crops, mainly lettuce, chicory and arugula (22%), spinach beet and spinach (13%), cabbage, broccoli and cauliflower (13%), tomato and pepper (15%), zucchini, gourd, and cucumber (10%) and other vegetables such as beet, radish, aubergine, leek and parsley in a much smaller percentage.

2. Use of pesticides

Horticulture is characterized by a more intensive use of agrochemicals by unit of area or surface than other types of agricultural production. A high-risk crop, such as tomato, receives nearly 40 treatments with insecticides and fungicides along its process of development. The application often includes three active simultaneous or sequenced ingredients (Castignani et al., 2004).
Tomato productivity is severely limited by plagues and diseases. Performance losses have been detected in tomato attacked by the red mite, *Aculops licopersisci* (Lin et al., 1999), the white fly, *Trialeurodes vaporariorum* (Lei et al., 1998), and the tomato moth (Miranda et al., 1998). The most known diseases are those caused by Verticillium and Fusarium. In addition, there are viruses that can cause plant death.
The most common pesticides used in tomato crops are Mancozeb, Cypermethrin, Deltamethrin, Buprofezin, Imidacloprid, Chlorpyrifos, Methamidophos and Copper oxychloride. By analyzing the results of our cross-country research through a survey of producers, we determined that most of the EAP use different types of biocides alone and that the application of fertilizers is scanty. This agrees with that found in other researches carried out in the same area, which show that 57% of the producers use only pesticides, and that the remaining 43% applies credit foliate, urea, adherents, and so on (Rodriguez & Lenardon, 2007).
The most frequently used biocides are insecticides, nematicides and acaricides (57%; Methamidophos, Chlorpyrifos, Cypermethrin, Lambdacialotrin, Chlorfenapyr, Carbofuran, Imidacloprid), herbicides (35%; Glyphosate, Trifluralin, Linuron), and fungicides (8%; Mancozeb, Zineb, Copper oxychloride) (Castignani et al., 2004; Rodriguez & Lenardon, 2007; Simoniello et al., 2008). Many of these agrochemicals are prohibited in developed countries such as the United Kingdom, the U.S.A. and China.

3. Pesticide mixture conflicts

The current social conditions of agrochemical use are far from laboratory conditions which determine their safety. In general, agrochemicals are rarely applied with suitable protection equipment. As a consequence, subjects who work and/or live near vegetable crops usually suffer from pesticide-induced illnesses, generally considered as conditions typical of their daily lives, since they do not lead to a significant incapacity for work.
Toxicity of pesticides, expressed by the LD50, is reported only for individual products and not for mixtures. It is widely known that active ingredients, when combined, can increase their individual ability to cause damage or generate new kinds of damages.
The vast majority of toxicological studies of chemicals have focused on the evaluation of exposures to single compounds. Humans are exposed to complex and variable mixtures of

chemicals, which may act independently as in a single exposure, but may also interact to modulate the effects of the mixture as a whole and the components therein. The risk assessment of real-life exposures is thus much more difficult than that of exposure to single agents. In assessing such risks from a public health perspective, it is necessary to assess whether the chemicals in a mixture interact to cause either an increased or a different overall response as compared with the sum of the responses of the individual chemicals present in the mixture, or whether the overall effect is simply a summation of the expected effect of each chemical (Hughes & Wood, 2002).

Two basic methodological strategies exist to study the toxicology of mixtures: component interaction analysis and whole mixture analysis. Component interaction analysis (bottom-up approach) can be applied to the analysis of simple mixtures with a small number of constituents and where the composition is clearly known. In the absence of specific knowledge of the composition of a mixture, or where there are numerous components (a complex mixture), whole mixture analysis may be more appropriate. However, such studies cannot define the extent of true interactions between components of the complex mixture without data on the fractions of the mixture (Carpenter et al., 2002).

When using a metabolite as a quantitative indicator of exposure it is important to be aware that various factors can affect the proportion of compound being metabolised by a particular route and therefore the amount of metabolite appearing in blood or urine. For example, chemicals other than the compound in question may either induce or inhibit cytochrome P450, which is involved in the metabolism of many chemicals including organophosphorus (OP) pesticides. Furthermore, if the hydrolytic pathways, important in the detoxification of OP pesticides, are inhibited, this can increase the toxicity although the excretion of dialkylphosphate metabolites (often used as biomarkers of exposure) may be lower. Also, genetic factors and age may influence metabolism. For example, the elimination of drugs may be lower in neonates and young children than in adults. The enzymes which can hydrolyse OPs (esterases) show a ten-fold variation in activity in humans and the main enzyme involved exhibits a genetic polymorphism. Thus, although measurement of a single metabolite may indicate that exposure has occurred, using it for precise exposure quantification may not always be appropriate or possible.

Biological monitoring and biological effect monitoring have been little used to study combined effects of exposures to pesticide mixtures. Measurement of pesticides or their metabolites in asymptomatic populations provides no information on the combined effects of pesticides, even if parent compounds or specific metabolites are measured in biological fluids. Group-specific metabolites are measured, as with OPs, much more frequently, and these are difficult to relate to the toxicity of specific pesticides. On the other hand, studies in human milk and fat surveys (Lenardon et al., 2000; Trossero et al., 2009) show that simultaneous exposure to more than one pesticide clearly occurs. A further limitation of biomonitoring is that strategies for biomonitoring the exposure are still strongly influenced by the availability of suitable biomarkers. In fact, for many pesticides, there are none. The alternative of biological effect monitoring may be more promising for the study of combined effects, when new techniques become more widely available. The present methods of biological effect monitoring are rather insensitive.

Humans are often exposed to different pesticides or pesticide mixtures, either simultaneously or in series, making it difficult to identify the effects of each one separately. Chronic exposure to pesticides involves exposure to complex mixtures of different types of chemicals, active ingredients and by-products, such as impurities, solvents and other

compounds produced during the storage procedure, present in technical formulations. Moreover, although inert ingredients have no pesticide activity, they may be biologically active and sometimes the most toxic component of a pesticide formulation.
It is important to consider that each active ingredient has a specific mode of action for controlling a pest, and has its own possible side effects on the wild-life and humans exposed to it. Dangerous effects of pesticides in the environment have been documented in many investigations, on soil microorganisms and aquatic flora and fauna. Occupational exposure to pesticides may increase the risk for adverse reproductive outcomes, brain and nervous system disturbances, may cause immunodepression and lead to cancer in later life and can also induce heritable changes. Three million cases of pesticide poisoning, about 220,000 of which are fatal, occur world-wide every year (Raipulis et al., 2009). The activities or circumstances in horticultural work that cause the greatest number of accidents are those that involve the preparation or application of agrochemicals (48%), and those that take place when using tractors and agricultural machinery, carrying boxes, and/or repairing greenhouses (13% each). Of those interviewed in a survey argentina, 13% did not provide an answer in relation with this issue. With regards to the place where the accidents took place, they found that 50% were in the greenhouse, 25% in the open field, and 25% in the shed. As to the body areas affected, in 52% of the cases, intoxication with agrochemicals affected the body in general, whereas in 13% of the cases, lesions were produced in the eyes, and 7% in other body areas (Paunero et al., 2009).
Genotoxicological biomonitoring of human populations is a useful tool to estimate the genetic risk posed by an integrated exposure to complex mixtures of chemicals. Cytogenetic studies refer to different typology of exposure and provide different information about the genetic risk associated with pesticide exposure. Few studies are available on acute pesticide exposure in poisoned subjects. The large majority of cytogenetic monitoring studies in human populations exposed to pesticides concern the genotoxic effects of chronic low doses of a single compound or of a complex mixture of chemicals (Bolognesi et al., 2003).

4. Biomonitoring and biomarkers

Human biomonitoring depends on the use of biomarkers, defined as quantitative indicators of molecular and cellular events in biological systems, relevant to human health, development and aging. Biomarkers are measured in biological material (generally blood or urine) collected from patients or volunteer subjects in observational or intervention studies (Collins & Dusinska, 2009). The molecular epidemiological approach, which measures molecular or cellular biomarkers as indicators of disease risk or of exposure to causative or preventive factors, has applications in studies of environmental and occupational exposure, disease etiology, nutrition, lifestyle, and so on. It is a valuable adjunct to conventional epidemiology, and has the advantage that it requires far fewer subjects and much less time (and is therefore more economical) than the conventional approach. In addition, the biomarkers, if carefully chosen, can give useful information about the molecular mechanisms involved in disease etiology, for example if they reflect an early stage in the progression of the disease (Collins & Dusinska, 2009).
To help planning a biomonitoring study, it is important to consider the following issues:
- it is always necessary to obtain an ethical approval;
- the sampling of subjects should be performed in the same way throughout the study;

- it is fundamental to be aware of the possibility of 'seasonal effects' and thus collect samples from both the controls and the exposed/treated subjects at the same time, rather than in consecutive phases;
- it is necessary to carry out a pilot study for every critical aspect in order to check for unforeseen problems and to assess experimental variation (and, if possible, control each biomarker evaluated);
- it is essential to use the same protocols and chemicals from the same company and avoid making any change in procedure, however slight it may seem, while carrying out a particular study;
- it is required to follow the principles of Good Laboratory Practice, as far as possible (Dusinska & Collins, 2008).

A working group formed by IARC (1997) has defined biomarkers as ''any substance, structure or process that can be measured in the body or its products and may influence or predict the incidence or outcome of disease''. This definition is further extended by the definition of WHO-ICPS (1993).

The primary purpose of using biomarkers of effect is surveillance, i.e., the identification of individuals or a population at risk of adverse health effects, so that preventive measures can be taken. Although a biomarker of effect is usually also related to exposure to a specific chemical, it is generally more closely related to the occurrence of an adverse health effect (De Zwart et al., 1999).

The selection of appropriate biomarkers is of critical importance because of the opportunity for greater precision in the assessment of risk in individuals or population sub-groups, with the consequent implications for mitigation and health protection. However, this selection will depend upon the state of scientific knowledge and be influenced by social, ethical and economic factors. The process of selection and validation requires careful consideration of the specificity and sensitivity of the biomarker as a measure of the contribution of the exposure to an observed adverse health outcome. A similar process must also be applied to establish the accuracy, precision and quality assurance of the analytical procedure for measuring the selected biomarker, evaluating the intra- and inter-individual variation for a non-exposed population, and reviewing ethical and social considerations. Subject to ethical considerations, the use of validated biomarkers to monitor exposed populations may provide the basis for early, health-protective intervention (EHC 155, 1993).

Sampling both exposed (treated) and control (reference) individuals on the same day reduce the likelihood of day-to-day experimental variation influencing results. This may not be feasible; but what should be definitely avoided is collecting samples from all exposed subjects and then from all controls (or vice versa), over different time frames (Dusinska & Collins, 2008).

A number of factors have been used to describe pesticide exposure in cytogenetic studies: pesticide consumption (kg per year), amount of toxic chemicals used, total number of pesticide formulations used, extension of the areas of pesticide application, and working conditions (greenhouse versus open field), exposure magnitude, the use of protective measures and the specific genotoxic potential of the pesticides used. In addition, the crop type and the environmental factors can influence the kind of pesticide formulations used as well as the chemical absorption. Also, the complex combination of formulations used depending on the region and season, the sample size, the exposure times and intervals after the exposure mainly in relation to the samplings, represents major factors of uncertainty in the comparison of results from different studies (Bolognesi, 2003).

A biomarker of effect can be objectively measured and evaluated as an indicator of normal biological or pathological processes, or toxicological responses to a chemical exposure. The most reliable biomarkers of effect are mechanistically based. The measurement of such biomarkers forms the basis of biological effect monitoring. Some biomarkers can be used as surrogate endpoints. These can substitute for a clinical endpoint, and should be able to predict clinical outcome.
Sensitive biomarkers of effect offer considerable potential for use in studies of individuals exposed to low levels of pesticides and may be invaluable as a bridge between studies in experimental animals and studies in humans and between those in cultured cells and in the intact organism. Much effort is now being devoted to the application of modern biological methods, including transcriptomics, proteomics, metabonomics and non- or minimally-invasive imaging, to identify and develop effective biomarkers of effect. Examples of the application of this approach have been published recently (Petricoin et al., 2002; Issaq et al., 2002). Any accessible biofluid or tissue can be used for biomarker assessment. Techniques now available offer high sensitivity and are applicable to a broad range of endpoints. However, for use in studies of pesticide interactions, it will be important to establish the mechanistic relationship between biomarkers of effect identified in this way and biological responses of concern. Adequate validation, demonstrating their reproducibility and reliability, will be necessary before adopting their widespread use in the study of the toxicology of mixtures.

5. Acetylcholinesterase and Butyrylcholinesterase

The existence of two types of cholinesterases has been proved: acetylcholinesterase (AChE), or 'true cholinesterase', which is found in erythrocytes and in cholinergic nerve terminals; and butyrylcholinesterase (BChE), or pseudocholinesterase, found in plasma, liver, smooth muscle and fat cells. It is well known that AChE can be an effect biomarker of organophosphorous (OP) and methyl-carbamic (MC) compounds. Also, there is evidence that AChE inhibition correlates with OP-induced symptoms of toxicity (Ranjbar et al., 2002).
The inhibition resulting in the accumulation of endogenous acetylcholine responsible for toxicity in the nervous system presents a dose–response pattern of relatively mild symptoms at a 50–60% inhibition of AChE, with weakness, headache, dizziness, nausea and salivation and a convalescence of 1–3 days. Moderate symptoms at 60–90% inhibition are reversed within periods of a few weeks and are characterized by sweating, vomiting, diarrhea, tremors, disturbed gait, pain in the chest and cyanosis of the mucous membranes. At 90–100% inhibition, the prognosis is death from respiratory or cardiac failure.
Biological effect monitoring could be an important component to study the interactive effects of pesticides and related compounds. To be effective, the biomarker should reflect a response (e.g. inhibition of AChE) that is common to several components of a mixture (e.g. OPs). This may be a more meaningful parameter than the measurement of a metabolite common to compounds of differing potencies. Biomarkers of effect in current use lack sensitivity. For example, alkyl phosphates can be detected in the urine of individuals after exposure to amounts of OP well below those causing depression of AChE activity, although this may also reflect the concentration-effect relationship that exists for such compounds (Moretto & Lotti, 1998).
Measurements of AChE activity in red blood cells have routinely been performed to survey exposures to OPs in exposed environments. It has also been established that if AChE activity

(based on individual pre-exposure level – baseline) decreases by 25%, a second measurement has to be carried out, and that if a decrease in AChE activity is confirmed, exposure has to be avoided for 14 days (Knudsen & Hansen 2007). Historically, the measurement of both cholinesterases in plasma and erythrocytes, which reflected the influence of absorbed OPs on the inhibition of these blood enzymes as surrogates of AChE in neural tissue and neuromuscular junctions, was carried out (Cocker et al., 2002). However, it is well recognized that this is a relatively insensitive indicator of an absorbed dose of OP (Reid & Watts, 1981; Drevenkar et al., 1991; Nutley & Cocker, 1993; Hardt & Angerer, 2000). Blood cholinesterase activity needs at least 15% depression from an individual's normal level of plasma or erythrocyte enzyme activity to be considered indicative of pesticide over-exposure.

Furthermore, due to the large inter-individual variability in cholinesterase activity, this approach requires the collection of both baseline and post-exposure samples from an individual and long-term precision of the methods as this directly influences the level of BChE or AChE depression that can be considered significant (Mason & Lewis, 1989).

In addition, the collection of blood samples is sometimes considered invasive and, in some occupational settings, logistically difficult. BChE and AChE measurements have been used for a number of years in cases of clinical poisoning and accidental OP exposure, and in monitoring workers with high risk of exposure. Depression of the plasma BChE enzyme activity is not necessarily associated with symptoms of anti-cholinergic toxicity and large depressions in BChE have been noted in the absence of any effect on erythrocyte AChE. Decreases in the red cell enzyme activity have been suggested to have closer relations to these symptoms. Therefore, in both clinical toxicology and monitoring high-risk occupational activities, the measurement of both enzymes has been recommended (HSE, 2000; Heath & Vale, 1992).

6. Oxidative status

Oxidative stress is a mechanism that could link pesticide exposures to a number of health outcomes observed in epidemiological studies. In blood, normal erythrocyte function depends on the intactness of cell membrane, which is the target for many toxic factors including pesticides (Banerjee et al., 1999).

Free radicals are generally very reactive molecules possessing an unpaired electron. They are produced continuously in cells either as by-products of metabolism, or for example, by leakage from mitochondrial respiration. The most important reactions of free radicals in aerobic cells involve molecular oxygen and its radical derivatives (superoxide anion and hydroxyl radicals), peroxides and transition metals. Cells have developed a comprehensive set of antioxidant defense mechanisms to prevent free radical formation and to limit their damaging effects. These mechanisms include enzymes that inactivate peroxides, proteins that sequester transition metals and a range of compounds that scavenge free radicals. Reactive free radicals formed within cells can oxidize biomolecules and this may lead to cell death and tissue injury (De Zwart et al., 1999).

Continuous exposure of aerobic organisms to prooxidant challenges has endowed living cells with efficient and sophisticated antioxidant systems. These can be divided into enzymatic antioxidant and non-enzymatic antioxidant systems. Superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSHpx) have been distinguished as the most important members of the enzymatic defense systems against oxygen radicals.

Obviously, assaying these enzymes can offer an indication of the antioxidant status of an individual. Besides measuring the enzymatic antioxidant systems in blood samples, non-enzymatic antioxidants, such as vitamin E and C, b-carotene, urate, retinyl esters and GSH, can be monitored as well (Jaeschke, 1995). The available data on experimental animals and humans, obtained both from *in vitro* and *in vivo* studies, indicate that the enzymes associated with antioxidant defense mechanisms are altered under the influence of pesticides (Barerjee et al., 1999, Ranjar et al., 2002, Gultekin et al., 2001).

Oxidative stress plays an important role in the toxicity of various xenobiotics, including pesticide mixtures. Lipid peroxidation is probably the most extensively investigated process induced by free radicals. The abundant presence of membrane phospholipids at sites where radicals in general and, more specifically, reactive oxygen species are formed, render them easily accessible endogenous targets rapidly affected by free radicals. The extent of lipid peroxidation in whole blood was evaluated by measuring the formation of thiobarbituric acid reactive substances (TBARS). The higher oxidative stress in pesticide sprayers is evidenced by an increased concentration of TBARS in plasma and red blood cells, changes in antioxidant status, and altered activities of cellular enzymes. The increased concentration of TBARS observed could be due to the increased peroxidation of membranes. However, oxidative stress is a balance between free radical production and antioxidant activity, and it is possible that the increased TBARS are due to a decreased antioxidant activity (Prakasam et al., 2001).

7. Comet assay

The Single Cell Gel Electrophoresis (SCGE) or Comet assay is a very sensitive method for measuring DNA strand breaks in individual cells. The assay is now a well-established, simple, versatile, rapid, visual, sensitive, and extensively used tool to assess DNA damage and repair, both quantitatively and qualitatively in individual cell populations (Dusinska & Collins, 2008).

The version of the Comet assay developed by Singh et al. 1988, electrophoresis under highly alkaline conditions (pH>13), has been found to be up to two orders of magnitude more sensitive than neutral version (Ostling & Johanson 1984). This enables the DNA supercoils to relax and unwind and allows the detection of alkali-labile sites and single-strand breaks in DNA during electrophoresis. This method measures low levels of strand breaks with high sensitivity.

The simplest types of DNA damage detected by the Comet assay are double-strand breaks (DSBs). DSBs result in DNA fragments and can be detected by merely subjecting them to electrophoretic mobility at neutral pH. Single-strand breaks (SSBs) do not produce DNA fragments unless the two strands of the DNA are separated / denatured. This is accomplished by unwinding the DNA at pH 12.1. It is also possible that single-strand breaks can relax the DNA and hence can also be detected with the Comet assay at neutral pH. Other types of DNA damage broadly termed alkali-labile sites (ALS) are expressed when the DNA is treated with alkali at a pH greater than 13. Breaks can also be introduced at the sites of DNA base modifications by treating the DNA with lesion-specific glycosylases / endonucleases and the fragments thus produced can also be detected by the Comet assay.

At the same time, by controlling the conditions that produce nicks at the sites of specific DNA lesions, the Comet assay can be used to detect various classes of DNA damage. While breaks increase DNA migration, DNA binding and crosslinks can retard DNA migration

and can also be detected by the Comet assay. Therefore, increased migration in the Comet assay can be attributed to strand breaks, alkali-labile sites and incomplete excision repair sites, while decreased DNA migration could be attributed to crosslinks, DNA–DNA or DNA–protein interactions. Some other lesions of DNA damage such as DNA cross-linking (e.g. thymidine dimers) and oxidative DNA damage may also be assessed using lesion-specific antibodies or specific DNA repair enzymes in the Comet assay.

The assay can be performed both *in vivo* and *in vitro* in a variety of samples. Peripheral blood lymphocytes, nasal and buccal epithelial cells have extensively been used to assess human genotoxicity in clinically or occupationally exposed population (Valverde et al., 1997). Also, *in vitro* studies have been conducted in cell lines and primary cell cultures for environmental biomonitoring using fish, earthworms and molluscs (Akcha et al., 2003). The *in vivo* assay with different tissues and organs from mice has also been used (Sasaki et al., 2000) for both DNA damage and repair and widely used in genetic toxicology (Dhawan et al., 2002), human epidemiology (Dhawan et al., 2001; Bajpayee et al., 2002), monitoring of human genotoxicity (Kassie et al., 2000; Palus et al., 2003; Basaran et al., 2003; Piperakis et al., 2003), patients undergoing radio/chemotherapy (Vaghef et al., 1997), and aging (Piperakis et al., 1998; Singh et al., 2003). Also, the *in vivo* assay has been used to monitor the dietary factors in various diseases such as diabetes (Raslova et al., 2000; Pitozzi et al., 2003) and thalassemia (Anderson et al., 2001; Ruf et al., 2003).

Single Cell Gel Electrophoresis has gained wide acceptance as a valuable tool in fundamental DNA damage and repair studies, genotoxicity testing and human biomonitoring. Human blood cells are particularly useful for biomonitoring purposes as they are easily acquired (Dusinska & Collins, 2008).

The biochemical changes induced after exposure to pesticides or their active metabolites include target cell/receptor binding, protein and DNA adduct formation, and induction or inhibition of enzymes (Lopez et al., 2007). DNA damage and oxidative stress have been proposed as mechanisms that could mechanistically link pesticide exposures with a number of health outcomes observed in epidemiological studies (Muñiz et al., 2008).

8. Biomonitoring of pesticide-exposed workers: DNA Damage (Part A)

Study population.

The Regional Ethical Committee established the regulations for the development of the study and informed consent was given by each individual prior to the beginning of the study. A face-to-face questionnaire was completed to obtain information on a) standard demographic data (age, gender, etc), b) individual lifestyle (diet, smoking habit, alcohol and medicine consumption), c) occupational aspects (working hours/days, years of exposure to pesticides, use of protective measures, etc), and d) pesticides used.

The study involved 84 subjects divided into three groups. The first group consisted of 27 pesticide sprayers and applicators and the second group of 27 agricultural workers and farmers. The control group consisted of 30 workers, from the same area, with no history of occupational exposure to pesticides or any potential genotoxic agent. Peripheral blood heparinized samples were obtained from all the subjects involved in the study.

Cell Viability using Fluorescent Dyes.

A cell suspension was mixed with fluorescent DNA-binding dyes an examined by fluorescent microscopy to visualize and count cells with aberrant chromatin organization

(Mercille & Massie, 1994). This mixture was examined with a 40 x objective using a fluorescent microscope. A minimum of 200 total cells was counted, recording the number of viable cells (V) and nonviable cells (NV). The percentages of each of these cellular states in relation to the total number of cells were obtained (Simoniello et al., 2008).

Alkaline Comet Assay.

The standard procedure originally described by Singh et al. (1988) was used with minor modifications. Two slides were processed for each sample, including negative and positive (H_2O_2 50 μM) controls. DNA strand breaks were measured with the Comet assay. One hundred randomly selected Comet assays from each of two duplicate gels were analysed visually on a scale of 0–4 (categories depending on DNA damage level). The overall score, between 100 and 400 arbitrary units, is related to the DNA break frequency and a comet-like image indicates the presence of DNA breaks (Simoniello et al. 2008). The Damage Index Comet Assay (DICA) was calculated.

DNA repair assay.

We tested lymphocytes for their resistance to oxidative DNA damage using Bowden et al. (2003), with modifications. Briefly, aliquots of cells were resuspended in RPMI 1640 medium. A cell suspension were mixed with hydrogen peroxide solution and and was generated oxidative damage. The reaction was quenched using DMSO solution in PBS. Each cell sample was centrifuged, washed again, and resuspended in RPMI 1640 medium supplemented with fetal bovine serum at 37 ºC for 30 min. The remainder of the alkaline Comet assay procedure was performed as previously reported. The Damage Index Repair Assay (DIRA) was calculated (Simoniello et al., 2008).

Part A, results.

The demographic characteristics were similar in the three groups evaluated, except for the occupational exposure (Table 1).

Parameter	Controls (n=30)	Pesticide Applicator Workers (n=27)	Non-pesticide applicator Workers (n=27)
Age (X±S.D.)	37.70±14.07	40,20±11,44	34,06±12,60
Gender (n)(%)			
Female	14 (47)	8 (30)	15 (44)
Male	16 (53)	19 (70)	12 (56)
Smoking (n)(%)			
Yes	7 (23)	3 (11)	6 (22)
No	23 (77)	24 (83)	21 (78)
Alcohol (n)(%)			
Yes	15 (50)	16 (59)	14 (52)
No	15 (50)	11 (41)	13 (48)

Table 1. Demographic characteristics of controls and exposed workers.

A summary of the pesticides most commonly used in horticulture zone, the CAS number, the IARC classification and the US EPA and WHO hazard classification is presented in Table 2.

Pesticides	Compound	CAS Number	Chemical Class	IARC	US EPA	WHO
Fungicide	Captan	133-06-2	Thiophthalimide	3	NL	U
	Copper	7440-50-8	Inorganic-Copper	NL	D	NL
	Mancozeb	8018 01 7	Dithiocarbamate-Inorganic Zinc	NL	B2	U
Insecticide-Nematicide	Chlorpyrifos	2921-88-2	Organophosphorus	NL	E	II
Insecticide	Cypermethrin	67375-30-8	Pyrethroid	NL	NL	II
	Dimethoate	60-51-5	Organophosphorus	NL	C	II
	Endosulfan	115-29-7	Organochlorine	NL	NL	II
	Imidacloprid	105827-78-9	Chloro-nicotinyl	NL	NL	II
	Malathion	121-75-5	Organophosphorus	3	Suggestive	III
	Methamidophos	10265-92-6	Organophosphorus	NL	E	Ib
	Parathion	56-38-2	Organophosphorus	3	C	Ia
	Permethrin	54774-45-7 51877-74-8	Pyrethroid	3	Suggestive	II
Herbicide	Glyphosate	1071-83-6	Phosphonoglycine	NL	NL	III

Table 2. List of pesticides most commonly used (questionnaire answers) by the exposed subjects, CAS number, IARC classification, US EPA classification, and WHO hazard classification. **IARC Classification**: 3: Not classifiable as to carcinogenicity to humans; NL: Not Listed. **US EPA Classification: Group B:** Probable human carcinogen; **B2**: Sufficient evidence of carcinogenicity from animal studies; **Group C**: Possible human carcinogen; **Group D**: Not classifiable as to human carcinogenicity; **Group E**: Evidence of non-carcinogenicity to humans. **WHO hazard classification: Ia:** Extremely hazardous; **Ib**: Highly hazardous; **II**: Moderately hazardous; **III**: Slightly hazardous; **U**: Unlikel to pose an acute hazard in normal use.

Both exposed groups revealed a significant increase in DICA when compared to controls ($P < 0.0001$; Table 3). Sensitivity of lymphocytes to oxidative damage by H_2O_2 *in vitro*, indicated by strand breakage evaluated by the repair assay, showed significant increases in DIRA in both cases when compared to controls ($P < 0.0001$; Table 3).

Individuals	Comet Assay (DICA)	Repair Assay (DIRA)
Control (n=30)	113.20±13.68	116.24±12.49
Pesticide applicators workers (n=27)	215.29±15.06*	218.22±20.89*
Pesticide non-applicators workers (n=27)	221.66±19.95*	224.14±19.99*

Table 3. Damage Index in Comet Assay and Repair Assay in control and exposed workers. Values are mean ±S.D. DICA: Damage Index Comet Assay, DIRA: Damage Index Repair Assay, $P< 0.05$. *t*-Test

When the Damage Index was analyzed with the Comet assay and the Repair assay in pesticide sprayers, no statistically significant differences were observed in relation to confounding factors such as age, gender, smoking and alcohol consumption. However, this

group exhibited a marginally significant difference in DICA when the years of exposure were analysed by the Mann Whitney's test ($P = 0.05$). At the same time, a significant difference ($P < 0.05$) was detected when the individual's protection was used as a comparison factor.

Also, in relation to the Damage Index obtained by the Comet assay and Repair assay for the non-exposed workers, no statistically significant differences ($P > 0.05$) were detected in relation to confounding factors such as age, gender, smoking, alcohol consumption, working years.

Figure 1 shows the behaviour of individual groups when analyzed before and after repair treatment. We observed an important difference between the exposed groups (both) and the control ($P < 0.0001$). The efficiency of leukocytes in repairing damaged DNA was assessed together with the evaluation of DNA damage, using the Comet assay. Moreover, using paired samples *t*-Test, the DNA repair efficiency for each group was estimated as the number of people in whom the damaged DNA was not reduced during incubation at 37 ^{o}C for 30 minutes ($P > 0.05$).

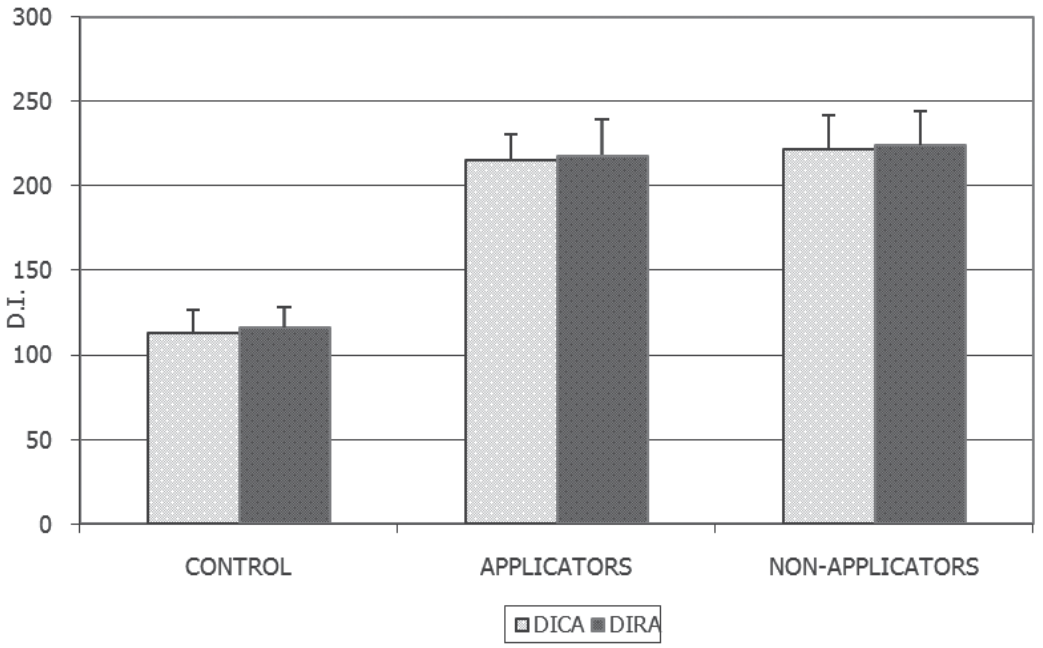

Fig. 1. Damage Index in Exposed (applicators and non-applicators) and control people using the Comet Assay and Repair Assay. DICA: Damage Index Comet Assay, P<0.0001 (ANOVA). DIRA: Damage Index Repair Assay, P<0.0001 (ANOVA)

Taking these results into account, we decided to consider both exposed populations as one. Figure 2 exhibits box-plots showing DICA (Damage Index in Comet Assay) and DIRA (Damage Index in Repair Assay) in control and all pesticide-exposed workers. In both cases, we observed significant differences ($P < 0.0001$) when compared to controls (analysed with *t*-Test).

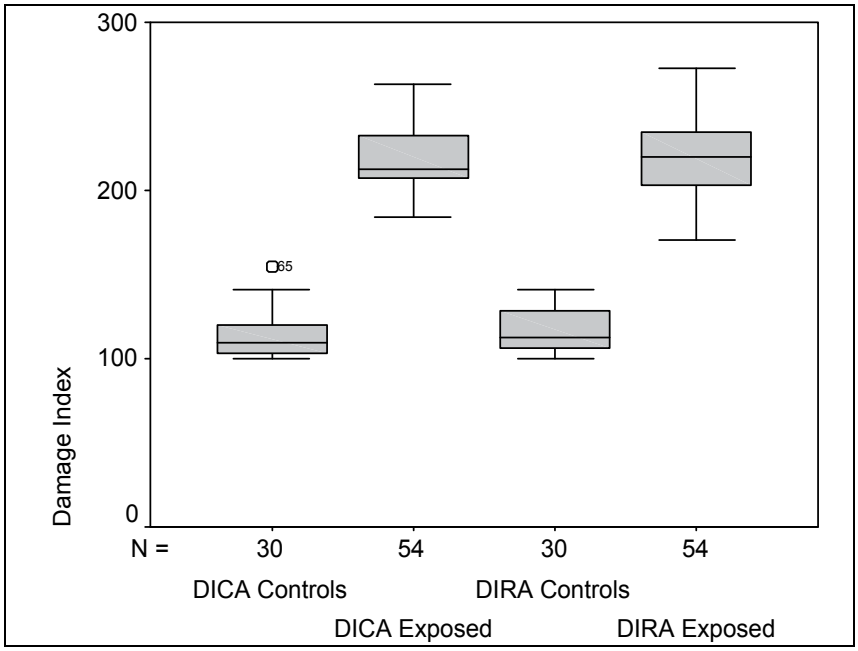

Fig. 2. Boxplots showing the Damage Index Comet Assay (DICA) and Damage Index Repair Assay (DIRA) in control and pesticide-exposed workers. Boxes are limited by 1st and 3rd quartiles divided by the median; thin vertical lines represent minimum and maximum values except when outliers (o) are present.

9. Biomonitoring of pesticide exposed workers: Markers of oxidative stress and genotoxicity (Part B)

Study subjects.

The Provincial Hospital Ethical Committee established the regulations for the development of the study. A face-to-face questionnaire was completed to obtain information on: standard demographic data, individual lifestyles, occupational aspects and pesticides used. The study involved 124 subjects divided into three groups: a group consisting of 18 pesticide sprayer workers directly exposed to pesticides, a group consisting of 23 non-applicator agricultural workers and farmers indirectly exposed to pesticides, and a control group consisting of 82 people from the same area without current or previous exposure to pesticides in their workplace (two unexposed were sought for each exposed subject). In addition, 41 horticultural workers between 18 and 65 years of age, who had been occupational exposed to pesticide mixtures in the previous month, with a minimum work history of 1 year and a maximum of 25 years, were included in the study.

Acetylcholinesterase activity in erythrocytes.

An aliquot of washed erythrocytes were haemolyzed by adding demineralized water at a 1:10 dilution. The hydrolysis rate of acetylthiocholine iodide (substrate) in erythrocyte dilution was measured at 405 nm with spectrophotometer by the reaction with DTNB to give the yellow 5-thio-2-nitrobenzoate anion. Enzyme activity was expressed as U/L of Red Blood Cells (RBC) (Ellman et al., 1961)

Plasmatic Butyrilcolinesterase.

The hydrolysis rate of butyrilthiocholine (substrate) in plasma was measured at 405 nm with spectrophotometer by the reaction of thiocholine iodide with DTNB, to give the yellow 5-thio-2-nitrobenzoate anion. Enzyme activity was expressed as U/L. (Ellman et al., 1961.)

Catalase activity in erythrocytes.

Erythrocytes were haemolyzed by adding ice-cold demineralized ultrapure water at a 1:100 dilution. CAT activity in haemolysate erythrocytes was measured by monitoring the decrease in H_2O_2 concentration spectrophotometrically over time (Aebi, 1984). The specific activity of each sample was calculated on the basis that one unit of enzyme activity was defined as the activity required to degrades 1 mole hydrogen peroxide during 60 s/g Hb.

Lipid peroxidation in erythrocytes.

MDA as a marker for lipid peroxidation in red blood cells (dilution 1:4 with ice-cold) was determined by measuring the formation of the colour produced during the reaction of thiobarbituric acid (TBA) with MDA (TBARS Assay) according to a modification of the method of Buege & Aust (1978). The sample absorbance was determined at 535 nm and the TBARS concentration was calculated using the extinction coefficient 1.56×10^5 M^{-1} cm^{-1}. MDA concentration in erythrocytes was expressed as nmol/g Hb.

Alkaline Comet Assay.

The standard procedure originally described by Singh et al. (1988) with modifications was used. Damage Index Comet Assay (DICA) was calculated for each sample (Simoniello et al., 2010).

Cell Viability using Fluorescent Dyes.

The same cell suspension used in the comet assay, was mixed with fluorescent DNA-binding dyes and examined by fluorescent microscopy to visualize and count cells with aberrant chromatin organization. The percentages of each of these cellular states in relation to the total cells were obtained (Simoniello et al 2010).

Part B, results.

Demographic features of both exposed groups and controls were analyzed; groups were similar regarding age and smoking habits (Table 4).

Parameter	Controls (n=82)	Pesticide Applicator Workers (n=18)	Non-pesticide applicator Workers (n=23)
Age (X±S.D.)	37.70±14.07	40,66±11,44	33,78±11,26
Gender (n)(%)			
Female	37 (45)	6 (33)	13 (56)
Male	45 (55)	12 (67)	10 (44)
Smoking (n)(%)			
Yes	20 (25)	3 (17)	3 (13)
No	62 (75)	15 (83)	20 (87)
Alcohol (n)(%)			
Yes	41 (50)	13 (72)	13 (56)
No	41 (50)	5 (28)	10 (44)

Table 4. Demographic characteristics of controls and exposed workers.

The levels (mean ± SD) of Comet Assay, BChE and AChE assays, CAT activity and TBARS assay, in control and exposed workers are shown in detail in Table 5.

Parameter	Controls (n=82)	Pesticide Applicator Workers (n=18)	Non-pesticide applicator Workers (n=23)
Comet Assay	113.56±16.01	212.94±14.79**	224.73±20.56**
BChE Assay	6993.31±1131.92	6777.77±1281.84	6313.86±1268.26
AChE Assay	9045.54±2191.56	6740.33±5.19**	7651.52±2062.07**
CAT Activity	187.12 ±23.71	72.60±30.48**	106.12±37.15**
TBARS Assay	151.14±30.26	192.74±42.13*	138.90±31.89

Table 5. Comet Assay, BChE and AChE assays, CAT Activity and TBARS assay, in control and exposed workers. Values are presented as mean ± S.D. Comet Assay (DICA); BChE Assay (U/L); AChE Assay (U/L RBC); CAT Activity (kU/g Hb); TBARS Assay (nmol/g Hb). *P: <0.05, **P: <0.001 (Mann Whitney's Test).

Statistical evaluations of the two exposed groups were contrasted in all cases with the control population. AChE decrease was significant ($P < 0.01$), showing an inhibition of 25 and 15 % in the directly and indirectly exposed group, respectively. BChE decrease was not significant ($P > 0.05$), showing an inhibition of 4 and 10 % in the directly and indirectly exposed group, respectively.

A significant increase (51 %) in the levels of TBARS was found in pesticide sprayers ($P < 0.001$), but no differences were observed in the indirectly exposed group ($P > 0.05$).

CAT activity decreased in the whole pesticide-exposed population (applicators and non-applicators). The decrease in CAT activity was 61 % in the directly ($P < 0.0001$) and 43 % in the indirectly exposed group ($P < 0.05$). The analysis of the Comet assay values (mean±SD) indicated a significant increase in DICA (approximately 50%) in both the directly and indirectly exposed groups ($P < 0.001$; Mann–Whitney's U-test). Cell viability (> 85%) was evaluated and expressed as a proportion of living cells.

The Spearman correlation analysis showed a significant inverse correlation between erythrocyte TBARS and AChE in both exposed groups. On the other hand, the Comet assay showed a positive correlation with TBARS in the indirectly exposed group. Figure 3 shows the significant linear regression between AChE and TBARS in both exposed groups: Pesticide Applicators ($r = - 0.33$, $P < 0.05$) and Non-pesticide Applicators ($r = - 0.1747$, $P < 0.05$).

We considered using Personal Protective Equipment (PPE) when at least two items/types of protection (gloves, breathing masks, glasses, impermeable boots, etc.) had been used, since 93% of the pesticide-exposed workers reported using only one kind of protection during the preparation and application of pesticides. None of them reported using the full protective equipment.

A significant correlation was found between age and CAT in agricultural and farmer workers (indirectly exposed), but no significant difference was obtained for other confounding factors.

According to the answers to our questionnaire, the great majority of the subjects in the exposed group were in contact with many pesticides, including Captan, Copper, Mancozeb Chlorpyrifos, Carbofuran, Cypermethrin, Dimethoate, Endosulfan, Imidacloprid, Malathion, Methamidophos, Parathion, Permethrin and Glyphosate. These pesticides were

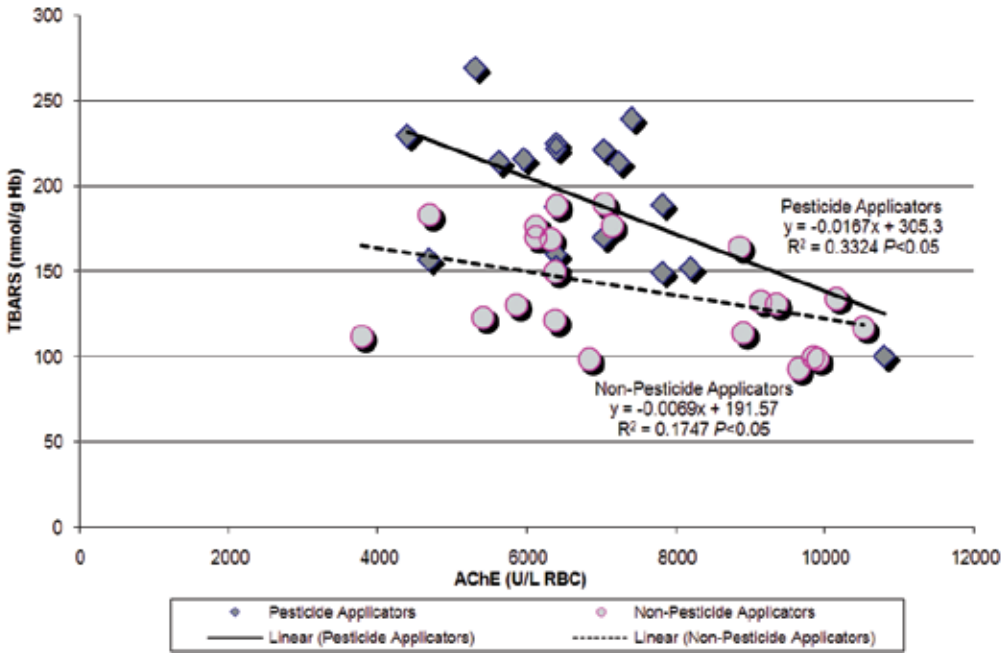

Fig. 3. Correlation of biochemical parameters (TBARS vs. AChE) in blood samples.

similar to those found in part A, but different pesticide mixtures were used in each treatment. For this reason, we could not associate the observed biochemical changes and DNA damage with a specific product or chemical class. In addition, no detailed data were available on the quantities of these pesticides used by the individuals.

10. Discussion

New, more selective and efficient pesticides, possibly ''safer'' for non-target organisms, have been produced in the past few years. So far, they have coexisted in the farming practice with agents in which one or more active principles have been found to be genotoxic and cytotoxic to various systems (De Marco et al., 2000). The primary objective of mutagen testing in genetic toxicology is to determine whether a chemical has the potential to cause genetic alterations in humans. The fundamental concern is the risk to future generations.

The association of mutagenesis with other endpoints such as carcinogenesis, teratogenesis, and aging has been noted. Hence, it is necessary to obtain quantitative data from clinical observations and epidemiological studies in order to predict virtually safe or tolerable levels of exposure (Bajpayee et al., 2005). Genotoxic monitoring in farming population could be a useful tool to estimate genetic risk from exposure to complex pesticide mixtures over extended lengths of time.

The agricultural workers included in this study were also exposed to a great number of pesticides (all of the subjects were exposed to more than two different pesticides), some of which are classified as being carcinogenic by the US Environmental Protection Agency (US-EPA) and hazardous by the World Health Organization (WHO), although not yet listed by the IARC (Table 2). In our study, the recommendation for the use of some agrochemicals to the producers come from technical advice in 35% and the rest consults different sources,

principally to sellers of these inputs, a situation already distinguished by Ringuelet & Laguens (2000) in Great La Plata, Buenos Aires Province, and by Bulacio & Panelo (2000) in the Horticultural Belt of Rosario, Santa Fe Province. Considering the chemicals used, it is important to note that some of these, such as metamidophos, have been banned in other countries because of their high toxicity, while in developing countries such a prohibition is limited (Castillo-Cadena et al., 2006). Assessment of the associations with individual pesticide exposure is very difficult because most occupations involve the regular use of a large number of different pesticides, together with other chemicals such as co-formulants, which vary greatly in their potential toxicities and potencies. Furthermore, measurements of systemic exposure to pesticides were not taken and therefore correlations between increased genotoxicity biomarkers and the degree of exposure were not possible to obtain (Bull et al., 2006).

Pesticides act selectively against certain organisms without adversely affecting others. However, absolute selectivity is difficult to achieve and most pesticides are a toxic risk also to humans. Pesticide application is still the most important method in self-poisoning in the developing world. The International Agency for Cancer Research (IARC) has reviewed the potential carcinogenicity of a wide range of insecticides, fungicides, herbicides and other similar compounds and classified several of them as carcinogenic to laboratory animals; in addition, the IARC has reported the association of different chemical agents, such as phenoxy acid herbicides, 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), lindane, methoxychlor, toxaphene and several organophosphates, with cancer in human studies (Bolognesi, 2003).

The mixtures of pesticides used included some that have proved to have genotoxic effects *in vivo* in human biomonitoring. Considering pesticide information and taking previous reports into account, we found a partial coincidence in the mixtures used in this study and those that had been used in other researches (Dulout et al., 1985; Carbonell et al,. 1995; Peluso et al., 1996; Gómez-Arroyo et al., 2000; Grover et al., 2003).

Multiple chemical exposures are of great interest in Toxicology and Public Health (US-EPA, 1998). Most of the research has been performed on individual chemical agents (Groten, 2000) without considering that the effect of a chemical mixture could be either more or less powerful than the exposure to the individual compounds. Multiple exposures are a rule and not an exception in agricultural practice: pesticide applicators spray large amounts of agrochemical mixtures including a significant number of genotoxic compounds. The pesticides most often used are chlororganics and, more recently, carbamates, organophosphates and pyrethroids, which have been reported to be positive for genotoxic effects in experimental studies in bacterial and in mammalian systems (Bolognesi, 2003).

Although several studies have reported that pesticide sprayers (applicators) represent the most exposed group of agricultural workers (Bolognesi, 2003), in our study, non-applicators were included in the exposed group since they were present during all working activities, including pesticide applications. This can be due to the misconception that non-applicators are not as exposed as applicators. Our study revealed similar frequencies in the Comet assay considering applicators and non-applicators in agreement with previous research (Costa et al., 2006). Therefore, occupational exposure to pesticides can fluctuate in time and the skin constitutes a significant exposure route of absorption mainly in agriculture (Jakubowski & Trzcinka-Ochocka, 2005). On the other hand, some reports consider para-occupational or take-home exposure although agricultural chemicals move from the work place to residential environments through the activities of farm workers (Curl et al., 2002). All farm

workers live close to the fields where chemicals are applied and these are taken home on workers´ bodies, clothing, and shoes, accumulating in the home environment.

Different researches have recognized the invaluable role of AChE monitoring in rural workers at high risk of exposure to OPs and MC pesticides (Mc Cauley et al., 2006). In our work, in agreement with different reports (Ranjbar et al., 2002; Singh et al., 2007), AChE showed a significant decrease in directly and indirectly pesticide-exposed workers. Measuring BChE activities is a frequent marker of exposure in pesticide sprayers, is easier to assay and is more widely available; in our case, BChE activity inhibition was not significant. This may be related to the differential profiles of cholinesterase inhibition that can be observed depending on the particular OP compound; for example, chlorpyrifos and malathion are preferential inhibitors of BChE whereas dimethoate is a preferential inhibitor of AChE. In the interpretation of cholinesterase monitoring results, we may consider that both groups had been exposed to different pesticide mixtures and take into account inter-individual variation and confounding factors in enzymatic activity.

Oxidative damage is thought to be an important mechanism of several pesticides (Banerjee et al., 1999, Prakasam et al., 2001). In blood, normal erythrocyte function depends on an intact cell membrane, which is the target for many toxics, including pesticides. The results of the present study indicate that CAT activity decreased significantly in both pesticide applicators and non-pesticide applicators ($P < 0.001$).

The available data on experimental animals (Seth et al., 2001), *in vitro* studies (Gultekin et al., 2000; Prasanthi et al., 2005) and *in vivo* studies (Ranjbar et al., 2002; Lopez et al., 2007) indicate that the enzymes associated with the antioxidant defence mechanism change under the influence of pesticides. These enzymes efficiently scavenge toxic free radicals and are partly responsible for protection against lipid peroxidation due to pesticide exposure (Banerjee et al., 1999). Hence, the increased level of TBARS observed in this work could be due to increased peroxidation of membranes and/or to decreased antioxidant activity, caused by exposure to pesticide mixtures.

Different OPs, such as phosalone, chlorpyrifos ethyl, and diazinon, have been reported to induce oxidative stress as shown by enhancement of MDA production (Gultekin et al., 2000; Prakasam et al., 2001; Altuntas et al., 2003; Catalgol et al., 2007). Carbamate pesticides may induce oxidative stress, which leads to the generation of free radicals and an alteration in antioxidant enzymes or OFR scavenging enzymes (Seth et al., 2001; Dettbarn et al., 2006). Some pyrethroids affect the flow of erythrocyte membrane due to increased lipid peroxidation (Kale et al., 1999, Gabbianelli et al., 2002; Nasuti et al, 2003). It is likely that the production of O_2^- or the direct action of pyrethroid on the production of GPx could be the cause of oxidative damage (Prasanthi et al., 2005; El Demerdash, 2007). The correlation between TBARS and AChE activity found in the present study (Figure 3) is similar that obtained by other authors (Ranjbar et al., 2002; Akhgari et al., 2003; Singh et al., 2007).

Several different pathways by which oxidative DNA damage occurs have been proposed. These include chemical modification of nucleotides (Cicchetti & Argentin, 2003), direct action of ROS on DNA, or indirect lipid peroxidation degradation products (Collins, 1999). The Comet assay has been used to determine the extent of DNA damage in leukocytes from rural workers occupationally exposed to a variety of pesticides (Garaj-Vrhovac & Zeljezic, 2000; Shadnia et al., 2005; Remor et al., 2008). Our results show that pesticide-spraying workers and farmers presented a significant increase in DICA as well as in DIRA when compared to controls ($P < 0.0001$ in both cases). However, the spraying group exhibited a marginally significant difference in DICA when years of exposure were considered

($P = 0.05$), and a significant difference ($P < 0.05$ in Part A, $P = 0.05$ in Part B) when we used the personal protective equipment (PPE) worn by individuals as a comparison factor.

The positive genotoxicity observed in the exposed workers of this study may be due to the lack of protective measures or protective clothing, gloves or boots in a few cases. In other works carried out in Argentina, 86% of the workers interviewed declared to use PPE, but the authors commented that only 20% had the complete equipment, existing cases in which they wore gloves only. In the present work, 35% of the workers interviewed admitted not using PPE (Panero et al., 2009); this finding agrees with the results indicated by Souza Casadinho (2003) with regard to the unawareness in relation to the danger of using agrochemicals, although it is widely admitted that horticultural activity is risky. In agreement with this, when asking about the aspects which they considered that should be improved, only 5 % of the workers interviewed mentioned aspects of hygiene and safety in the work.

An increase in micronuclei was seen in pesticide-exposed people who did not wear protective gloves (Bolognesi et al., 2002). At the same time, increases in the frequencies of chromosomal aberrations and micronuclei have been found in some studies where the population exposed to pesticides wore no protection during work activities (Costa et al., 2006) or little or no protective clothes (Dulout et al., 1985). Interestingly, several studies that reported a majority of workers using protective measures (>60%) concluded that the results were negative (Bolognesi et al., 2004; Pastor et al., 2001 and 2002; Piperakis et al., 2003 and 2006), suggesting the importance of PPE for preventing exposure. Therefore, field workers may be affected by a lack of available work-site laundering facilities, prolonging their exposure to pesticides and other farm chemicals.

DMSO inhibited H_2O_2-induced DNA damage (Klein et al., 1981). Oxidative DNA damage, as measured in lymphocytes, is maintained in a dynamic steady-state by antioxidant defences, which control input of damage, together with cellular DNA repair, which removes the damage that occurs in spite of the antioxidant protection. In the present work, neither of the exposed groups showed statistically significant differences in DNA damage before and after the repair process, when compared to controls (Figure 1). Palus et al. (1999) reported a significant reduction in the number of cells with DNA damage after a 1-hour repair process in workers of a wooden furniture plant, whereas Piperakis et al. (2003; 2006) observed that the repair efficiency was similar in the studied groups, in agreement with our findings.

Results from *in vitro* and *in vivo* studies can be influenced by individual sensitivity, status of the immune system, genetic predisposition, metabolic or DNA repair differences and simultaneous exposure to other environmental toxicants. These factors may contribute to the variation of the individual response in each subject (Islas-Gonzalez et al., 2005). The individual genetic variability in the enzymes that metabolize agricultural chemicals may also be involved in this process. When these enzymes are not efficient in detoxification, metabolic products accumulate, contributing to a carcinogenic process.

The influence of confounding factors, such as age, gender, smoking and alcohol consumption, on the genotoxic effects of occupational exposure to pesticides was investigated and no significant differences were observed in relation to DICA and DIRA ($P > 0.05$). Other authors have reported similar results when evaluating micronucleus frequency in pesticide-exposed workers (Sailaja et al., 2006). Likewise, smoking failed to have a significant influence on the number of CA (Zeljezic & Garaj-Vrhovac, 2001), level of MN (Bolognesi et al., 2002) and increase in comet tail-length values (Garaj-Vrhovac & Zeljezic, 2000; Liu et al., 2006). However, the discrepancy in some reports is not surprising since the failure to show an effect of smoking could be due to the kind of exposure, target

tissue, and individual susceptibility of subjects in the population. When individuals are exposed to mixtures, it is difficult to predict the final genotoxic effect because of the interaction that could occur between the agents involved, either maximizing or antagonizing the effect (Castillo-Cadena et al., 2006).
A significant correlation was found between age and CAT in indirectly exposed workers, which could be due to random deleterious effects of free radicals produced during aerobic metabolism causing DNA, lipid and protein damage and accumulation over time (Valko et al., 2007).

11. Conclusion

Our study shows that, under the conditions of this experimental work, subjects directly and indirectly exposed to pesticides have enzymatic alterations, modifications in oxidative balance and genotoxic damage when compared to controls. Further studies should be carried out enlarging the sample size and conducting a serial and routine monitoring of populations exposed to pesticide mixtures, using effect and exposure biomarkers.

12. Future perspectives

Cells are continuously exposed to endogenous and exogenous agents that damage DNA. One of the most common kinds of damage is oxidation. A great variety of oxidized bases have been identified in nuclear DNA but 8-oxo-7,8-dihydroguanine (8-oxo-G) is one of the most abundant and readily formed oxidized DNA lesions (Azqueta et al., 2009). Comet assay was adapted to measure oxidized purines and oxidized pyrimidines by the incubation of the nucleoids with bacterial DNA repair enzymes. Formamidopyrimidine glycosylase (FPG) is used to detect oxidized purines, mostly 8-oxo-G, and endonuclease III (EndoIII) is used to detect oxidized pyrimidines. FPG and EndoIII will be employed to investigate environmental or occupational exposure of humans to pesticides associated with oxidative stress.
On the other hand, oxidative DNA damage, as measured in lymphocytes, is maintained in a dynamic steady-state by antioxidant defences, which control input of damage, together with cellular DNA repair, which removes the damage that occurs in spite of antioxidant protection. It would be important to assess the extent of inter-individual variation in repair capacity, or the susceptibility of repair in humans pesticide exposed. The most obvious new direction for molecular epidemiological studies is in dealing with the availability of new technologies (Bonassi & Au, 2002). New information coming from the genome-derived methods can be of paramount importance in human studies. Since the role of susceptibility genes can be investigated more efficiently, their influence on specific effects of exposures and response to genotoxic agents will be elucidated.
Also, diet plays an important role in preventing cancer, but the mechanisms still not clear. Convincing epidemiological evidence links consumption of fruits and/or vegetables with decreased risk of cancer of the lung, mouth, pharynx, oesophagus, stomach, colon and rectum (Collins et al., 2003). These foods are rich in antioxidants such as vitamins C and E, carotenoids and flavonoids, which are capable of decreasing oxidative damage to DNA and thus might prevent mutation and cancer. Knowledge of specific responses and how these responses are affected by nutrimental status, in each region, will facilitate the development of new clinical strategies for the prevention of cancer and other pathological conditions.

13. References

Aebi, H. (1984). Catalase in vitro, *Method. Enzymol*., 105, 121-6, ISSN 0076-6879.

Akcha, F.; Leday, G. & Pfohl-Leszkowicz, A. (2003). Potential value of the Comet assay and DNA adduct measurement in dab (Limanda limanda) for assessment of in situ exposure to genotoxic compounds, *Mutat. Res*., 534, 21–32 ISSN 0921-8262.

Akhgari, M. (2003). Biochemical evidence for free radical induced lipid peroxidation as a mechanism for subchronic toxicity of malathion in blood and liver of rats, *Hum. Exp. Toxicol*., 22, 4, 205-211, ISSN 0960-3271.

Altuntas, I.; Delibas, N.; Doguc, D.K.; Ozmen, S. & Gultekin, F. (2003). Role of reactive oxygen species in organophosphate insecticide phosalone toxicity in erythrocytes in vitro, *Toxicol. in Vitro,* 17, 153–157, ISSN 0887-2333

Anderson, D. (2001) Factors that contribute to biomarker responses in humans including a study in individuals taking vitamin C supplementation, *Mutat. Res*., 480–481, 337–47, ISSN 0921-8262.

Azqueta, A.; Shaposhnikov, S. & Collins, A.R. (2009). Detection of Oxidised DNA Using DNA Repair Enzymes, In: *The Comet Assay in Toxicology,* Dhawan, A. & Anderson (Ed.), D., 57-78, RSC Publishing, ISBN 978-0-85404-199-2, Cambridge, U.K.

Bajpayee, M.; Pandey, A. K.; Parmar, D. & Dhawan, A. (2005) Current Status of Short-Term Tests for Evaluation of Genotoxicity, Mutagenicity, and Carcinogenicity of Environmental Chemicals and NCEs. (Review), *Toxicology Mechanisms and Methods*, 15: 155–180, ISSN 1537-6516

Banerjee, B.D.; Seth, V.; Bhattacharya, A.; Pasha, S.T. & Chakraborty A.K. (1999). Biochemical effects of some pesticides on lipid peroxidation and free-radical scavengers, *Toxicol. Lett*, 107, 33-47, ISSN 0378-4274.

Basaran, N.; Shubair, M.; Undeger, U. & Kars, A. (2003). Monitoring of DNA damage in foundry and pottery workers exposed to silica by the alkaline Comet assay, *Am. J. Ind. Med*., 43, 6, 602–10, ISSN 0271-3586.

Beuge, J.A. & Aust SD. (1978). Microsomal lipid peroxidation. *Method. Enzymol*. 52, 302–10, ISSN 0076-6879.

Bolognesi, C. (2003). Genotoxicity of pesticides: a review of human biomonitoring studies, *Mutat. Res*., 543, 251–272, ISSN 0921-8262.

Bolognesi, C.; Landini, E.; Perrone, E. & Roggieri P. (2004). Cytogenetic biomonitoring of a floriculturist population in Italy: micronucleus analysis by fluorescence in situ hybridization (FISH) with an all-chromosome centromeric probe, *Mutat. Res.,* 557, 109–117, ISSN 0921-8262.

Bolognesi, C.; Perrone, E. & Landini, E. (2002). Micronucleus monitoring of a floriculturist population from western Liguria, Italy, Mutagenesis, 17, 391–397, ISSN 0267-8357.

Bonassi, S. & Au, W. (2002). Biomarkers in molecular epidemiology studies for health risk prediction. Review, *Mutat. Res.,* 511, 73–86, ISSN 0921-8262.

Bowden, R.D.; Buckwalter, M.R.; McBride, J.F.; Johnson, D.A.; Murray, B.K. & O´Neill, K.L. (2003). Tail profile: a more accurate system for analyzing DNA damage using the comet assay, *Mutat. Res*., 537, 1-9, ISSN 0921-8262.

Bulacio, L.G. & Panelo, M.S. (2000). Cinturón Hortícola de Rosario: Situación actual en el manejo de fitosanitarios, *Horticultura Argentina*, 19, 46, 5-14, ISSN 0327-3431.

Bull, S.; Fletcher, K.; Boobis, A.R. & Battershill, J.M. (2006). Evidence for genotoxicity of pesticides in pesticide applicators: a review, *Mutagenesis,* 21, 93-103, ISSN 0267-8357.

Carbonell, E.; Valbuena, A.; Xamena, N.; Creus, A. & Marcos, R. (1995). Temporary variations in chromosomal aberrations in a group of agricultural workers exposed to pesticides, *Mutat. Res*. 344, 127–134, ISSN 0921-8262.

Carpenter, D.O.; Arcaro, K. & Spink, D.C. (2002). Understanding the Human Health Effects of Chemical Mixtures, *Environ Health Perspect* 110, 1, 25–42 ISSN 0091-6765.

Castignani, M.I. ; Arregui, M.C.; Pelatti, N.S. (2004). Estimación de contaminación por plaguicidas con indicadores ambientales y económicos en huertas de tomate en Santa Fe, *Revista FAVE Ciencias Agrarias*, 3 ,1-2, ISSN 1669-1555.

Castillo-Cadena, J.; Tenorio-Vieyra, L.E.; Quintana-Carabia, A.I.; Garcia-Fabila, M.M.; Ramirez-San Juan, E. & Madrigal-Bujaidar, E. (2006). Determination of DNA Damage in Floriculturists Exposed to Mixtures of Pesticides, *Jour. Biomedicine and Biotechnology*, 1–12, ISSN 1110-7251.

Catalgol, B.K.; Ozden, S. & Alpertunga, B. (2007). Effects of trichlorfon on malondialdehyde and antioxidant system in human erythrocytes, *Toxicol. in Vitro*, 21, 1538–1544, ISSN 0887-2333.

Cicchetti, R. & Argentin, G. (2003). The role of oxidative stress in the in vitro induction of micronuclei by pesticides in mouse lung fibroblasts, *Mutagenesis,* 18, 127-32, ISSN 0267-8357.

Cocker, J.; Mason, H.J.; Garfitt, S.J. & Jones K. (2002). Biological monitoring of exposure to organophosphate pesticides, *Toxicology Letters*, 134, 97–103, ISSN 0378-4274

Collins, A.R. & Dusinska, M. (2009) Applications of the Comet Assay in Human Biomonitoring, In: *The Comet Assay in Toxicology,* Dhawan, A. & Anderson, D. (Ed.), 201-227, RSC Publishing, ISBN 978-0-85404-199-2, Cambridge, U.K.

Collins, A.R. (1999). Oxidative DNA damage, antioxidants, and cancer, *Bioassays*. 21, 238-46.

Collins, A.R.; Harrington, V.; Drew, J. & Melvin, R. (2003). Nutritional modulation of DNA repair in a human intervention study, *Carcinogenesis,* 24, 3, 511–515, ISSN 0143-3334.

Costa, C.; Teixeira, J.; Silva, S.; Roma-Torres, J.; Coelho, P.; Gaspar, J.; Alves, M.; Laffon, B.; Rueff, J. & Mayan, O. (2006). Cytogenetic and molecular biomonitoring of a Portuguese population exposed to pesticides, *Mutagenesis,* 21, 343-350, ISSN 0267-8357.

Curl, C.; Fenske, R.; Kissel, J.; Shirai, J.; Moate, T.; Griffith, W.; Coronado, G. & Thompson, B. (2002). Evaluation of Take-Home organophosphorus pesticide exposure among agricultural workers and their children, *Environ Health Perspect*, 110, 787-792, ISSN 0091-6765.

De Marco, A.; De Salvia, R.; Polani, S.; Ricordy, R.; Sorrenti, F.; Perticone, P.; Cozzi, R.; D'Ambrosio, C.; De Simone, C.; Guidotti, M.; Albanesi, T.; Duranti, G.; Festa, F.; Gensabella, G. & Owczarek, M. (2000). Evaluation of Genotoxic and Cytotoxic Properties of Pesticides Employed in Italian Agricultural Practices, *Environ. Res*. Section A., 83, 311-321, ISSN 0013-9351.

De Zwart, L.; Meerman, J.H.N.; Commandeur, J.N.M. & Vermeulen, N.P.E. (1999). Biomarkers of free radical damage applications in experimental animals and in humans, *Free Radical Biology & Medicine,* 26, 1, 2, 202–226, ISSN 0891-5849.

Dettbarn, W.D.; Milatovic, D. & Gupta, R. (2006). Oxidative Stress in Anticholinesterase-induced Excitotoxicity, In: Toxicology of Organophospahate and Carbamate Compounds, Gupta, R. (Ed), Elsevier: 511-532, ISBN 0-12-088523-9, Academic Press, Hopkinsville, KY, USA.

Dhawan, A.; Mathur, N. & Seth, P. K. (2001). The effect of smoking and eating habits on DNA damage in Indian population as measured in the Comet assay, *Mutat. Res.*, 474,1, 2, 121–8, ISSN 0921-8262.

Dhawan, A.; Anderson, D.; de Pascual-Teresa, S.; Santos-Buelga, C.; Clifford, M.N. & Ioannides, C. (2002). Evaluation of the antigenotoxic potential of monomeric and dimeric flavanols, and black tea polyphenols against heterocyclic amine-induced DNA damage in human lymphocytes using the Comet assay, *Mutat. Res*., 515, 1,2, 39–56, ISSN 0921-8262.

Drevenkar, V.; Radic, Z.; Vasilic, Z. & Reiner, E. (1991). Dialkylphosphate metabolites in the urine and activities of esterases in the serum as biochemical indices for human absorption of organophosphorus pesticides, *Arch. Environ. Contamin. Toxicol.,* 20, 417–422, ISSN 0090-4341.

Dulout, F.N.; Pastori, M.C.; Olivero, O.A.; Gonzalez Cid, M.; Loria, D.; Matos, E.; Sobel, N.; de Bujan, E.C. & Albiano, N. (1985). Sisterchromatid exchanges and chromosomal aberrations in a population exposed to pesticides, *Mutat. Res.,* 143, 237–244, ISSN 0921-8262.

Dusinska, M. & Collins, A. R. (2008). The Comet assay in human biomonitoring: gene–environment interactions, *Mutagenesis,* 23, 191–205, ISSN 0267-8357.

El-Demerdash, F.M. (2007). Lambda-cyhalothrin-induced changes in oxidative stress biomarkers in rabbit erythrocytes and alleviation effect of some antioxidants, *Toxicol. in Vitro*, 21, 392–397, ISSN 0887-2333.

Ellman, G.L.; Curtney, K.D.; Andrews, V. & Featherstone, R.M. (1961). A new and rapid colorimetric determination of acetyl cholinesterase activity, *Biochem. Pharmacol*., 7, 88–95, ISSN 0006-2952

Environmental Health Criteria 155. (1993) International programme on chemical safety. Biomarkers and Risk Assessment. Concepts and Principles. (http://www.inchem.org/pages/ehc.html)

Environmental Protection Agency (EPA). (2006). Integrated Risk Information (IRIS). Washington, DC (http://www.epa.gov/iris).

Gabbianelli, R.; Falcioni, G.; Nasuti, C. & Cantalamessa, F. (2002). Cypermethrin-induced plasma membrane perturbation on erythrocytes from rats: reduction of fluidity in the hydrophobic core and in glutathione peroxidase activity, *Toxicology,* 175, 91-101, ISSN 0300-483X

Garaj-Vrhovac V. & Zeljezic D. (2001). Cytogenetic monitoring of croatian population occupationally exposed to a complex mixture of pesticides, *Toxicology,* 165: 153–162, ISSN 0300-483X

Giunta, C.R. (2004) La Provincia de Santa Fe en el total agropecuario nacional. Una aplicación del Censo Nacional Agropecuario 2002. Dirección Gral. de Programación y Coordinación Económica Financiera, Ministerio de la Producción. (www.santafe.gov.ar/index.php/web/)

Giunta, C.R.; Zalesky, M. & Sona G. (2005) Cambios registrados en el sector agropecuario santafesino entre 1988 y 2002.Análisis de las variables más significativas. (www.santafe.gov.ar/index.php/web/)

Gomez-Arroyo, S.; Noriega-Aldana, N.; Osorio, A.; Galicia, F.; Ling, S. & Villalobos-Pietrini, R. (1992). Sister-chromatid exchange analysis in a rural population of Mexico exposed to pesticides, *Mutat. Res.,* 281,173–179, ISSN 0921-8262.

Groten, J.P. (2000). Mixtures and interactions. *Food Chem. Toxicol*. 38, 1, S65–S71, ISSN 0278-6915.

Grover, P; Danadevi, K.; Mahbood, M.; Rozati, R.; Saleha Banu, B. & Rahman, M.F. (2003). Evaluation of genetic damage in workers employed in pesticide production utilizing the Comet Assay, *Mutagenesis,* 18, 201-205, ISSN 0267-8357.

Gultekin, F.; Ozturk, M. & Akdogan, M. (2000). The effect of organophosphate insecticide chlorpyrifos-ethyl on lipid peroxidation and antioxidant enzymes (in vitro), *Arch. Toxicol*, 74, 9, 533-8, ISSN 0090-4341.

Hardt, J. & Angerer, J. (2000). Determination of dialkyl phosphates in human urine using gas chromatography-mass spectrometry, *J. Anal. Toxicol*., 24, 678–684, ISSN 0146-4760.

Heath, A. & Vale, J. (1992). Clinical presentation and diagnosis of acute orgnophosphate insecticide and carbamate poisoning. In: *Clinical and Experimental Toxicology of Organophosphates and Carbamates,* Ballantyne, B. & Marrs, T. (Ed.), 513–519, ISBN 0750602716, Butterworth, Oxford.

HSE (2000). Medical Aspects of work-related exposure to organophosphates Guidance note MS17: Health and Safety Executive, HSE Books, ISBN 0 7176 1977 X, Sudbury Suffolk.

Hughes, I. & Wood, H.F. (2002). Risk Assessment of Mixtures of Pesticides and Similar Substances. Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment. Hughes, I. & Wood, H.F. (Ed.), 1-298, Food Standards Agency.

IARC. International Agency for Research on Cancer, 2008. Agents reviewed by the IARC monographs. Vols 1–88, IARC, Lyon. (http://monographs.iarc.fr).

Islas-Gonzalez, K.; Gonzalez-Horta, C.; Sánchez-Ramirez, B.; Reyes-Aragon, E. & Levario-Carrillo M. (2005). In vitro assessment of the genotoxicity of ethyl paraoxon in newborns and adults, *Human Experim. Toxicology.* 24, 319-324, ISSN: 0960-3271.

Issaq, H.J.; Veenstra, T.D.; Conrads, T.P. & Felschow, D. (2002) The SELDI-TOF MS approach to proteomics: proteinprofiling and biomarker identification, *Biochem Biophys Res Commun*, 292, 587-592, ISSN 0006-291X.

Jaeschke, H. (1995) Mechanisms of oxidant stress-induced acute tissue injury, *Proc. Soc. Exp. Biol. Med.* 209,104–111, ISSN 0037-9727.

Jakubowski, M. & Trzcinka-Ochocka M. (2005). Biological Monitoring of exposure: Trends and key developments. *J. Occup. Health*. 47, 22-48, ISSN 1341-9145.

Kale, M.; Rathore, N.; John, S. & Bhatnagar, D. (1999). Lipid peroxidative damage on pyrethroid exposure and alterations in antioxidant status in rat erythrocytes: a possible involvement of reactive oxygen species, *Toxicol. Lett.*, 105, 197–205, ISSN 0378-4274.

Kassie, F.; Parzefall W. & Knasmuller, S. (2000). Single-cell gel electrophoresis assay: a new technique for human biomonitoring studies, *Mutat. Res*., 463, 1, 13–31, ISSN 0921-8262.

Klein, S.M.; Cohen, G. & Cederbaum, A. (1981). Production of formaldehyde during metabolism of dimethyl sulfoxide by hydroxyl radical generating system, *Biochemistry*, 20, 6006-6012, ISSN 0302-4369.

Knudsen, L.E. & Hansen, A.M. (2007) Biomarkers of intermediate endpoints in environmental and occupational health, Int. J. Hyg. Environ. Health, 210, 461–470, ISSN 1438-4639.

Lei, H.; Tjallingii, W. F. & Van Lenteren, J. C. (1998). Probing and feeding characteristics of the greenhouse whitefy in association with host-plant acceptance and whitefy strains, *Entomologia Experimentalis et Applicata*, 88, 73-80, ISSN 0013-8703.

Lenardon, A.; Maitre, M.I.; Lorenzatti, E. & Enrique, S. (2000). Plaguicidas organoclorados en leche materna en Santa Fe, *Acta Toxicologica Argentina,* 8, 1, 2-4, ISSN 1851-3743.

Lin, T.; Schwartz, A. & Saranga, Y. (1999). Non-stomatal factors limit cotton photo-synthesis under silverleaf whitefy stress, *Physiologia Plantarum*, 107, 303-31, ISSN 0031-9317.

Liu, Y.J.; Huang, P.L.; Chang, Y.F.; Chen, Y.H.; Chiou, Y.H.; Xu, Z.L. & Wong, R.H. (2006). GSTP1 Genetic Polymorphism Is Associated with a Higher Risk of DNA Damage in Pesticide-Exposed Fruit Growers, *Cancer Epidemiol Biomarkers Prev*., 15 ,4, 659–66. ISSN 1055-9965.

Lopez, O.; Hernandez, A.F.; Rodrigo, L.; Gil, F.; Pena, G.; Serrano, J. L.; Parron, T.; Villanueva, E. & Pla, A. (2007). Changes in antioxidant enzymes in humans with long-term exposure to pesticides, *Toxicol. Lett.,* 171, 146-153, ISSN 0378-4274.

Mason, H.J. & Lewis, P. (1989). Intra-individual variation in plasma and erythrocyte cholinesterase activities and the monitoring of uptake of organophosphorus pesticides, *J.Soc. Occup. Med.,* 39, 121–124, ISSN 0301-0023.

McCauley, L.A.; Anger, W.K.; Keifer, M.; Langley, R.; Robson, M.G. & Rohlman, D. (2006). Studying Health Outcomes in Farmworker Populations Exposed to Pesticides, *Environ. Health Perspect*., 114, 953–960, ISSN 0091-6765.

Mercille, S. & Massie, B. (1994). Induction of apoptosis in nutrient-deprived cultures of hybridoma and myeloma cells, *Biotechnology and Bioengineering,* 44, 1140-1154, ISSN 0006-3592.

Miranda, M. M. M.; Picanco, M.; Zanuncio, J. C. & Guedes R. N. C. (1998). Ecological life table of Tuta absoluta (Meyrick) (Lepidoptera: Gelechiidae), *Bio-control Sci,. & Technol*., 8, 597-606, ISSN 0958-3157.

Moretto, A. & Lotti, M. (1998) Poisoning by organophosphorus insecticides and sensory neuropathy, *J Neurol Neurosurg Psychiatry,* 64, 463-468, ISSN 00223050.

Muniz, J.F.; McCauley, L; Scherer, J.; Lasarev, M.; Koshy, M.; Kow, Y.W.; Nazar-Stewart, V. & Kisby, G.E. (2008). Biomarkers of oxidative stress and DNA damage in agricultural workers: A pilot study, Toxicology and Applied Pharmacology, 227, 97–107, ISSN 0041-008X.

Nasuti, C.; Cantalamessa, F.; Falcioni, G. & Gabbianelli, R. (2003). Different effects of Type I and Type II pyrethroids on erythrocyte plasma membrane properties and enzymatic activity in rats, *Toxicology,* 191, 233-244, ISSN 0300-483X.

Nutley, B. & Cocker, J. (1993). Biological monitoring of workers occupationally exposed to organophosphorus pesticides, *Pesticide Sci,*. 38, 315–322, ISSN 1096-9063.

Ostling, O. & Johanson, K.J. (1984). Microelectrophoretic study of radiation induced DNA damages in individual mammalian cells, *Biochem. Biophys. Res. Commun.,* 123, 1, 291–8, ISSN 0006-291X.

Palus, J.; Dziubaltowska, E. & Rydzynski, K. (1999). DNA damage detected by the comet assay in the white blood cells of workers furniture plant, *Mutat. Res*., 444, 61-74, ISSN 0921-8262.

Palus, J.; Rydzynski, K.; Dziubaltowska, E.; Wyszynska, K.; Natarajan, A.T. & Nilsson, R. (2003). Genotoxic effects of occupational exposure to lead and cadmium, *Mutat. Res*., 540, 1, 19–28, ISSN 0921-8262.

Pastor, S.; Creus, A.; Xamena, N.; Siffel, C. & Marcos R. (2002). Occupational exposure to pesticides and cytogenetic damage: results of a Hungarian population study using the micronucleus assay in lymphocytes and buccal cells, *Environ. Mol. Mutagen*., 40, 101–109, ISSN 0893-6692.

Pastor, S.; Gutierrez, S.; Creus, A.; Xamena, N.; Piperakis, S. & Marcos R. (2001). Cytogenetic analysis of Greek farmers using the micronucleus assay in peripheral lymphocytes and buccal cells, *Mutagenesis*, 16, 539–545, ISSN 0267-8357.

Paunero, I.E.; Mitidieri, M.; Ferratto, J.; Giuliani, S.; Bulacio, L.; Panelo, M.; Amoia, P.; Strassera, M.E.; Granitto, G.; del Pino, M., Martínez, S., Fortunato, N.; Tangorra, M.; Andreau, R.; Garbi, M. & Martínez-Quintana, O. (2009). Identifying the primary types of accidents that occur to horticultural workers in Argentina, *Agricultura, Sociedad y Desarrollo*, 6, 2, 177-182, ISSN 1870-5472.

Peluso, M.; Merlo, F.; Munnia, A.; Bolognesi, C.; Puntoni, R. & Parodi, S. (1996). (32)P-postlabeling detection of DNA adducts in peripheral white blood cells of greenhouse floriculturists from western Liguria, Italy, *Cancer Epidemiol. Biomarkers Prev*., 5, 361–369, ISSN 1055-9965.

Petricoin, E.F.; Ardekani, A.M.; Hitt, B.A.; Levine, P.J.; Fusaro, V.A.; Steinberg, S.M.; Mills, G.B.; Simone, C.; Fishman, D.; Kohn, E.C. & Liotta, L.A. (2002) Use of proteomic patterns in serum to identify ovarian cancer, *Lancet*, 35, 9, 572-577, ISSN 0140-6736.

Piperakis, S. M.; Visvardis, E. E.; Sagnou, M. & Tassiou, A. M. (1998). Effects of smoking and aging on oxidative DNA damage of human lymphocytes, *Carcinogenesis,* 19, 4, 695–698, ISSN 0143-3334.

Piperakis, S.M.; Kontogianni, K.; Piperakis, M.M. & Tsilimigaki, S. (2006). Effects of Pesticides on Occupationally Exposed Humans, *The Scientific World Journal*, 6, 1211-1220, ISSN 1537-744X.

Piperakis, S.M.; Petrakou, E.; Tsilimigaki, S.; Sagnou, M.; Monogiudis, E.; Haniotakis, G.; Karkaseli, H. & Sarikaki, E. (2003). Biomonitoring with the comet assay of Greek greenhouse workers exposed to pesticides, *Environ. Mol. Mutagen*., 41, 104–110, ISSN 0893-6692.

Pitozzi, V.; Giovannelli, L.; Bardini, G.;. Rotella, C. M & Dolara, P. (2003). Oxidative DNA damage in peripheral blood cells in type 2 diabetes 42 Chapter 1mellitus: higher vulnerability of polymorphonuclear leukocytes, *Mutat. Res*, 529(1–2), 129–33, ISSN 0921-8262.

Prakasam, A.S.; Sethupathy, S. & Lalitha, S. (2001). Plasma and RBCs antioxidant status in occupational male pesticide sprayers, *Clin. Chim. Acta*, 310, 107–112, ISSN 0009-8981.

Prasanthi, K.; Muralidhara & Rajini, P.S. (2005). Morphological and biochemical perturbations in rat erythrocytes following in vitro exposure to Fenvalerate and its metabolite, *Toxicol. in Vitro,* 19, 449–456, ISSN 0887-2333.

Raipulis, J.; Maija, M. & Balode M. (2009). Toxicity and Genotoxicity Testing of Roundup. *Proceedings of the latvian academy of sciences Section B*, 63, 29–32, ISSN 1407-009X.

Ranjbar, A.; Pasalar, P. & Abdollahi, M. (2002). Induction of oxidative stress and acetylcholinesterase inhibition in organophosphorous pesticide manufacturing workers. *Hum. Exp. Toxicol*. 21, 179-82, ISSN 0960-3271.

Raslova, K.; Nagyova, A.; Dobiasova, M.; Ptackova, K. & Dusinska, M. (2000). Effect of ciprofibrate on lipoprotien metabolism and oxidative stress parameters in patients with type 2 diabetes mellitus and atherogenic lipoprotein phenotype. *Acta. Diabetol*. 37, 3, 131–134, ISSN 0940-5429.

Reid, S.J. & Watts, R.R. (1981). A method for the determinationof dialkyl phosphate residues in urine. *J. Anal. Chem.* 5,126–132, ISSN 0937-0633.

Remor, A.P.; Totti, C.C.; Moreira, D.A.; Dutra, G.P.; Heuser, V.D. & Boeira, J.M. (2008). Occupational exposure of farm workers to pesticides: Biochemical parameters and evaluation. *Environ. Int.* 35, 2, February 2009, 273-278, ISSN 0160-4120.

Ringuelet, R. & Laguens, J. (2000). Notas sobre el uso de agroquímicos. Espacio tecnológico, población y reproducción social en el sector hortícola de La Plata. Universidad Nacional de La Plata. Biblioteca del Congreso de la Nación, (http://consulta.bcn.gov.ar/bcn/Catalogo)

Rodriguez, A.R. & Lenardon, A.L. (2007). Provincia Santa Fe Sur, In: *La problemática de los agroquímicos y sus envases, su incidencia en la salud de los trabajadores, la población expuesta y el ambiente. Estudio colaborativo multicéntrico.* Ministerio de Salud. Secretaría de Ambiente y Desarrollo Sustentable, Secretaría de Ambiente y Desarrollo Sustentable: OPS: AAMMA, 247-268, ISBN 978-987-96256-7-5, Buenos Aires.

Ruf, A.A.; Jerwood, D.; Webb, J. & Anderson, D. (2003). Sensitivity of different thalassaemia genotypes to food mutagens in the Comet assay. *Teratog. Carcinog. Mutagen*. Suppl 2, 83–91, ISSN 0270-3211.

Sasaki, Y.F.; Sekihashi, K.; Izumiyama, F.; Nishidate, E.; Saga, A.; Ishida, K. & Tsuda, S. (2000) The Comet assay with multiple mouse organs: comparison of Comet assay results and carcinogenicity with 208 chemicals selected from the IARC monographs and US NTP Carcinogenicity Database, *Crit. Rev. Toxicol.*, 30, 629, ISSN 1040-8444.

Seth, V.; Banerjee, B.D. & Chakravorty, A.K. (2001). Lipid peroxidation, free radical scavenging enzymes, and glutathione redox system in blood of rats exposed to Propoxur, *Pest. Biochem. Phys*, 71, 133-139, ISSN 0048-3575.

Shadnia, S.; Azizi, E.; Hosseinil, R.; Khoei, S.; Fouladdel, S.; Pajoumand, A.; Jalali, N. & Abdollahi, M. (2005) Evaluation of oxidative stress and genotoxicity in organophosphorus insecticide formulators. *Hum.Exp. Toxicol*. 24, 439- 445, ISSN 0960-3271.

Simoniello, M.F.; Kleinsorge, E.C.; Scagnetti, J.A.; Grigolato, R.A.; Poletta, G.L. & Carballo, M.A. (2008) DNA damage in workers occupationally exposed to pesticide mixtures. *J. Appl. Toxicol*. 28, 8, 957-965, ISSN 0260-437X.

Simoniello, M.F.; Kleinsorge, E.C.; Scagnetti, J.A.; Mastandrea, C.; Grigolato, R.A.; Paonessa, A.M. & Carballo, M.A. (2010). Biomarkers of cellular reaction to pesticide exposure in a rural population, *Biomarkers*, 15, 1, 52-60, ISSN 1354-750X.

Singh, N.P.; McCoy, M.T.; Tice, R.R. & Schneider, E.L. (1988). A simple technique for quantitation of low levels of DNA damage in individuals cells, *Exp. Cell. Res,* 175, 1, 184-191, ISSN 0014-4827.

Singh, N.P.; Muller, C.H. & Berger, R.E. (2003) Effects of age on DNA double-strand breaks and apoptosis in human sperm, *Ferti. Steril*., 0, 6, 1420–30, ISSN 0015-0282.

Singh, V.K.; Jyoti; Krishna Reddy, M.M.; Kesavachandran, C.; Rastogi, S.K. & Siddiqui, M.K.J. (2007). Biomonitoring of organochlorines, glutathione, lipid peroxidation and cholinesterase activity among pesticide sprayers in mango orchards, *Clin. Chim. Acta*, 377, 268–272, ISSN 0009-8981.

Souza Casadinho, O. & Bocero, S. (2008). Agrotóxicos: Condiciones de utilización en la horticultura de la Provincia de Buenos Aires (Argentina), *Revista Iberoamericana de Economía Ecológica*, 9, 87-101, ISSN 1390-2776.

Trossero, S.M; Trossero, N.; Scagnetti, J.; Portillo, P. & Kleinsorge E. (2009). Plaguicidas en leche materna y su potencial efecto disruptor endocrino en gestantes del cordón hortícola de Santa Fe (Argentina) (Informe Preliminar), *Revista FABICIB*, 13, 97-103, ISSN 0329-5559.

Vaghef, H.; Nygren, P.; Edling, C.; Bergh, J. & Hellman, B. (1997). Alkaline single-cell gel electrophoresis and human biomonitoring for genotoxicity: a pilot study on breast cancer patients undergoing chemotherapy including cyclophosphamide, *Mutat. Res*, 395, 2–3, 127–138, ISSN 0921-8262.

Valko, M.; Leibfritz, D.; Moncola, J.; Cronin, M.T.D.; Mazura, M. & Telser, J. (2007). Review Free radicals and antioxidants in normal physiological functions and human disease, *Intern. J. Biochem. Cell Biology,* 39, 44–84. ISSN 1357-2725.

Valverde, M.; Lopez, M.; Lopez, I.; Sanchez, I.; Fortoul, T.; Ostrosky-Wegman P. & Rojas, E. (1997). DNA damage in leukocytes and buccal and nasal epithelial cells of individuals exposed to air pollution in Mexico City, *Environ. Mol. Mutagen*., 30, 147–152, ISSN 0893-6692.

WHO, World Health Organization, 2009. The WHO Recommended classification of pesticides by hazard and guidelines to classification. World Health Organization, Geneva. ISSN: 1684-1042.
http//www.who.int/ipcs/publications/pesticide_hazard_2009

Zeljezic, D. & Garaj-Vrhovac, V. (2001). Chromosomal aberration and single cell gel electrophoresis (comet) assay in the longitudinal risk assessment of occupational exposure to pesticides, *Mutagenesis*, 16, 4, 359–363, ISSN 0267-835.

18

In-Vivo and In-Vitro Methods for Evaluation of Pesticides on DNA Structure

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1. Introduction

It is well known that the growth of the population and convenience of human's life depends on industry and agriculture. In this context, the pesticides have contributed greatly to the increase of yields in agriculture by controlling pests and diseases. The pesticides currently in use include a wide variety of compounds belonging to different chemical classes. More than 800 chemicals marketed as multiple formulations are used in the European Union as insecticides, herbicides, fungicides. There is a large range of positive outcomes or benefits from the pesticides. Reduced crop loss resulting from spraying fungicides is an obvious benefit. The benefits of pesticides can be classified in three stages such as: immediate effects, primary, and secondary benefits. The outcomes of pesticides use is immediate, and the main effects of pesticides can be divided as follows: (1) controlling agricultural pests (including diseases and weeds) and vectors of plant disease; (2) controlling human and livestock disease vectors and nuisance organisms; (3) preventing or controlling organisms that harm other human activities and structures.

The secondary benefits are the consequences of the pesticides, effects. From the three main effects listed above, 26 primary benefits have been identified ranging from protection of recreational turf to saved human lives. The secondary benefits are less immediate, less intuitively obvious or longer term consequences. It follows that for secondary benefits, it is more difficult to establish causes and effects nevertheless, and they can be powerful justifications for pesticide use. These classifications are summarized in Fig. 1.

However, the effectiveness of pest management is the wide application of pesticides throughout the world. Unfortunately, the result of these widespread uses is the contamination of soils, surface and specially ground waters. In fact, due to the macropore structure of soils and rapid and not uniform leaching via preferential flow paths, a fraction of the pesticides percolates into ground water before it can degrade or be adsorbed by the soil. Although this leaching through the vadose zone to ground water is a complex process and controlled by a variety of processes such as soil water flow, solute transport, heat transport, pesticide sorption, transformation and degradation, volatilization, crop uptake, and surface run off (Fig. 2), the result is the wide contamination of drinking waters and food chain (Abhilash, & Singh,2009).

The Food and Agricultural Organization (FAO, 1988) has been concerned about various reports of ill health arises of pesticides. The World Health Organization had estimated that

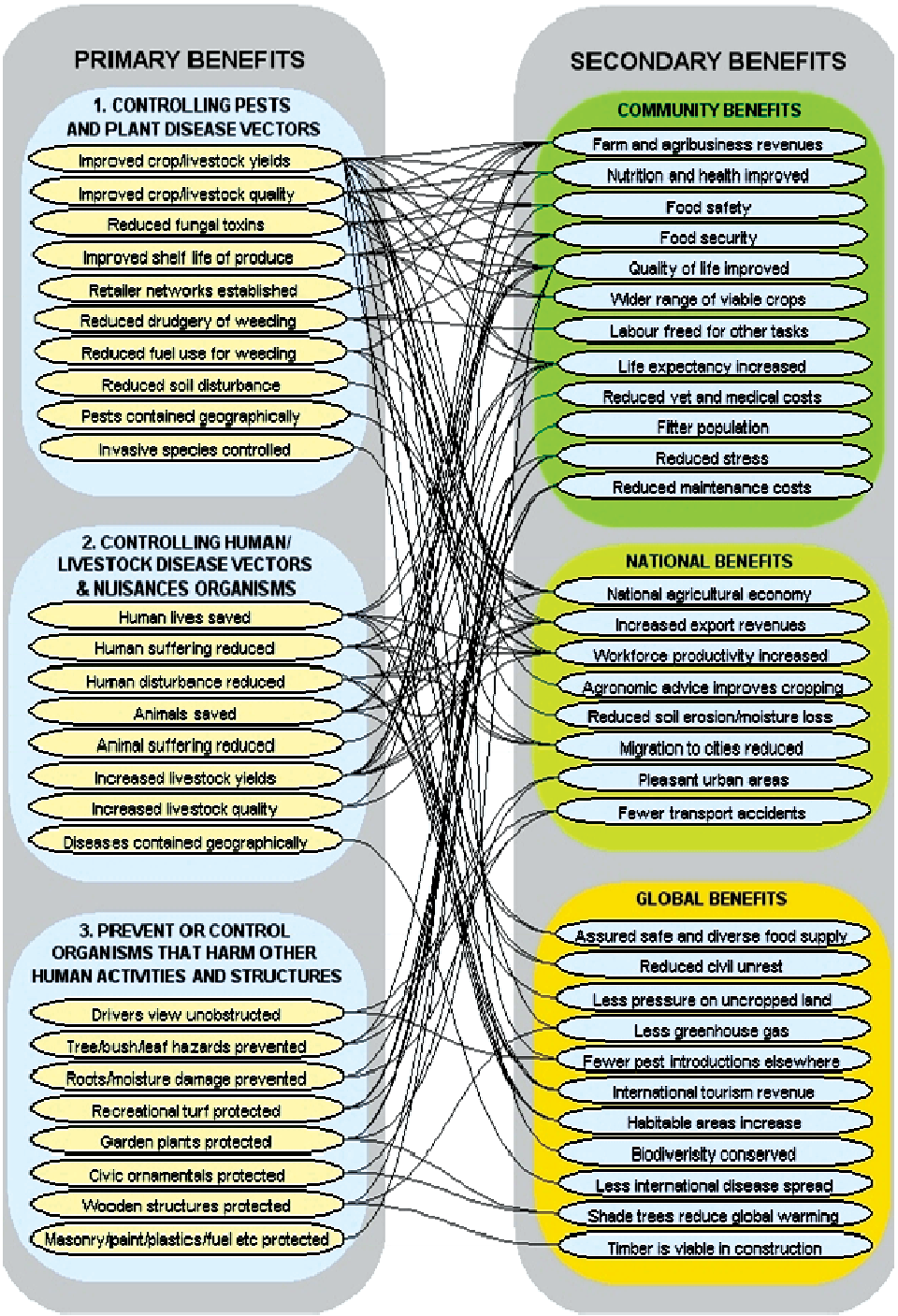


Fig. 1. The benefits of pesticides that classified in three stages such as: immediate effects, primary, and secondary. The linkages are not easy to follow, but serve to illustrate the complexity of the interactions between them. With permission (Cooper,. & Dobson, 2007).

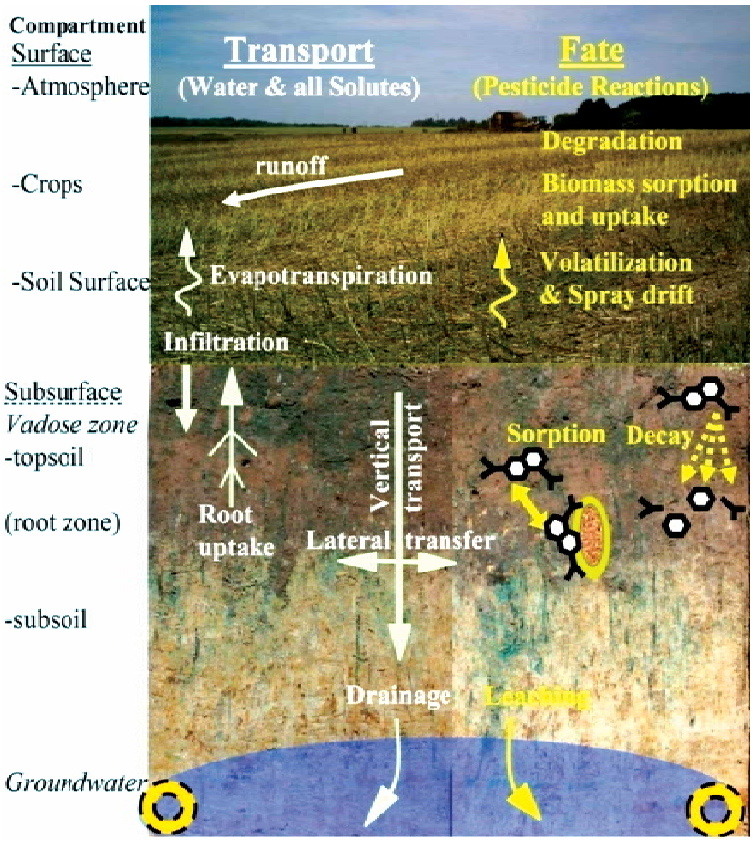


Fig. 2. Principal processes governing pesticide transport and fate in agricultural structured soil systems. With permission (Köhne, Köhne, & Simůnek, 2009).

several millions people were being poisoned via pesticides (WHO, 1986). Pesticides have been considered potential chemical mutagens. Experimental data revealed that various agrochemical ingredients possess mutagenic properties inducing gene mutation and chromosomal alteration or DNA damage (Bolognesi, & Morasso, 2000). Increasing incidence of cancer, chronic kidney diseases, suppression of the immune system, sterility among males and females, endocrine disorders, neurological and behavioral disorders, especially among children, have been attributed to chronic pesticide poisoning. Human health hazards vary with the extent of exposure. Moderate human health hazards from the exposure to pesticides include mild headaches, flu, skin rashes, blurred vision and other neurological disorders while rare, but severe human health hazards include paralysis, blindness and even death. Pesticide pollution to the local environment also affects the lives of birds, wildlife, domestic animals, fish and livestock. Although it is documented that, the pesticides are toxic for living organisms with variable specificity for targeted species, the chronic toxicity in humans such as neurotoxicity, endocrine disruption, immunotoxicity or carcinogenicity, are still to be explored (Hodgson, & Levi, 1996; Carpy, Kobel, & Doe, 2001). The risk for cancer is frequently discussed since some of these compounds have demonstrated carcinogenic potential in-vivo, more particularly in rodents (Cueto Jr., 1980). In some epidemiological studies conducted on farmers, it is thought that occupational

exposure to pesticides contributes to an increased incidence of various cancers at specific sites. These concern especially tumors of the lip, skin, prostate or brain, Hodgkin disease and non-Hodgkin lymphoma (NHL) (Blair, & Zahm, 1991; Georgellis, and et al., 1999). Several chemical classes of pesticides, like organochlorine insecticides (Leary and et al., 2004) or phenoxyacetic acid herbicides have been more specifically incriminated (Zahm, & Blair, 1992). Cancer is the third leading cause of death in adults and is the second cause in the children, with heart failure being the most frequent one. Leukemia is the group of childhood malignancies with by far the highest incidence, representing close to a third of all cases; childhood brain tumors (CBT) and lymphomas are the second and third most frequent groups (Rull, 2009). Knowledge about the causes of childhood cancer is scant, with ionizing radiation and some therapeutic drugs being the only well-established causal factors. Several other factors, however, have been suggested, comprising natural factors such as infections, and man-made factors such as electromagnetic fields, traffic exhaust, and pesticides. The existing epidemiological studies on associations between childhood cancer and either parental or child exposure to pesticides have been recently reviewed (Daniels,

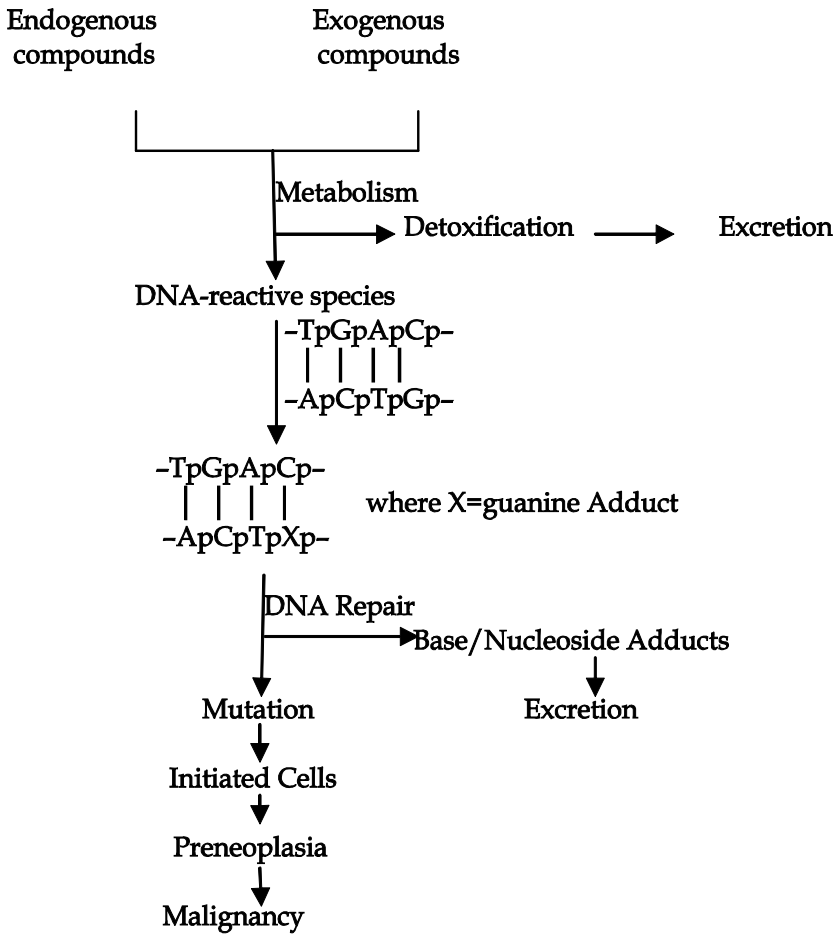


Fig. 3. The role of DNA adducts in mutation and cancer.

1997; Zahm, & Ward, 1998; Nasterlack, 2006; Nasterlack, 2007). Nonetheless, there are a variety of mechanisms by which pesticides may potentially initiate or promote cancer, including mutagenicity, tumor promotion immunotoxicity and hormonal disruption. It is completely clear that when DNA interacts with carcinogenic and/or mutagenic compounds it can play a key role in the advent of cancers, unusual proliferation and metastasis of malignant cells. For example aromatic amines, nitro aromatic amines, nitrosamines, hydrazine, aflatoxins, pesticides, and halogenated hydrocarbons can be activated by P-450 to form compounds capable of covalent binding to DNA. Alkylation of DNA is believed to be the first step in the initiation of chemically induced carcinogenesis and cancer (Prakash and et al., 1998). DNA adducts, if not repaired before the onset of DNA replication or misreported, are capable of inducing gene mutations and putatively initiate the conversion of exposed natural cells to irreversibly altered preneoplastic states. Figure 3 depicts sequence of the main events that are considered crucial in chemically induced carcinogenesis.
However, to discover the mutagenic and carcinogenic effects of pesticides, the development of accurate, reliable and rapid methods is of interest for evaluating effects of these compounds and analyzing their mechanisms of action especially on DNA.

2. DNA conformations and modes of interaction

DNA is found in cells, and usually is as a right-handed double helix. The two chains (strands) of the double helix have complementary sequences of nucleic bases. The structure and numbering of nucleic bases are shown in Fig. 4.

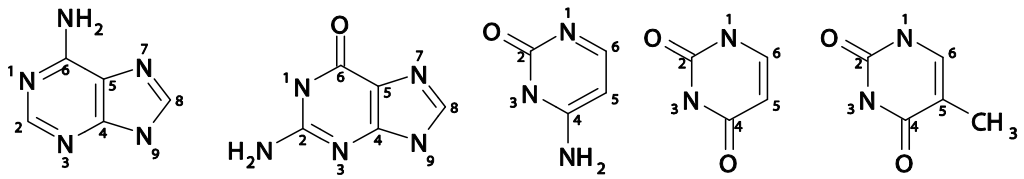


Fig. 4. Structure and numbering of the bases adenine, guanine (purines) and cytosine, uracil, thymine (pyrimidines). Hydrogen's are not shown.

The double helix of DNA is of great length and short diameter. The minor groove is narrow and shallow, only about 10 Å in width. The major groove is deeper and wider, approximately 24 Å in width. The grooves form because the patterns of hydrogen bonding between complementary bases of DNA cause sugar groups to stick out at 120˚ angles from each other instead of 180˚. It has been known from early X-ray diffraction studies on fibers that DNA can exist in more than one conformation depending on the fiber salt concentration, the degree of hydration, the metal ion, etc. It was found from X-ray or spectroscopic data that in A-DNA and also RNA the sugar pucker exists in C3'-*endo* conformation, while in B-DNA the conformation is converted to C2'-*endo* (Fig. 5).
It is known that in solutions there is an equilibrium between the C2'-*endo* and C3'-*endo* pucker in the deoxyribose. Since nucleic acids are polyanions, they require counter ions in order to neutralize the negatively charged phosphate groups. DNA can be interacting with other compounds through a variety of modes and each may be exploited to stabilize the bound ligand at a DNA site. For example the metal complexes are known to bind to DNA

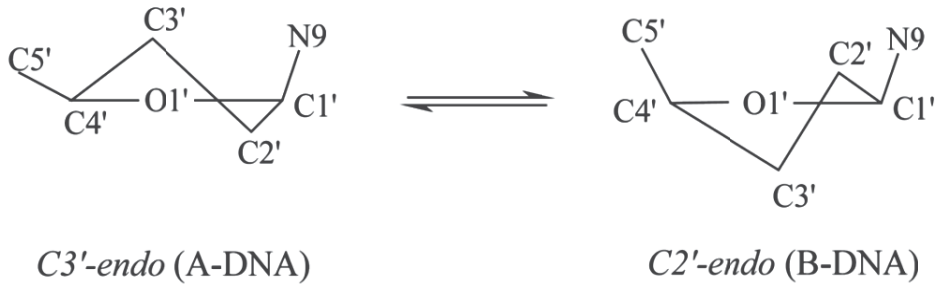


Fig. 5. The numbering of the pentose ring and C2'-*endo*, C3'-*endo* sugar puckering in DNA.

through a series of interactions, such as Z-stacking interaction associated with intercalation of aromatic heterocyclic groups between the base pairs. Intercalators are small molecules that contain a planar aromatic heterocyclic functionality which can insert and stack between the base pairs of double helical DNA, while the groove binders bind DNA within either grooves of the double helix. In general, the binding of an intercalator to DNA is driven entirely by a large favorable enthalpy reduction but with an unfavorable entropy decrease, and the binding of a groove binder to DNA is driven by a large favorable increase in entropy. Once a pesticide binds DNA within either grooves of the double helix, a variety of non-covalent molecular interactions, such as hydrophobic interactions between the bound pesticide and either groove floor/walls, specific hydrogen bonds and van der Waals contacts between the pesticide and DNA, may occur, and the apparent enthalpy increase should be the summation of all of these molecular interactions. In principle, there are six modes for reversible binding of molecules with double-helical DNA: (i) electrostatic attractions with the anionic sugar-phosphate backbone of DNA, (ii) interactions with the DNA major groove, (iii) interactions with the DNA minor groove, (iv) intercalation between base pairs via the DNA major groove, (v) intercalation between base pairs via the DNA minor groove, and (vi) a threading intercalation mode. Depending on structural features of both the molecule and DNA, many molecules show more than a single interaction mode with DNA. Notwithstanding, the strong interactions have binding modes which fall into two categories: intercalation and specific hydrogen-bonding interactions in the DNA grooves. The development of high throughput screening techniques for study of molecular interactions and biological activities of pesticides with DNA has been an increasingly popular area of research. Two most approaches to study pesticides/DNA interactions are widely used *in-vivo* and *in-vitro* strategies. The following sections we will review and introduce the basic procedures that are especially used for in-vivo study of DNA interactions such as: ^{32}P postlabeling analysis of DNA-adducts, alkaline single cell gel electrophoresis (Comet assay) and alkaline elution (or unwinding) techniques, and techniques that are used for in-vitro study of DNA interactions such as: spectroscopy of Uv/Vis, quenching fluorescence, competitive fluorescence and circular dichroism(CD); electrochemistry containing the linear sweep voltammetry (LSV), cyclic voltammetry (CV) and differential pulse voltammetry (DPV).

3. In-vivo methods for studying DNA-pesticides interactions

Some pesticides are genotoxic/carcinogenic or are converted into genotoxicin during metabolism. Thus DNA damage has the potential as a biomarker and can act as endogenous

or exogenous compounds. The actual damage and alterations to DNA when interact with pesticides occur in two basic forms, the detection of which employs specific techniques:

1. Pesticides bind covalently to DNA to form "DNA adducts". The DNA adducts are usually detected by the non-specific ^{32}P postlabeling techniques.
2. Strand breaks can be measured by two methods, the alkaline single cell gel electrophoresis (Comet assay) and alkaline elution (or unwinding).

3.1 ^{32}P postlabeling analysis of DNA-adducts

The sites susceptible to adduct formation include nucleic acid bases, as well as the sugar-phosphate backbone. For example, there are 17 potential sites for reaction in DNA treated with N-nitroso alkylating agents, and of these sites the phosphate and ribose moieties each has one nucleophilic site. The exocyclic oxygen and amino groups of nuclei acid bases, N1, N3, and N7 positions of purines, and N3 position of pyrimidines are frequently involved in adduct formation. The N3 and N7 positions of guanine are most susceptible to electrophilic attack, compared with the exocyclic oxygen. The DNA adducts, if not repaired, have the

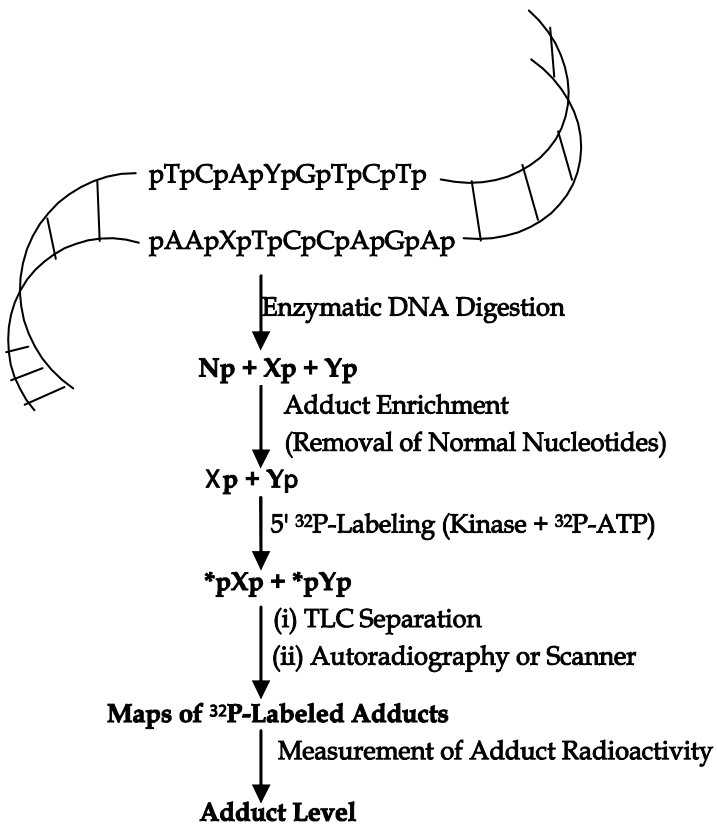


Fig. 6. The ^{32}P-postlabeling assay for DNA adducts detection. The assay involves six steps: digestion of DNA to 3'-mononucleotides of normal (Np) and adducted (Xp, Yp) deoxyribonucleotides; enrichment of adducts; ^{32}P-labeling of adducts; separation, detection, and quantization of adducts. Asterisks indicate the position of the ^{32}P label (Phillips, & Arlt, 2007).

potential to interfere with normal DNA replication, which leads to base substitution/frame shift mutations and/or cell killing. DNA-adducts in the cells are effective molecular dosimeters of genotoxic contaminant exposure, and the ^{32}P-postlabeling assay has been used to measure covalent DNA-pesticides adducts. Figure 6 gives an outline of various steps involved in the analysis of DNA adducts by the ^{32}P-postlabeling assay.
In the ^{32}P-postlabeling method, DNA is enzynmatically digested to 3´-monophosphates of normal deoxynucleosides (Np) and adducts (Xp, Yp). Adducted nucleosides are enriched by extracting them with butanol or converting Np to N by 3´-dephosphorylation with nuclease P1 treatment. These two adduct enrichment procedures are applicable to the enrichment of bulky or aromatic DNA adducts, but not to the most simple alkylated adducts like methyl- and ethyl-substituted products. Simple alkylation of purines at the N7 position creates a positive charge and such adducts can be enriched as a group or class by an anion-exchange column chromatography. The adducted nucleotides are subsequently labeled enzymatically with polynucleotide kinase and [γ-^{32}P]ATP at the 5´-end (Fig. 7), separated by thin-layer chromatography (TLC), detected by exposure to X-ray films or scanning by electronic autoradiography, and quantified by measuring the ^{32}P incorporation.

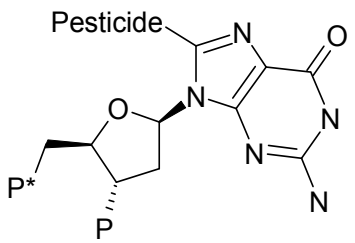


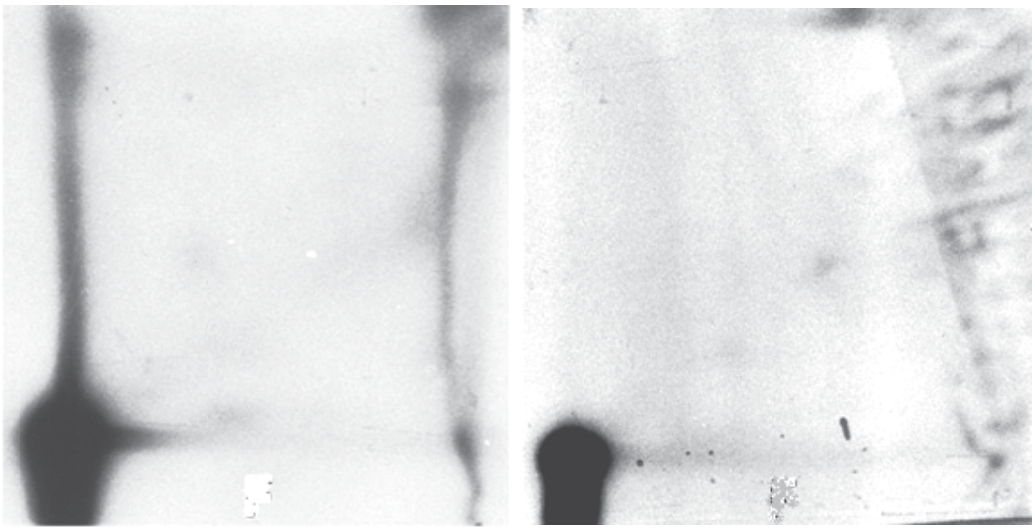
Fig. 7. Structures of the ^{32}P-labeled DNA adducts formed by interaction with pesticide.

As it was said, two procedures are used for adduct enrichment: butanol extraction and nuclease P1. The butanol method allows the extraction of aromatic adducts into 1-butanol in the presence of a phase-transfer agent (tetrabutylammonium chloride). The nuclease P1 method is based on the finding that certain adducted nucleotides are resistant to 3'-dephosphorylation and remain as substrates for kinase, while normal nucleotides are hydrolyzed to nucleosides which are not substrates for kinase due to the lack of a phosphate at the 3'-end. Both methods have been shown to be suitable for the enrichment of most aromatic adducts derived from chemicals containing one or more aromatic rings, such as polycyclic aromatic hydrocarbons, aromatic amines, and pesticides. Some aromatic amine adducts, however, are sensitive to nuclease P1 activity, resulting in their poor recovery and weak detection. On the other hand, some adducts are not extractable by butanol, but they are resistant to nuclease P1-catalyzed 3'-dephosphorylation. Because of the difference in adduct recovery by the two methods, it is recommended that both methods be used for the enrichment of unknown adducts. In addition to these two procedures, an anion-exchange chromatography method should be considered for the purification of adducts containing a positive charge, such as N7-alkylpurines, N1 adenine, and N3 cytosine. The purification on an anion-exchange column is based on the positive charge on the adducts, because these are less retained and readily eluted in a low salt buffer, while unmodified nucleotides are strongly retained on the column under these conditions. For separation of ^{32}P adducts the thin layer chromatography (PEI-(polyethyleneimine) cellulose) is a suitable method. Based on the polarity of adducts, different TLC techniques such as two dimentional and

multidirectional TLC procedure (MD-TLC) are used. The ^{32}P postlabeling method has an advantage over the others in that prior structural knowledge is not required for adduct detection. Furthermore, this technique could be used for detecting adducts formed by chemicals of diverse structures, such as alkylating agents, polycyclic aromatic hydrocarbons, aromatic amines, and pesticides. The ^{32}P-postlabeling assay is very sensitive and can detect a single adduct in 10^9–10^{10} nucleotides from 1 to 10 μg of DNA. Potential pitfall of the postlabeling technique includes the lack of structural identification of the adduct. The assay may give false-negative results due to either adduct loss or lower sensitivity for some adducts. The loss of adducts could occur because of the instability of adducts or the inefficiency of certain steps in the assay. In addition, some small adducts are difficult to analytically resolve from unmodified nucleotides, making the assay less sensitive for the detection of these adducts than bulky aromatic adducts. A false-positive response could be due to the phosphorylation of nonnucleic acid components, such as the metabolites containing hydroxyl groups, if they are present as contaminants in DNA preparations at high levels. It is, therefore, important to purify the DNA well, particularly that from *in vitro* exposures, where the test article is generally investigated at higher concentrations than in *in vivo* studies. It appears that a standard DNA isolation procedure using solvent extraction methods is adequate to purify DNA from contaminants. Another concern about the ^{32}P-postlabeling assay is the detection of I-compounds or endogenous adducts on the TLC plates. These adducts, if present, can contribute to background spots and interfere with the detection of exposure-related adducts. Since I-compounds increase with aging, it is recommended that younger animals be used to reduce background activity to the extent it is permissible in the experimental design. Also, different TLC solvents or different versions of the ^{32}P-postlabeling assay may be explored to resolve adducts of interest from I-compounds.

3.1.1 Sample preparation

For in-vivo study of Pesticides-DNA adduct formation by ^{32}P-postlabeling assay, the DNA is commonly isolate from the rat tissues and liver rats, calf thymus, and the tissues of fish or plants. In general, the tissues are homogenized by a solvent and the cells are lyses. After that, the nuclei are isolated by centrifugation. The DNA is extracted with a chloroform/isoamyl alcohol mixture, and then treated with RNAses A and T1 before final extraction by chloroform/isoamyl alcohol mixture. The isolated DNA is digested to mononucleotides with micrococcal nuclease and spleen phosphodiesterase. The adducted nucleotides of digested sample are concentrated by either 1-butanol extraction or nuclease P1. Adducted nucleotides are preferentially extracted over the normal bases into the organic phase during the butanol extraction, while nuclease P1 is able to cleave the 3'phosphate group from the normal bases due to the resistant bulk of adducted bases. The pervious studies conducted on Chlordane (CLD) as an organochlorin pesticide demonstrated that the CLD increases liver tumor incidences in rodents and is a tumor promoter. However, Whysner and coworker by using the ^{32}P postlabeling assay on male and female $B6C3F_1$ mice, concluded that, the mode of action for tumorigenesis by CLD is not based on adduct formation (Whysner and et al., 1998). Results showing typical TLC separations for liver DNA of mice exposed to CLD for 2 weeks are illustrated in Fig. 8a and 8b. As it is observed no spots were identified by either the 1-butanol extraction or nuclease P1 enrichment methods. Furthermore, the pathology findings demonstrated no association between liver cancer and CLD exposure (International Agency for Research on Cancer, 1991).



(a) (b)

Fig. 8. ^{32}P-Postlabeling results using **(a)** 1-butanol extraction **(b)** nuclease P1 showing typical TLC plates of B6C3F$_1$ mouse liver DNA for CLD-DNA adduct formation after 2-week.

Shah and coworkers used the ^{32}P postlabeling assay in order to study adduct formation between the Guthion (azinphos methyl), Sencor (metribuzin), Lorox (linuron), Reglone (diquat), Daconil (chlorothalonil) and Admire (imidacloprid) pesticides with calf thymus DNA (Shah and et al., 1997). The pesticides were reacted with calf thymus DNA. In addition the metabolites of the pesticides were obtained enzymatically using arochlor induced rat liver S9 fraction, in an NADPH generating system. The resulting metabolites were reacted with calf thymus DNA and the DNA was analyzed for the presence of adducts by either the nuclease P1 or butanol enrichment. Nuclease P1 enrichment resulted in adducts for all the pesticides. Compared to the level of adducts in control DNA, the levels in pesticide-treated DNA were higher for all the pesticides, except Daconil. The increase in adduct numbers for pesticide-treated DNAs ranged from 4.9–12.4 times the control-DNA indicating pesticide genotoxicity in this *in vitro* system. Enrichment using butanol extraction gave three adducts unique to Sencor-DNA. These adducts were different from those obtained with nuclease P1 enrichment of the same. B(a)P was the positive control for the *in vitro* metabolism, and two adduct enrichment procedures were nuclease P1 digestion and butanol extraction (Fig. 9).

3.2 Alkaline single cell gel electrophoresis (comet assay)

One of the other procedures that enable us to assess the DNA damage is the alkaline single cell gel electrophoresis or comet assay. In general, a small number of cells suspended in a thin agarose gel on a microscope slide are lyses, electrophoreses and stained with a fluorescent DNA finding dye. The technique is based on the fact that broken DNA migrates more easily in an electric field than intact molecules. When the slide is visualized with a fluorescence microscope, the observed objects resemble comets with a head region containing undamaged DNA and a tail containing the broken DNA (Fig. 10). The amount of DNA able to migrate and the distance of migration indicate the number of strand breaks present in that cell. Greater migration of the chromosomal DNA from the nucleus is an indication of higher level of DNA damage. Two versions of the Comet assay are currently in use; one introduced by Singh et al. (Singh, and et al., 1988), who used alkaline

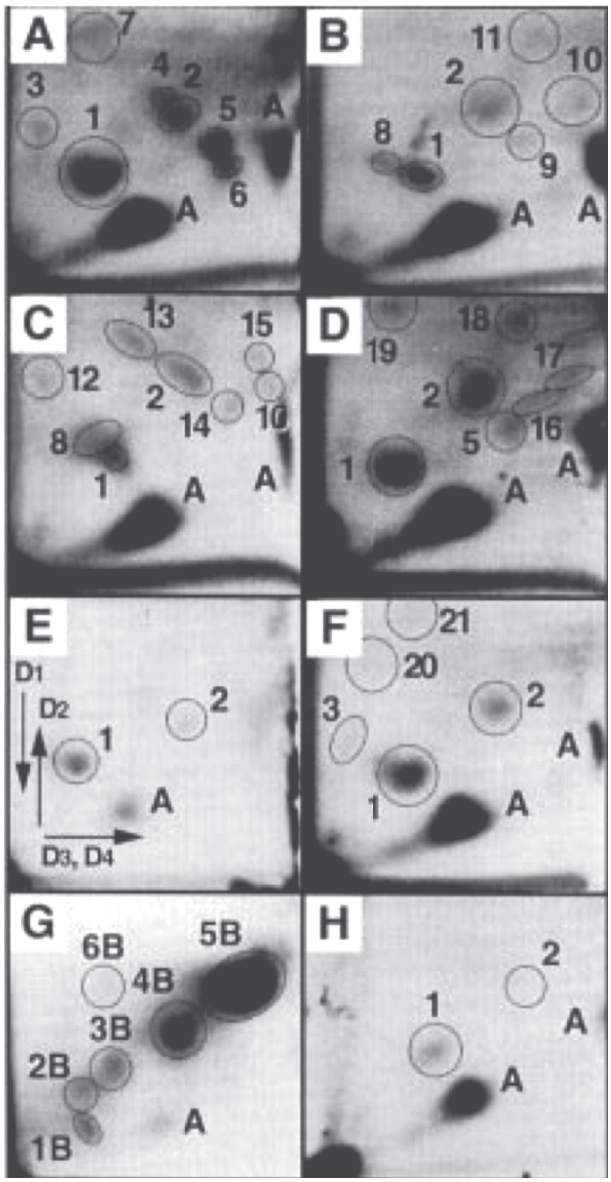


Fig. 9. Autoradiograph of PEI cellulose TLC maps of nuclease P1 enriched and postlabeled DNA, modified *in vitro* with metabolites of (A) Guthion, (B) Lorox, (C) Sencor, (D) Reglone, (E) Daconil, (F) Admire, (G) B(α)P and (H) negative control. Calf thymus DNA was reacted with the S9 metabolites of the above mentioned compounds. The adducts were enriched with nuclease P1 and labeled. The labeled adducts were separated on PEI cellulose plates using solvents for bulky adducts. Adducts are highlighted by encircling and numbered based on their position on the plate. All the autoradiograms were exposed for 18 h at –80°C with intensifying screens except for B(α)P which was exposed for 3 h. 'A' represents spots arising from ATP. The arrows represent the direction of migration with the solvents D1, D2, D3 and D4.

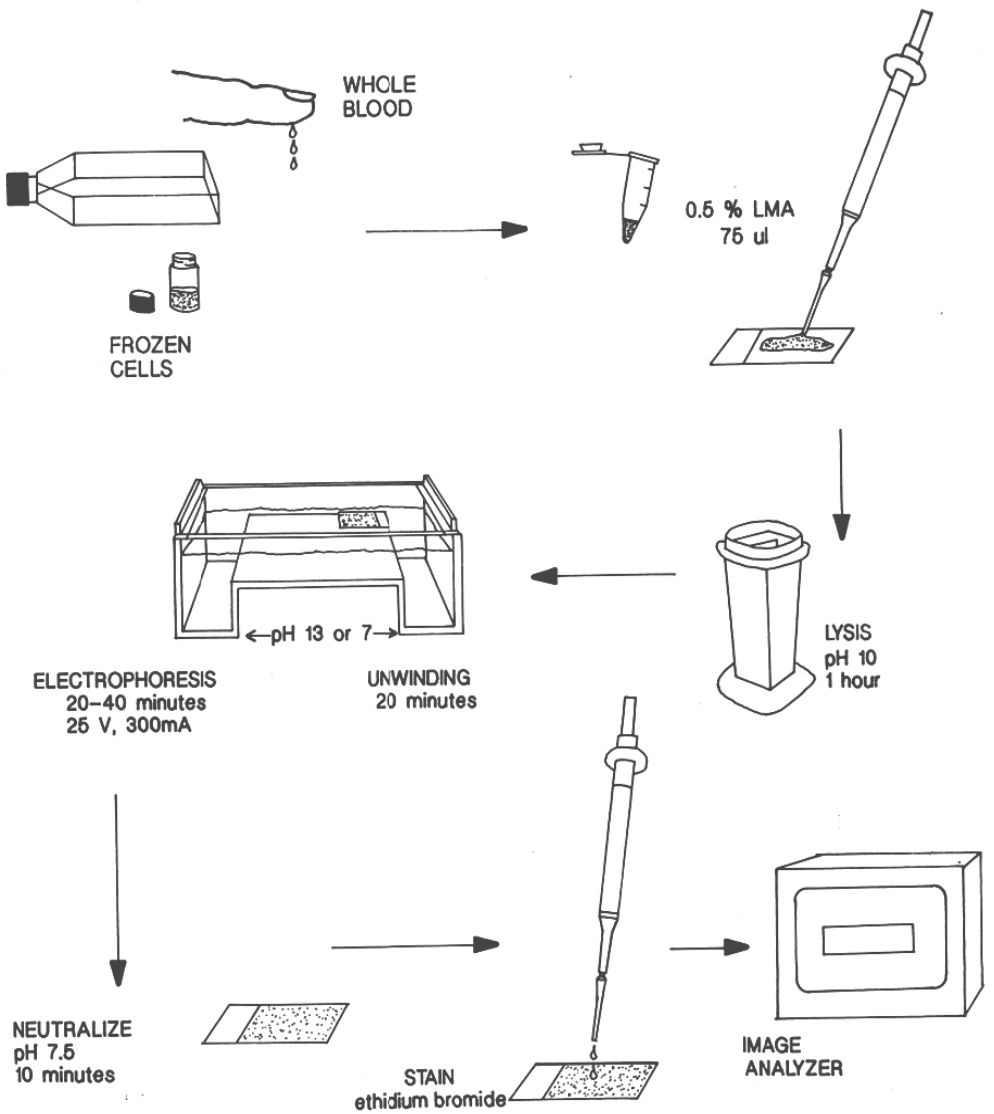


Fig. 10. SCGE Protocol.

electrophoresis (pH>13) to analyze DNA damage after treatment with X-rays or H_2O_2, which is capable of detecting DNA single-strand breaks and alkali labile sites in individual cells. This version is known as the ''*single cell gel electrophoresis (SCGE) technique*'', although for historical reasons many investigators refer to this method as the ''*Comet assay*''. Subsequently, Olive and co-workers developed versions of the neutral technique of Ostling and Johanson, which involved lyses in alkali treatment followed by electrophoresis at either neutral ([a] Olive, and et al., 1990) or mild alkaline (pH 12.3) conditions ([b] Olive and et al., 1990) to detect single strand breaks. The Singh and Olive methods are identical in principle and similar in practice, but the Singh method appears to be at least one- or two-orders of magnitude more sensitive. In the Singh version of the assay, a single cell suspension of the mammalian cell culture or tissue under study is embedded in low melting-point agarose in an agar gel sandwich on a microscope slide, lysed by detergents and high salt concentration at pH>10 and then electrophoresed for a short time under alkaline conditions. Lyses remove the cell contents except for the nuclear material. DNA remains highly super coiled in the presence of a small amount of non-histone protein but when placed in alkali, it starts to unwind from sites of strand breakage. Cells with increased DNA damage display increased migration of the DNA from the nucleus towards the anode under an electrical current, giving the appearance of a ''comet tail''. Depending on pH conditions for lyses and electrophoresis, the sensitivity of the technique can change. Employing neutral conditions for both variables allows us to detect DNA double strand breaks, but the pH 12.3 detects single strand breaks and delays DNA repair sites, while at pH>13 the sensitivity allows the evaluation of alkali labile sites, single strand breaks and delay repair sites of DNA. Therefore it is important to know the purpose of the study.

3.2.1 Slide preparation

Generally, the cells are mixed with 0.5% low-melting agarose at 37˚C and then placed on a microscope slide coated with 0.5% normal agarose. When the agarose is solidified, an additional layer of agarose is added. After the preparation of the three layers of this material (Fig. 11), the cells are lysed in a solution containing 2.5mM NaCl, 100mM Na_2-EDTA, 10mM Tris, pH 10, with 10% DMSO and 1% Triton X-100 for 1-24 h, and then the slides are put into an alkaline (300mM NaOH, 1mM Na_2-EDTA, and 0.2% DMSO, pH 13.5) or neutral buffer for double and single strand breaks detection, respectively, in a electrophoresis chamber, allowing the DNA unwinding. The electrophoresis is carried out, resulting in the migration of small pieces from the core of DNA toward the electric field. After electrophoresis, the slides are rinsed with neutralization buffer such as Tris-Hcl or PBS, and cells are stained with a fluorochrome dye such as Ethidium Bromide (5µg ml^{-1}).
A wide range of pesticides were studied by Comet assay for determination of their ability to induce in vivo genotoxic effects on DNA (see Table 1).

3.2.2 How to evaluate DNA damage by comet assay

For the evaluation of DNA damage by comet assay, the basic method is the image analysis of individual cells using the commercial software programs (such as SPSS) for collecting data. The parameters selected from the images for the quantification of DNA damage are length of DNA migration, tail length, tail moment, tail DNA% and head DNA%.
The length of DNA migration, usually presented in mm. Migration length, is related directly to fragment size and would be expected to be proportional to the extent of DNA damage. The migration length could be measured using different approaches; with a micrometer in

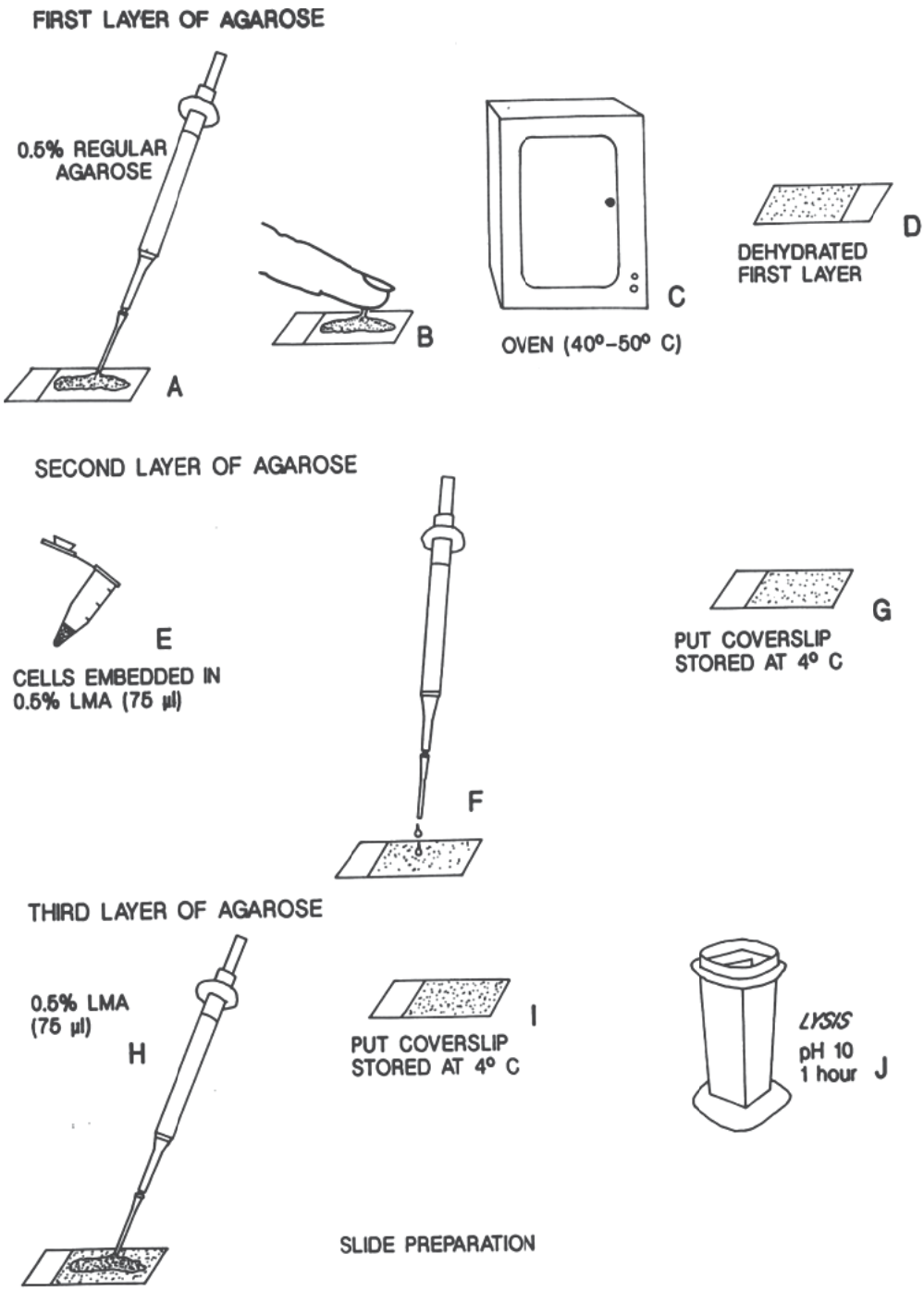
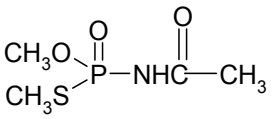
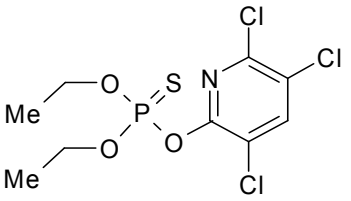
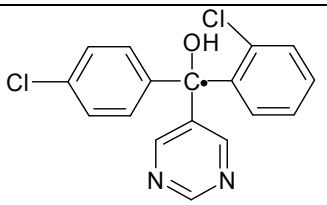
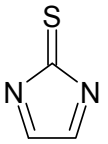
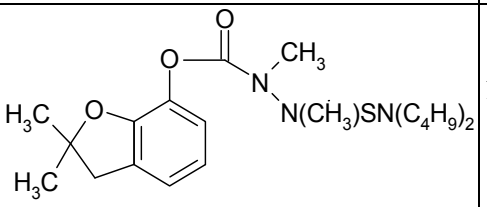
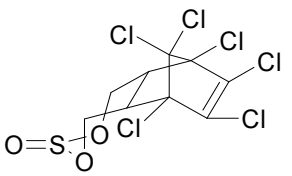
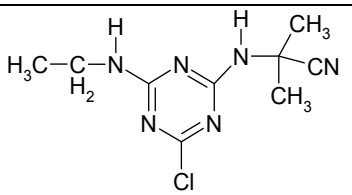
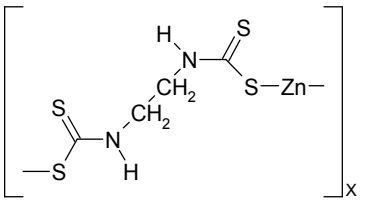
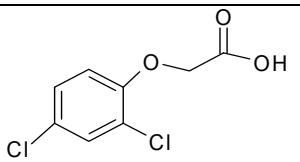
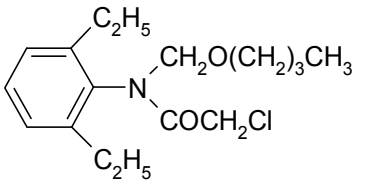
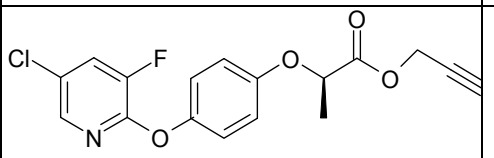
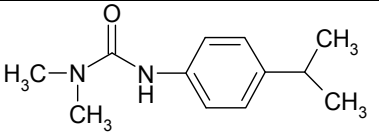
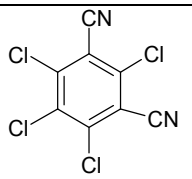
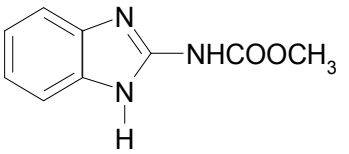
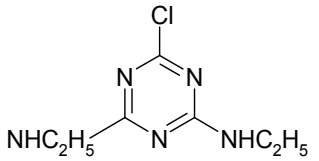


Fig. 11. Protocol for permanent slide preparation.

Reagent Name	Structure Formula	Type of Cells	Ref.
Acephate		Leucocytes of Swiss albino mice	(Rahman & et al., 2002)
Chloropyriphos		Leucocytes of Swiss albino mice and Lymphocyte and gill cells of *Channa punctatus* fish	Rahman & et al., 2002; Ali & et al., 2008)
Fenarimol		Leukocytes of two different rodent species (rat and mouse)	(Poli & et al., 2003)
2-Imidazolidinethione		Liver, lung, spleen, kidney, bone marrow from mouse	(Sasaki & et. al., 1997)
Carbosulfan		Erythrocyte and gill cells of *Channa punctatus* fish	(Nwani & et. al., 2010)
Endosulfan		Gill and kidney tissues of *Channa punctatus* fish	(Pandey , & et a., 2006)
Cyanazine		Leukocytes of mice	(Tennant & et al. 2001)

Reagent Name	Structure Formula	Type of Cells	Ref.
Zineb		Chinese hamster ovary (CHO) cells	(González , & et al., 2003)
2,4-D		Erythrocytes of *Clarias batrachus* catfish	(Ateeq & et al., 2005)
Butachlor		Erythrocytes of *Clarias batrachus* catfish	(Ateeq & et al., 2005)
Clodinafopp ropargyl		Hemocytes of *Bombyx mori* silkworm	(Yin , & et al., 2008)
Isoproturon		Chinese Hamster Ovary (CHO) cells	(Vigreux & et al., 1998)
Chlorothalonil		Chinese Hamster Ovary (CHO) cells	(Vigreux & et al., 1998)
Carbendazim		Chinese Hamster Ovary (CHO) cells	(Vigreux & et al., 1998)
Atrazine		Human blood samples	(Garaj-Vrhovac, & Zeljezic, 2000)

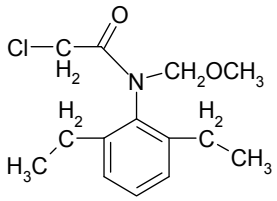
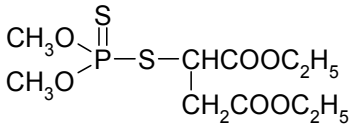
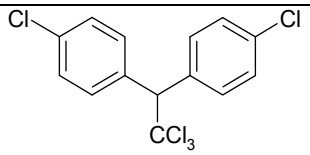
Reagent Name	Structure Formula	Type of Cells	Ref.
Alachlor		Human blood samples	(Garaj-Vrhovac, & Zeljezic, 2000)
Malathion		Cerebral tissue and peripheral blood of rats	(Reus, & et al., 2008)
P,P′-DDT		Human peripheral blood mononuclear cells (PBMC)	(Yanez & et al., 2004)

Table 1. The DNA damage by pesticides using the SCGE or Comet assay

the microscope eyepiece, a rule on photographic negatives / positives of cell images or in the camera monitor, and by using the image analyzer. Currently, the criteria used to identify the trailing and leading edge of the migrating DNA seem to depend on the investigator and/or software program. Furthermore, some investigators use the term ''DNA migration'' to describe total image length while others apply the term to migrated DNA only. A variant of this parameter is to present the ratio of length /width or width/ length, with cells exhibiting no damage having a ratio of approximately 1. (Olive, & Durand, 1992) discounted the utility of DNA migration as a parameter for DNA damage in the neutral or pH 12.3 alkaline assays, based on the observation that the length of DNA migration reached a plateau while the percentage of migrated DNA continued to increase. However, this limitation in migration length is not a characteristic of the pH.13 alkaline assay, where length has been reported to be the best parameter for this version of the assay. The computerized image analysis system to collect SCGE/Comet data, favors the evaluation of relative amount of migrated DNA, presented either as the percentage of migrated DNA or as the ratio of DNA in the tail to DNA in the head. This parameter assumes signal linearity in quantifying the amount of DNA ranging over multiple orders of magnitude and that the staining efficiency of the fluorescent dye is identical for migrated and non-migrated DNA. The concept of tail moment (tail length×tail intensity or percentage migrated DNA) as a parameter for DNA migration was introduced by Olive ([b]Olive, & et al., 1990). In a study conducted on damaging effects of pesticides on CHOK1 cells by Vigreux and co workers((Vigreux & et al., 1998) using SCGE, twenty-five randomly selected cells per slide were visually analyzed and submitted to image analysis (Fig. 12). Cells were eye-graded into three categories depending on DNA migration level: intact cells (IC) and slightly damaged cells (SDC), damaged cells (DC), and highly damaged cells (HDC) . These HDC, characterized by a small head and exceeding DNA fragmentation, may represent apoptotic cells.

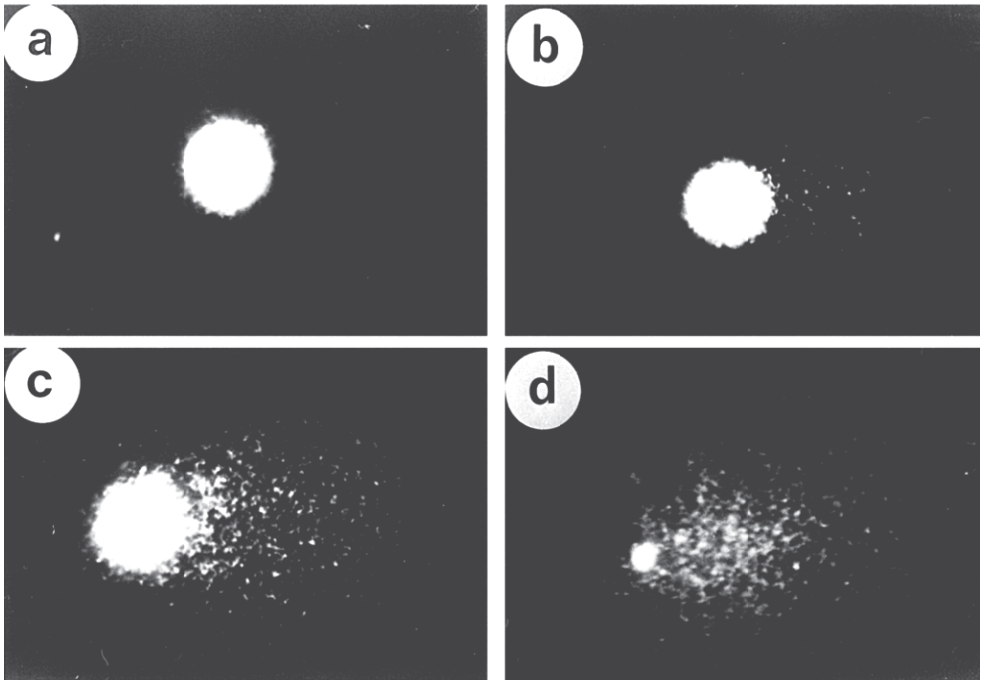


Fig. 12. Individual CHOK1 cells with various degrees of DNA damage: **(a, b)** intact cell (IC) and slightly damage cells (SCD) (tail moment 0–5) ; **(c)** damage cell (DC) (tail moment 5–60) and **(d)** highly damage cell HDC tail moment was not calculated with permission (Vigreux & et al., 1998).

Yin and et al., to evaluate DNA damage by pesticides and score each cell, used the relative intensity of the head and tail fluorescence of images (from undamaged DNA, stage 0, to maximally damaged DNA, stage D; Fig. 13a) (Yin , & et al., 2008). The visual analysis revealed that the exposure of hemocytes of the fourth instars silkworm, *B. mori*, to clodinafop-propargyl induced significantly elevated levels of DNA damage, and by increasing clodinafop-propargyl concentration the percentage of damaged DNA increased. Analysis of comet percentage of hemocytes in the second and fifth instars larvae of silkworm was skipped. The representative images (Fig. 13) illustrate how the damaged DNA was increasingly allowed to migrate to form a larger comet tail with the increase in the dose. The comet tail length of the hemocytes was 10.14, 9.5 and 6.6 times, and the tail moment was 86.8, 40.17 and 22.5 times compared with that of the control at the same dosages, respectively.

3.3 Alkaline elution or unwinding procedure

The DNA alkaline elution technique in combination with fluorometric measurement of the eluted DNA is a sensitive and suitable method for measuring single and double-strand DNA breaks, alkali-labile sites, and with minor modifications, protein associated strand breaks, DNA–protein and DNA–DNA cross links induced by physical, chemical and enzymatic processes in a mammalian test system. The alkaline elution method was developed by Kohn (Kohn, & Ewig, 1973), and the fluorometric method was developed by Cesarone.

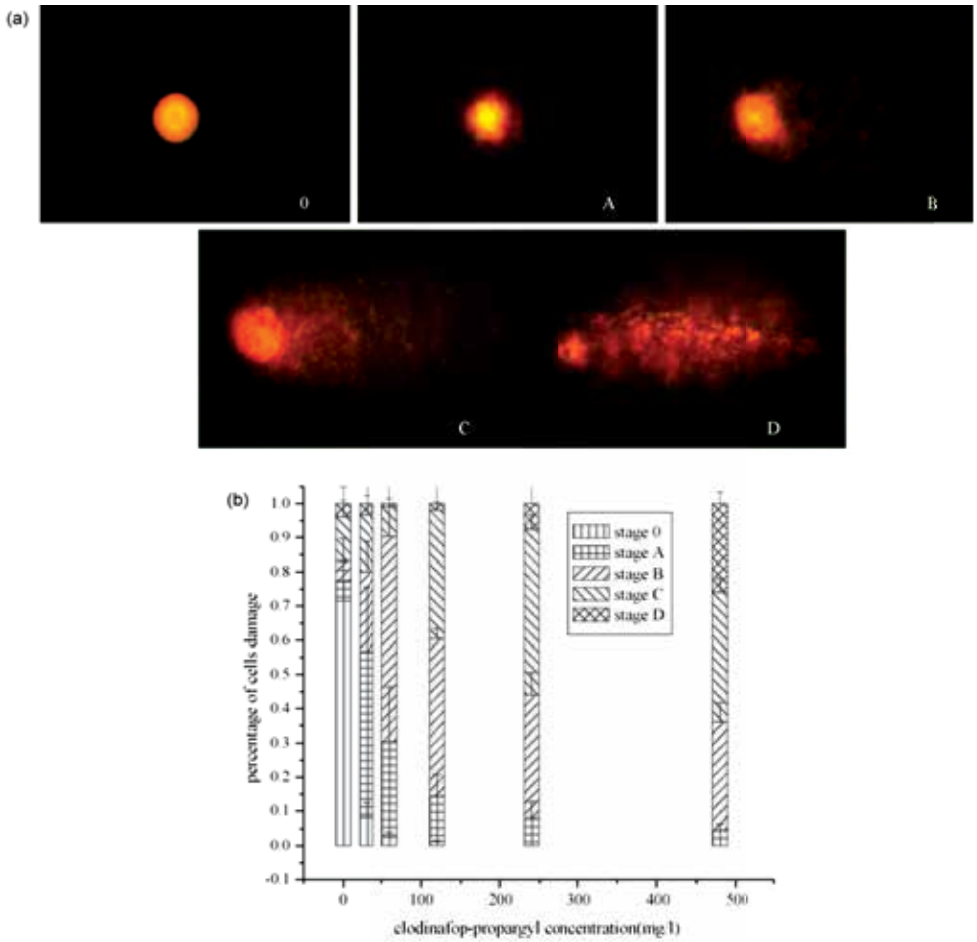


Fig. 13. **(a)** The different cell damage stages (0–D) used for scoring the SCGE by visual approach. **(b)** The percentage of cells damage stages (0–D) in the fourth instars larvae of silkworm, *B. mori* exposed to clodinafop-propargyl by visual approach. *Y* bars represent percentage of cells damage. *X* bars represent concentration of clodinafop-propargyl (Yin , & et al., 2008).

According to this method, cells are lysed on membrane filter and large nonelutable double strand-DNA is released. The filter is then eluted with an alkaline solution; single strand-DNA is released and slowly pumped through while fractions are collected to determine the elution kinetics. The elution rate of the single strand-DNA was proven to be size dependent and used as a measure of the DNA single strand breaks. Initially, for quantification of the DNA amount eluted during the alkaline elution procedure, prelabeling of cells with radioactive probes was used. Soon after, DNA quantification using fluorometric methods was considered preferable to avoid the use of radioactive materials. In original procedure, after extraction of crude nuclei from the tissues and centrifuge, the pelleted nuclei are resuspended in saline solution (EDTA, pH 7.4), an appropriate of solution containing a minimum of 1×10^6 cells or nuclei is placed in the membrane filter (25 mm diameter, 2.5 - 5 μm pore size). Then the nuclei or cells are lysed (2-5 mL solution containing 2% SDS

(Biorad), 0.025 M EDTA, 0.1 M glycine and 0.5 mg mL^{-1} proteinase K (BRL), pH 10) on the surface of the filter and single-stranded DNA fractions are elute with a solution containing EDTA and 10% tetraethylammonium hydroxide, pH 12.3, at low flow rate (0.1-0.3 mL min^{-1}). In a period of intervals, the fractions are collected. The fluorometric determination of DNA is performed with Hoechst 33258 reagent. This reagent, when complexed with DNA, enhanced the fluorescence yield.

The assay can be completed in 2.5 days, and for the diaminobenzoic acid dihydrochloride (DABA) method, it is labor-intensive for measuring the DNA in elution fractions. In DABA method, DNA cannot be assayed directly from the elution fractions and must be recovered by precipitation; also in order to get a sufficient amount of DNA, a minimum of 1×10^6 cells are required. In DABA method, it must be notified that the elution filters must have sufficient surface area so that they will not clog, and the fraction volumes must be large enough to obtain a sufficient amount of DNA per fraction. To extract the DNA from the eluting solution, a standard ethanol precipitation method is used which requires increasing the fraction volume 3-fold and incubating the samples at −20 ◦C for several hours. Therefore, wide attempts are made to minimize and automate the procedure along with elevation the efficiency (Gealy & et al., 2007). However, by this assay the elution rate constant, *K*, can be calculated from the equation (1):

$$K(mL^{-1}) = \frac{-Ln\ fraction\ of\ DNA\ retained\ on\ the\ filter}{eluted\ volume} \tag{1}$$

Bolognesi and coworkers (Bolognesi, & et al., 1997) evaluated the genotoxicity effects of Glyphosate (*N*-phosphonomethylglycine) and its technical formulation (Roundup) on liver and kidney of Swiss CD1 mice by alkaline elution assay. Figure 14 shows the results

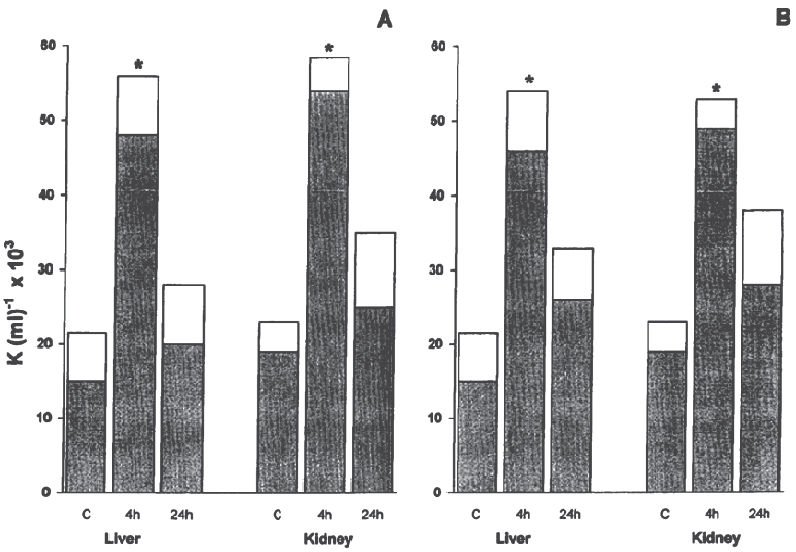


Fig. 14. Induction of DNA single-strand breaks and alkali labile sites by glyphosate (A) and Roundup (B). Dose was 300 mg/kg expressed as glyphosate. Mean data with SD of at least four independent experiments (8 animals) in treated animals and at least six experiments in controls (12 animals) are shown. * indicates $p < 0.05$.

obtained with the alkaline elution assay in treated liver and kidney with glyphosate or Roundup. A significant increase in the elution rate constant was observed 4 h after treatment with the technical formulation or its active ingredient, glyphosate. The increase in the elution rate was consistently higher in kidney of mice treated with glyphosate or Roundup. After 24 h, the elution rate constant returned to control values (data not shown). This transient effect could be attributed to the rapid elimination of this compound from the body and/or to repair of the DNA damage. No significant difference was observed in the extent of damage for the two compounds.
Pino (Pino & et al., 1988) reported that atrazine given orally to rats caused DNA damage in the stomach, kidney, and liver as measured by an alkaline elution assay. Thus, there is agreement that atrazine can cause DNA damage as measured by the alkaline elution-type DNA damage assays, but more than likely, the small amount of DNA damage that occurs is repaired.

4. In-vitro procedures for study of DNA-pesticides interactions

The in-vivo procedures described above are applicable for detection of Adduct-DNA complexes and dose not covers the non-covalent interactions. Therefore, recently the in-vitro methods have been developed. The Ultraviolet-visible spectrophotometry (Uv-vis), Fluorescence, circular dichroism (CD), and voltammetry are common procedures that are usable for analysis of non-covalent complexes including for DNA or RNA-targeting specially drugs. These techniques have now found important applications as a screening tool in drug discovery. Recently in our laboratory for the first time, we developed the in-vitro procedures for assessment of pesticides-DNA interactions to achieve a model of interaction for evaluation of genotoxicity effects of pesticides. Therefore, in this part we will discuss the procedure and the interpretation of data for assessing the mode and damaging effect of pesticides on DNA conformations. The data interpretations and how to use these procedures make the large body of this section.

4.1 The preparation of DNA solution and measuring concentration

Generally, the highly polymerized calf thymus-DNA (CT-DNA) is used for evaluation of damaging effect of pesticides. The stock solution of DNA is prepared by dissolving appropriate amount of DNA in a suitable buffer such as HEPES, phosphat, Tris–HCl and/or 10 mM NaCl solutions at pH 7.2 and dialyzing exhaustively against the same buffers for 24 h. The purity of the DNA is checked by monitoring the absorption ratio at 260/280 nm (A_{260}/A_{280}). The ratio must be more than 1.8, which indicate that DNA is fully free of protein. DNA concentration (Molar per nucleotide) of the stock solution is determined by UV spectrophotometery using the molar absorption coefficient $7000 M^{-1} cm^{-1}$ at 258 nm (Ahmadi, & et al., 2010).

4.2 Uv-vis titration

Electronic absorption spectroscopy is very suitable for interaction studies related to DNA. DNA has an absorption peak at 260nm, which arises due to the π–π* transitions of DNA bases. However, when DNA and pesticides are interacting, a clear change in DNA spectrum absorbance is observed. In other words, the change of stacking pattern, disruption of the hydrogen bonds between complementary strands, covalent bonding of DNA bases and intercalation reactions caused the hypochromism and/or hyperchromism, red and/or blue

shifts of this band. Hypochromism is due to the contraction of DNA in the helix axis and changes in the conformation of DNA, while hyperchromism results from the damage of the DNA double helix structure. Several studies revealed that interaction of foreign molecules with DNA by an intercalation mode causes significant elevation in absorbance (>0.07 Abs.) and a high red shift in wavelength (>6 nm), and the extent of hypochromism is commonly consistent with the strength of the intercalative interaction. In a study by author and Bakhshandeh conducted on the in-vitro study of damaging effects of 2,4-Dichlorophenoxyacetic Acid on DNA structure, the Uv/vis spectrum shows a weak hyperchromic and red shift (Fig.15a) (Ahmadi, & Bakhshandeh, 2009). We concluded that 2,4-D neither covalent nor classical intercalate binding with DNA bases and these Uv/vis results may be due to the conformation changes of DNA structure via the groove binding interaction with 2,4-D. In another study on effect of clodinafop-propargyl herbicide on DNA structure by author and co workers, the Uv/vis titration shows a significant increase in absorbance (>0.1 Abs.) and red shift (~7nm) (Fig. 15b). The author concludes that the clodinafop-propargyl herbicide interacts with calf thymus DNA by an intercalative mode of binding (Askari, Ahmadi & et al., 2008).

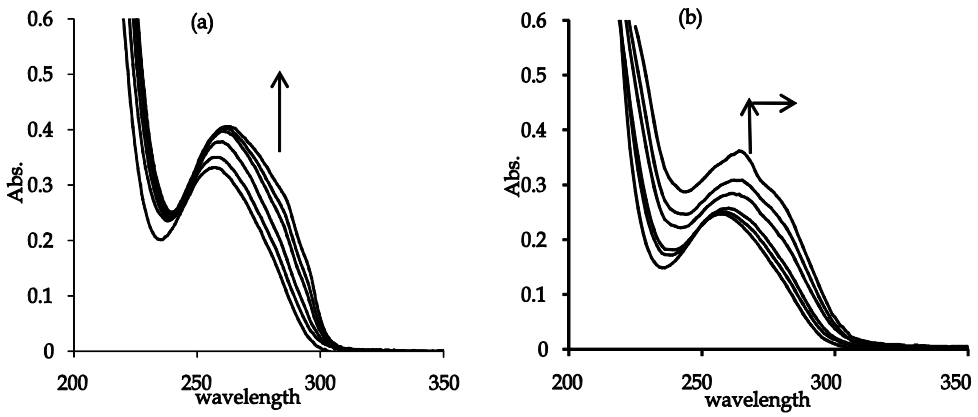


Fig. 15. Change in Uv/Vis absorption spectra of 5.0× 10^{-5} M of DNA in the presence of **(a)** 2,4-D; **(b)** clodinafop-propargyl.

From the Uv/vis titration spectra, the bonding constant of pesticide-DNA can be calculate as follows:

Generally a solution of DNA (5.0×10^{-5} M, 2 ml) is place in a Uv cell, thermo state at a given temperature, and the spectrum of the solution measuring. Then the pesticide (1.0×10^{-2} M) can be transfer step-by-step to the titration cell using a pre-calibrated micropipette, and the spectrum of the solution is measure after each transfer. Addition of the pesticide solution is continue until the desired r_i=[pesticide]/[DNA]=1.0 is achieved. To calculate the Pesticide-DNA bonding constant, the data can be treated according to the following equations:

$$\text{DNA} + \text{pesticide} \leftrightarrow \text{DNA} - \text{pesticide complex} \tag{2}$$

$$K = \frac{[\text{DNA} - \text{pesticide complex}]}{[\text{DNA}]_{\text{uncomplexed}}[\text{pesticide}]_{\text{uncomplexed}}} \tag{3}$$

For weak binding affinities, the data can treat using linear reciprocal plots based on the equation (4):

$$\frac{1}{A - A_0} = \frac{1}{A_\infty - A_0} + \frac{1}{K(A_\infty - A_0)} \cdot \frac{1}{[\text{pesticide}]} \tag{4}$$

Where, A_0 is the absorbance of DNA at 260nm in the absence of pesticide, A_∞ is the final absorbance of the pesticide-DNA complex and A is the recorded absorbance at different pesticide concentrations. The double reciprocal plot of 1/ (A – A_0) versus 1/[pesticide] is linear and the bonding constant (K) can be estimated from the ratio of the intercept to the slope. The author calculated the bonding constant of several pesticides such as Chloridazon, Fenitrothion and 2-Imidazolidinethione with DNA. If any pesticide has an obvious and sharp absorbance at higher wavelength of 260 nm, the K_b can be calculated from the spectrophotometric titration by keeping the concentration of pesticide constant and varying the DNA concentration. Based on the variations in the absorbance spectra of pesticide upon binding to DNA, the binding constant, K_b, can be calculated according to the equation (5):

$$\frac{A_0}{A - A_0} = \frac{\varepsilon_P}{\varepsilon_{P-D} - \varepsilon_P} + \frac{\varepsilon_P}{\varepsilon_{P-D} - \varepsilon_P} \frac{1}{K[DNA]} \tag{5}$$

Where A_0 and A are the absorbance of pesticide in the absence and presence of DNA, ε_P and ε_{H-P} are the absorption coefficients of pesticide and its complex with DNA, respectively. The plot of $A_0/(A-A_0)$ versus 1/[DNA] is linear and the bonding constant (K) can be estimated from the ratio of the intercept to the slope

4.3 Competitive fluorescence measurements

To estimate further the binding affinity of the pesticides with DNA, the variation of quantum yield of DNA fluorescence with pesticides can be monitored by stern-Volmer quenching constant. Fluorescence quenching refers to any process which decreased the fluorescence intensity of a sample. A variety of molecular interactions can result in quenching, including excited-state reactions, molecular rearrangement, energy transfer, ground-state complex formation, and collision quenching. In fact, two quenching processes are known: static and dynamic quenching. Both of them require molecular contact between the fluorophore and the quencher. Static quenching refers to formation of a nonfluorescent fluorophore–quencher complex. Dynamic quenching refers to the quencher diffusion to the fluorophore during the lifetime of the excited state and upon contact, the fluorophore returns to the ground state, without emission of a photon. Both static and dynamic processes are described by the Stern–Volmer equation (6):

$$\frac{F_0}{F} = 1 + K_{SV}[Q] \tag{6}$$

Where F_0 and F are the steady-state fluorescence intensities in the absence and presence of quencher (Pesticides), respectively, K_{SV} is the stern-Volmer quenching constant and [Q] is the concentration of quencher. It is well known that the fluorescence intensity of DNA itself is very weak; therefore, direct use of DNA fluorescence emission properties to monitor the interaction of pesticides with DNA is not possible. The standard method for fluorescence

enhancement of DNA is based on Ethidium bromide (EB) usage. EB is one of the sensitive fluorescence probe that with plane structure can bind to DNA. The interaction pattern of EB with DNA belongs to the intercalation model with the binding affinity in the order of 10^7. However, when EB intercalated into DNA, its emission intensity and excited-state lifetime dramatically increased. An experimental strategy for determining the quenching constant for the pesticide molecule interacting with DNA based on the quenching of EB fluorescence via a competition for binding sites in DNA has become a standard method in nucleic acid chemistry. Recently, due to carcinogenic properties of EB, the methylene blue (MB) was replaced and has become a safe reagent in nucleic acid chemistry. Methylene blue (MB) is one of phenothiazine dyes with a planar structure. The interaction of MB with DNA has been studied with various methods. Most studies indicated that at a low ionic strength buffer and a low concentration of DNA, the major binding mode of MB with DNA is through intercalation; otherwise, MB interacts with DNA by nonintercalative binding. However, two modes of interactions were postulated for MB with DNA: intercalate, and nonintercalate or groove binding.

In our laboratory we found that the addition of CT-DNA to an MB solution caused significant quenches of fluorescence of MB, and in [DNA]=[MB] ratio of 1 it reaches a minimum, and the spectrum is constant even when adding more DNA. By the addition of pesticide to the DNA–MB solution, the fluorescence of MB should increase. If the formation of pesticide– DNA complex is complete, the probe fluorescence intensity should be sufficiently close to the corresponding pure MB fluorescence intensity. This enhancement in fluorescence is due to the release of bonded MB molecules from DNA molecule. The formation constant of pesticides with DNA can be calculated based on the recorded fluorescence data using the modified Benesi–Hildebrand equation (7):

$$\frac{1}{(F - F_0)} = \frac{1}{K(LQ[MB - DNA]_0[Pesticide]_0)} + \frac{1}{(LQ[MB - DNA]_0)} \tag{7}$$

Where F_0 and F represent the fluorescence signals of MB–DNA in the absence and presence of pesticide, $[MB\text{-}DNA]_0$, and $[pesticide]_0$ represents the initial concentration of MB–DNA complex and pesticide, L is the instrumented constant, K is the formation constant of the pesticide–DNA complex, and Q is the quantum yield for the pesticide–DNA complex. By plotting $1/(F\text{-}F_0)$ versus $1/[pesticide]_0$, K can be obtained from the ratio of the intercept to the slope of the resulted curve. The interaction of DNA with Fenitrothion, Diazinon, has been studied by quenching competitive fluorescence, (see Fig16a, b).

Figures 16a and 16b shows the fluorescence emission spectrum of EB with DNA and the effect of the addition of Fenitrothion and Diazinon to EB bound DNA. As it is observed, by addition of the Fenitrothion and/or Diazinon a significant quenching of EB-DNA fluorescence occurred, reflecting an intercalative mode of binding between DNA and Fenitrothion or Diazinon.

Also, the interaction of DNA with clodinafop-propargyl have been studied by enhancement competitive fluorescence (see Fig17). As it is observed by addition of clodinafop-propargyl to the DNA–MB solution, the fluorescence of MB increased. The increase in the fluorescence intensity should be due to a greater amount of free MB molecules; in other words, the MB molecules were released after the addition of clodinafop-propargyl and the fluorescence of solution increased.

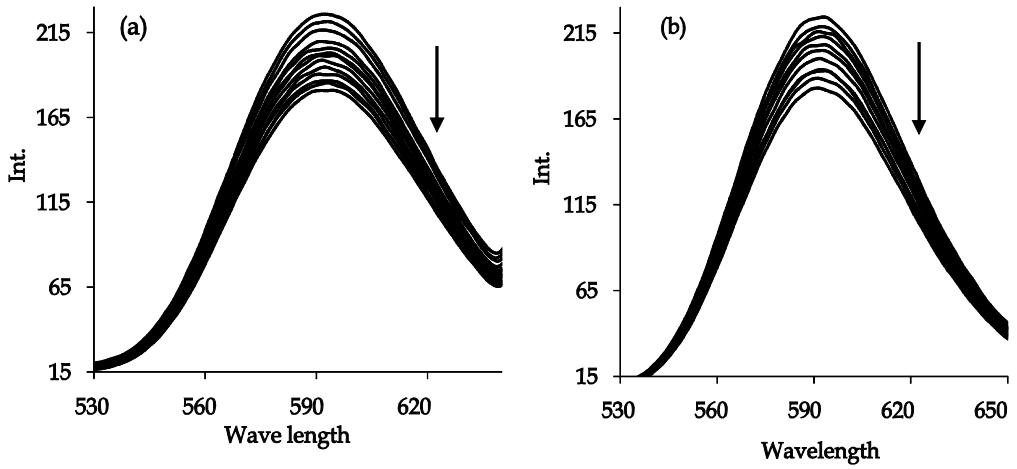


Fig. 16. Fluorescence quenching of DNA (5.0×10^{-5} M) with **(a)** Fenitrothion, **(b)** Diazinon with varying concentration (0.0, 0.492, 0.984, 1.476, 1.968, 2.46, 3.444, 4.428, 5.421, and 6.396×10^{-5} M) at 25˚C. Fluorescence was excited at 320 nm, and the monitoring wavelength was 590 nm.

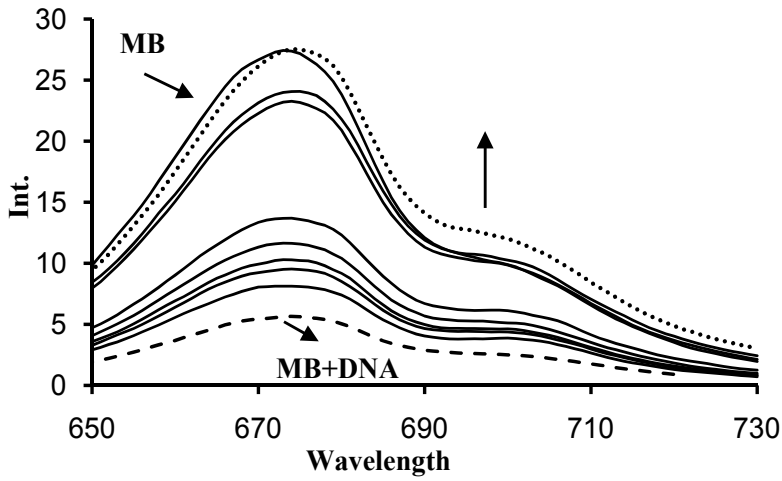


Fig. 17. Emission spectra of the MB–DNA complexes in the presence of the increasing clodinafop-propargyl (CP) concentrations in 0.01 M HEPES at room temperature. r_i=[CP]/[DNA+MB]=0.0(MB+DNA), 0.01, 0.02, 0.06, 0.2, 0.6, 1.0, 1.4, and 2.

4.4 Circular Dichroism (CD) spectroscopy

Circular dichroism (CD) spectroscopy CD has been extensively used in analysis of DNA conformation. In fact the CD provided essential information about the conformational properties of nucleic acids in solution. In the past decade, most DNA conformation studies included CD spectral data because they are informative, specific for various conformations of DNA and relatively easy and inexpensive to measure. The pervious studies revealed that CD resolved an apparent contradiction regarding the A-like conformation of (G-C)-rich

molecules of DNA, which was not solved by NMR spectroscopy, Raman spectroscopy, or other methods that are informative about the details of local DNA conformation. Also CD can distinguish between groove binders and intercalators but cannot identify individual binding sites. CD spectroscopy is complementary because it reflects the global properties of base stacking in DNA in a surprisingly realistic and sensitive way. The CD spectrum of free CT DNA is conservative and consists of a negative band at 245 nm and a positive one at 275 nm. The negative spectrum corresponds to the helical structure of DNA (helicity), and the positive spectrum corresponds to stacking of the base-pair that is characteristic of DNA in the right handed B-form. The effects of several pesticides such as 2,4-D, Chloridazon, fenitrtion, Diazinon, Clodinafop-propargyl, permetrin, 2-Imidazolidinethione, on DNA conformation have been reported by CD techniques. Depending on the structure formula of pesticides, the effects on DNA conformation were different.

For example, the CD spectra of Diazinon-DAN were measured at various ratios of Diazinon to DNA. In Figure 18, the changes in the CD spectra of DNA in the presence of increasing concentrations of Diazinon are depicted. The positive band (at 275 nm) in the CD spectrum has a very small change with addition of Diazinon. The negative band (at 245 nm) in the CD spectrum of DNA has an increase in the ellipticity to negative values with no shift in the band maximum.

These observations suggest that Diazinon binding to DNA induces certain conformational changes, such as condensation of DNA molecule.

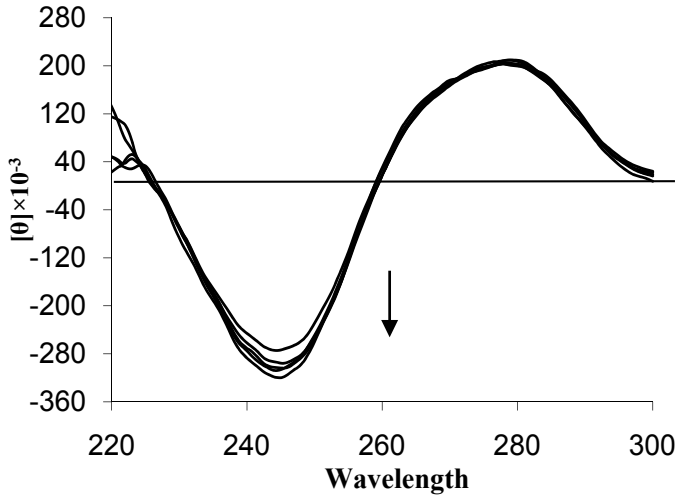


Fig. 18. CD spectra of the DNA in the presence of different amount of Diazinon (r¼0.0, 0.05, 0.2, 0.5, and 0.1) in 0.01M HEPES buffer (pH 7.3).

Also the bonding constant of DNA with pesticides can be calculated using the equation (8):

$$\frac{1}{\Delta A} = \frac{1}{(\varepsilon_b - \varepsilon_f)L_T} + \frac{1}{(\varepsilon_b - \varepsilon_f)L_T K_a} \times \frac{1}{M} \tag{8}$$

Where ε is the extinction coefficient; the subscripts b, f, and T denote bound, free, and total; and L is the DNA concentration. M is the concentration of pesticide and ΔA is the change of the CD response at a particular wavelength.

4.6 Voltammetric techniques in biomolecules

Recently, the electrochemical techniques were used as rapid and inexpensive methods for the study of DNA interactions with different compounds. The advantages of this technique are low cost, ease of use, high sensitivity, and a wide dynamic range of operations. Today the voltammetric methods enable us to estimate and predict the conformational changes of DNA by pesticides and evaluation of carcinogenic and mutagenic effects of pesticides or other molecules on DNA. The changes of electrochemical signals provide a very interesting evidence for elucidation of interaction mechanism. The common voltammetric techniques that usable for study effects of pesticides on DNA are linear sweep voltammetry (LSV), differential pulse voltammetry (DPV), cyclic voltammetry (CV), and alternative current voltammetry (ACV). In general, under specific conditions such as DNA concentration and ionic strength of background electrolyte, DNA can produce transmetic response around -1.2 and -1.4V versus SCE; however, the pure DNA is electrochemically inactive in potential range of 0.0 to -1.2 V at Hanging Mercury Drop Electrode (HMDE), Pt, Au and Glassy Carbon (GC) versus Ag/AgCl at pH=5-9. Therefore all voltammetric studies are based on considerable diminution of reduction/oxidation peak current and positive or negative shift in peak potential of electroactive pesticides when DNA is added to the voltammetric cell. This decrease in current is due to the decrease of diffusion of pesticide–DNA complex, not due to the increased viscosity of the solution or the blockage of the electrode surface by DNA adsorption. In this section we will discuses the application of these procedures in evaluating of in-vitro pesticides-DNA interactions and the basic of mesurment of interaction parameters such as bonding constant, site size bonding, number of redox electrons, and diffusion coefficient of molecules. The basic theory of voltammetric procedures are presented in literature (Bard, & Faulkner, 2001).

4.6.1 Linear Sweep Voltammetry (LSV)

To determine the composition of the supramolecular complex and the equilibrium constant of the binding reaction, the linear sweep voltammetry is a suitable procedure. Linear sweep voltammetry is sometimes abbreviated to LSV. In this method, a static indicator electrode (A cm^2 in area) is used and its potential is scanned at constant rate v ($V\ s^{-1}$) from an initial value (E_i) in the positive or negative direction. After reaching a peak, the current decreases again. The current decrease after the peak occurs, because the thickness of the diffusion layer increases with time. Even if the potential scan is stopped after the peak, the current continues to decrease with time in the same way (Fig. 19).

In order to improve the sensitivity of detection, minimizing the reading error the second order derivative linear sweep voltammetric (DLSV) peaks is used. In this manner it should be assumed that pesticide molecules interacted with DNA only to form a single complex of DNA-n Pesticide. The binding number and the equilibrium constant of the binding reaction can be deduced as follows:

$$\text{DNA} + \text{n Pesticide} \leftrightarrow \text{DNA} - \text{n Pesticide} \tag{9}$$

The equilibrium constant is:

$$\beta_n = \frac{[\text{DNA} - \text{n Pesticide}]}{[\text{DNA}][\text{Pesticide}]^n} \tag{10}$$

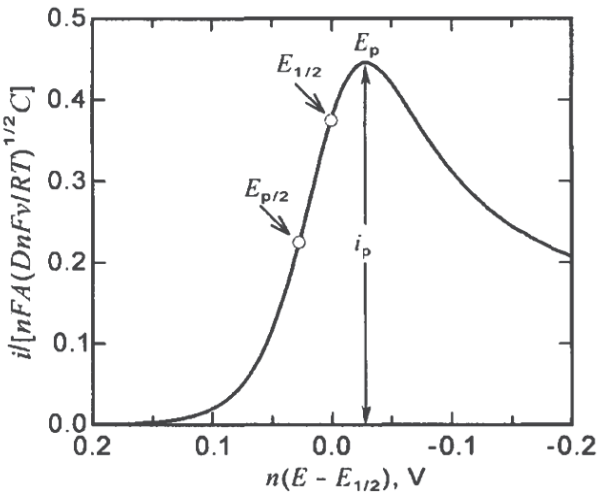


Fig. 19. Linear sweep voltammogram for a reversible process. E_p, peak potential; $E_{p/2}$, half-peak potential; $E_{1/2}$, half wave potential; i_p, peak current.

And the following can be deduced:

$$\Delta I_{max} = KC_{DNA} \tag{11}$$

$$\Delta I = K[DNA - n\ Pesticide] \tag{12}$$

$$[DNA] + [DNA - n\ Pesticide] = C_{DNA} \tag{13}$$

Therefore:

$$\Delta I_{max} - \Delta I = K(C_{DNA} - [DNA - n\ Pesticide]) = K[DNA] \tag{14}$$

Introducing Eqs. (10), (11), (13), and (14) gives:

$$\frac{1}{\Delta I} = \frac{1}{\Delta I_{max}} + \left(\frac{1}{\beta \Delta I_{max}}\right)\left(\frac{1}{[Pesticide]^n}\right) \tag{15}$$

or

$$Log\left[\frac{\Delta I}{(\Delta I_{max} - \Delta I)}\right] = \log \beta_n + n \log[Pesticide] \tag{16}$$

Where ΔI is the difference of peak current of reduction or oxidation of pesticide in the presence and absence of DNA and ΔI_{max} correspond to the obtain value when the concentration of pesticide is extremely higher than that of DNA. The C_{DNA}, [DNA], and [DNA-n Pesticide] correspond to the total, free, and bound concentrations of DNA in the solution, respectively. From the linear relationship between $\log[\Delta I/(\Delta I_{max}-\Delta I)]$ versus log[Pesticide], the stoichimetry and log β_n can be calculate from intercept and slope, respectively. The stoichiometry and log β_n of diazinon with DNA were reported using the

LSV. In Fig. 20 curve a was the relationship of Ip with the concentration of Diazinon, curve b represented the change of peak current after the addition of 1.0 ×10^{-5} M on varying the concentration of Diazinon, and curve c showed the differences between curve a and curve b, which represented the relationship between ΔIp (Ip_a-Ip_b.) and the concentration of Diazinon. From Eq. (16) the relation of log[ΔI/(ΔI$_{max}$ - ΔI)] with log[Diazinon] was calculated and from the intercept and slope the n=1.798 and log β_2=8.42 were deduced, which indicated that a stable 1 : 2 complex of DNA-2Diazinon was formed under the selected conditions.

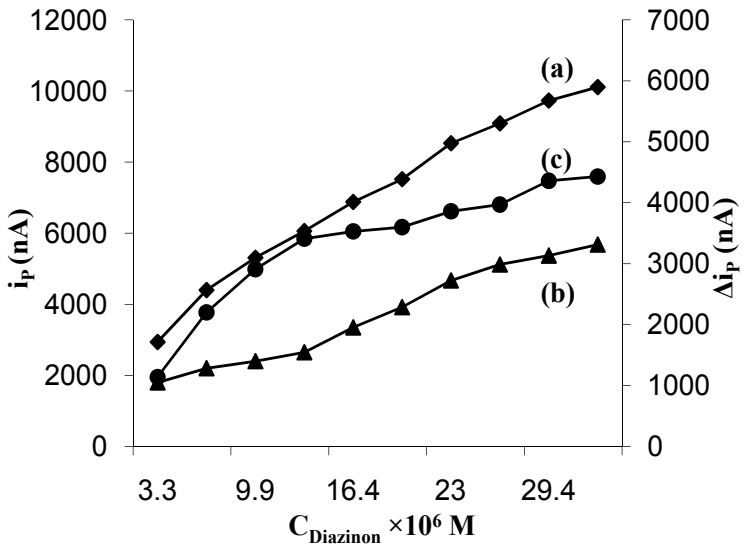


Fig. 20. Relationship between Ip and $C_{Diazinon}$ (a, b), ΔIp and $C_{Diazinon}$(c); (a) C_{DNA}= 0; (b) C_{DNA}= 1.0 ×10^{-5} M; (c) ΔIp= Ip_a-Ip_b (Ahmadi & et al.,2008).

4.6.2 Cyclic Voltammetry (CV)

In CV, the potential is linearly scanned forward from E_1 to E_2 and then backward from E_2 to E_1, giving a triangular potential cycle. Figure 21 shows some examples of cyclic voltammograms for the process, Ox + ne ↔ Red where only Ox is in the solution. Curve 1 is when the process is reversible. In the forward scan, a cathodic peak is obtained by the reduction of Ox to Red, as in LSV. In the backward scan, an anodic peak appears, due to the re-oxidation of the Red, which was generated during the forward scan. For a reversible process, the cathodic and anodic peak currents are equal in magnitude ($|i_{pc}| = |i_{pa}|$) and the cathodic peak potential (E_{pc}) is (*58/n*) mV more negative than the anodic peak potential (E_{pa}). These are important criteria for reversibility. Moreover, the half-wave potential, which is used to obtain the formal redox potential, is obtained by $E_{1/2}=(E_{pc}+E_{pa})/2$.

By decreasing the reversibility, the difference between the two peak potentials increases. Curve 2 is for a process that is considerably irreversible. Compared with curve 1, the cathodic peak appears at much more negative potential, the anodic peak at much more positive potential. If the process is completely irreversible, the anodic peak does not appear in the measurable potential region. From the irreversible CV curve, we can obtain kinetic parameters (rate constant and transfer coefficient) for the electrode reaction, the bonding formation and bonding site size usually by a simulation method. Curve 3 is for the case in

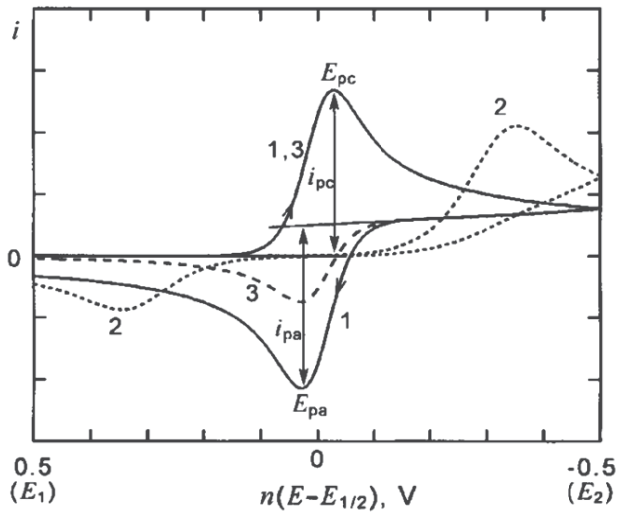


Fig. 21. Cyclic voltammograms for the electrode reaction Ox+ne ↔Red, which is reversible (curve 1), irreversible but α=0.5 (curve 2), and reversible but accompanied by a conversion of Red to an electroinactive species (curve 3). $E_{1/2}$ is for reversible process.

which $Ox \leftrightarrow Red \underline{k} A$ i.e. Red can be reversibly re-oxidized to Ox but, before the re-oxidation, some part of the Red is converted to non-electroactive species A. In CV, the voltage scan rate can be varied over a wide range, 0.001 to 2 V s^{-1} or more.

The application of cyclic voltammetry for interaction study of electroactive pesticides with DNA provides valuable information's. In voltammetric studies before all the electrochemical behavior of electroactive species at the surface of electrode should be assessment. Generally the CV is a common technique that widely used. From the cyclic voltammogrames the reversibility and/or irreversibility of electrochemical reaction is observable. Also, from the changes of the peak current (i_p) and peak potential (E_p) at the electrode surface versus scan rate (ν), $\nu^{1/2}$ and $ln\ \nu$ the absence and presence of an excess of DNA the type of mass transport of species to the surface (adsorption or diffusion), $(\alpha n)_f$ and $(\alpha n)_b$, K_s and E^0 can be calculated. The symmetry of the energy barrier (α) and all electrons (n), can be calculate, using the Anson equation (17):

$$E_p = E^0 - \frac{RT}{\alpha nF}\left[ln\left(\frac{K_s}{D^{\frac{1}{2}}}\right) + 0.5ln\left(\frac{\alpha nF\nu}{RT}\right) + 0.78 \right] \quad (17)$$

The α is obtained from the slope of the linear between E_p and $ln\nu$. As the $n\Delta E_p$ should be less than 200mV, the Laviron equation (18) also is usable for calculation of K_s and E^0.

$$E_p = E^\circ + \left(\frac{RT}{\alpha nF}\right)\left[ln\left(\frac{RTK_s}{\alpha nF}\right) - ln\,\nu \right] \quad (18)$$

Where E^0 (V) is the formal potential, R is the universal gas constant (8.314 J K^{-1} mol^{-1}), T (K) is the Kelvin temperature, K_s (s^{-1}) is the electrochemical rate constant and F is the Faraday constant (96,487 C mol^{-1}). According to Laviron equation the plot of E_p vs. $ln\nu$ is linear with a

slope that allows *an* being determined, and an intercept from which K_s can be calculated if the value of E^0 (V) is known. The value of E^0 in Laviron equation can be obtained from the intercept of the E_p vs. ν curve by extrapolation to the vertical axis at ν=0.

4.6.3 Cyclic voltammetric titration of pesticides with DNA

The electrochemical titration is more valuable to quantify the interaction parameters of an electroactive molecule with DNA than other methods. By addition of different amounts of DNA to the voltammetric cell containing an electroactive pesticide (5×10^{-5} M), the cathodic or oxidative peak current pesticide begin to decrease and the formal potential shifts to more negative or positive values which suggest the interaction of the pesticide with DNA. The addition of DNA to voltammetric cell continue untile the $R = \frac{[DNA]}{[Pesticide]} \geq 10$, and the CV currents of pesticide decrease and stabled. If an electroactive molecule (*E*) nonspecifically reacts with a DNA duplex at a binding site, which is composed of base pairs (S), a DNA-electroactive molecule complex (E-S) is produced as follows:

$$E + S \leftrightarrow E\text{-}S \tag{19}$$

As the pesticides non-specifically binds to the DNA duplex and covers S consecutive base pairs (i.e. one binding site), the binding constant, *K*, can be given by the equation (20):

$$K = \frac{C_b}{C_f C_s} \tag{20}$$

Where C_b, C_f, and C_s represent the equilibrium concentrations of the DNA-Pesticide complex, free Pesticide and free binding site, respectively. The total concentration of pesticide, C_t, is:

$$C_t = C_b + C_f \tag{21}$$

The average of number of binding sites (x) along a DNA duplex molecule with an average total number of base pairs *L* can be described by the following form: $x = \frac{L}{s}$, where *s* is the binding site size of the electroactive molecule interacting with DNA. It means that, the number of DNA base pairs is occupied (or covered) by a binding molecule. Thus, the total concentration of binding sites (xC_{DNA}) can be expressed as follows:

$$xC_{DNA} = C_b + C_s \tag{22}$$

where

$$C_{DNA} = \frac{C_{NP}}{2L} \tag{23}$$

C_{NP} represents the concentration of nucleotide phosphate, which is determined by the UV absorption at 260 nm. The total concentration of binding sites can also be expressed as follows:

$$\frac{C_{NP}}{2s} = C_b + C_s \tag{24}$$

The ratio of the NP concentration and the total concentration of electroactive molecules can be defined as *R*:

$$R = \frac{C_{NP}}{C_t} \tag{25}$$

For an irreversible reaction in CV, the total cathodic current (I_{pc}) under the fixed potential with any R can be calculated:

$$i_{pc} = B\left[(\alpha n)_f^{\frac{1}{2}} D_f^{\frac{1}{2}} C_f + (\alpha n)_b^{\frac{1}{2}} D_b^{\frac{1}{2}} C_b \right] \tag{26}$$

In fact B represents the appropriate, concentration-independent terms in the voltammetric expression. A Nernstain reaction in CV at 25 ^{o}C is shown as follows:

$$B = 2.99 \times 10^5 n A v^{\frac{1}{2}} \tag{27}$$

Where n is the number of electron transferred, A the electrode area, v the scan rate, D_f and D_b are the diffusion coefficients for free and bound molecules, α_f and α_b are the electron transfer coefficients for free and bound molecules, and C_f and C_b are the bulk concentrations of the free and bound irreversibly electroactive species.
Based on Carter et al., the binding constant, K, can be expressed as the following form:

$$K = \frac{C_b}{C_f\left(\frac{[NP]}{2s} - C_b\right)} \tag{28}$$

Where s is the size of binding site in terms of base pairs. Making appropriate substitutions and eliminating C_b and C_f from Eq. (26), a new equation was obtained:

$$i_{pc} = B\left\{ (\alpha n)_f^{\frac{1}{2}} D_f^{\frac{1}{2}} C_t + \left[(\alpha n)_b^{\frac{1}{2}} D_b^{\frac{1}{2}} - (\alpha n)_f^{\frac{1}{2}} D_f^{\frac{1}{2}} \right] \times \left[\frac{b - \left(b^2 - \frac{2K^2 C_t^2 R}{s} \right)^{\frac{1}{2}}}{2K} \right] \right\} \tag{29}$$

Where $b = 1 + KC_t + \frac{KRC_t}{2s}$.

Since i_{pc}, C_t and [NP] are experimentally measurable and $(\alpha n)_f$, $(\alpha n)_b$, have already been acquired as mentioned above, the binding constant (K) and binding site size (s) of the pesticide-DNA, D_f and D_b can be obtained from a nonlinear regression analysis of the experimental data (i_{pc} versus [R] plot) according to Eq. (29).
The in-vitro interaction of Fenitrothion with DNA by CV technique and nonlinear fit analysis using the equation (29) (see Fig. 22) yielded K =1.03×10^4, s =1.204, $D_f = 5.2\times10^{-2}$ and $D_b=1.72\times10^{-5}$.

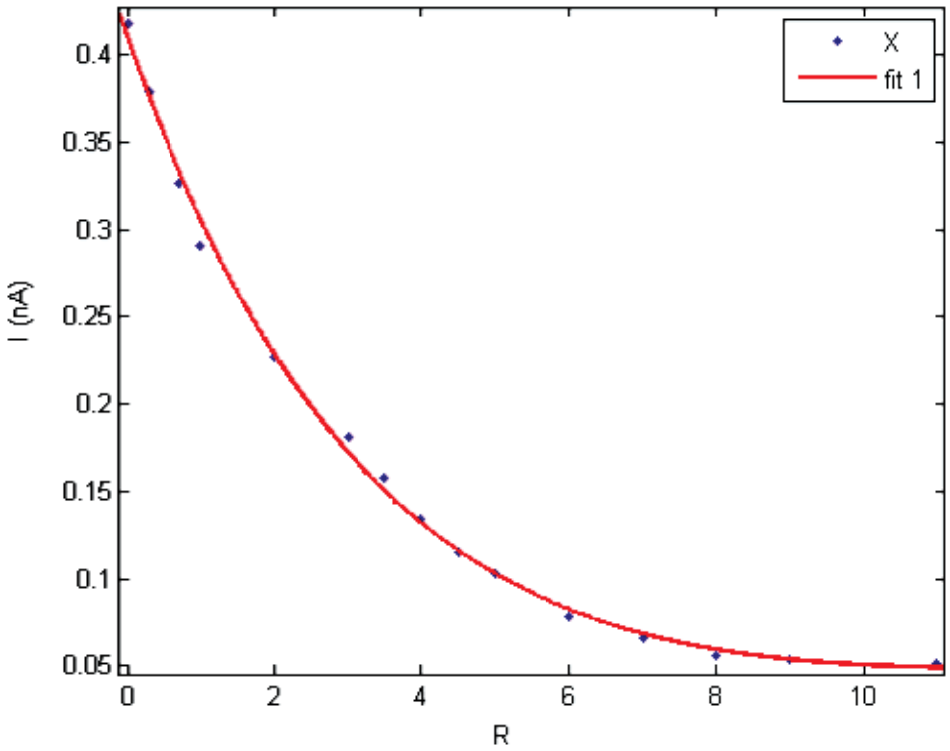


Fig. 22. Dependence of i_p of 5×10^{-5} M Fenitrothion on the concentration of added DNA by cyclic voltammetry(blue dots), and nonlinear fiting (solid Red line) using the equation (29).

4.6.4 Differential Pulse Voltammetry (DPV)

Differential pulse voltammetric titration is another valuable technique for monitoring of in-vitro DNA interactions. It is performed by keeping the concentration of electroactive species constant while varying the concentrations of DNA. The current titration equation can be described by equation (30):

$$\frac{1}{C_{DNA}} = K_f \frac{(1-A)}{1-\frac{i}{i_0}} - K_f \tag{30}$$

Where C_{DNA} is the concentration of DNA, K_f is the bonding constant of Pesticide-DNA, i_0 and i are the peak currents of electroactive species without and with DNA, respectively, and A is the proportional constant. The condition of using this Eq. (30) is that a 1:1 association complex is formed and C_{DNA} is much larger than the total concentration of electroactive pesticide in solution.

The interaction of 2-Imidazolidinethione (ETU) with guanine in binary mixtures of water-acetonitril were studied by DPV (Fig. 23, Table). In this study the hydrogen bonding were proved between DNA and ETU by electrochemical procedur.

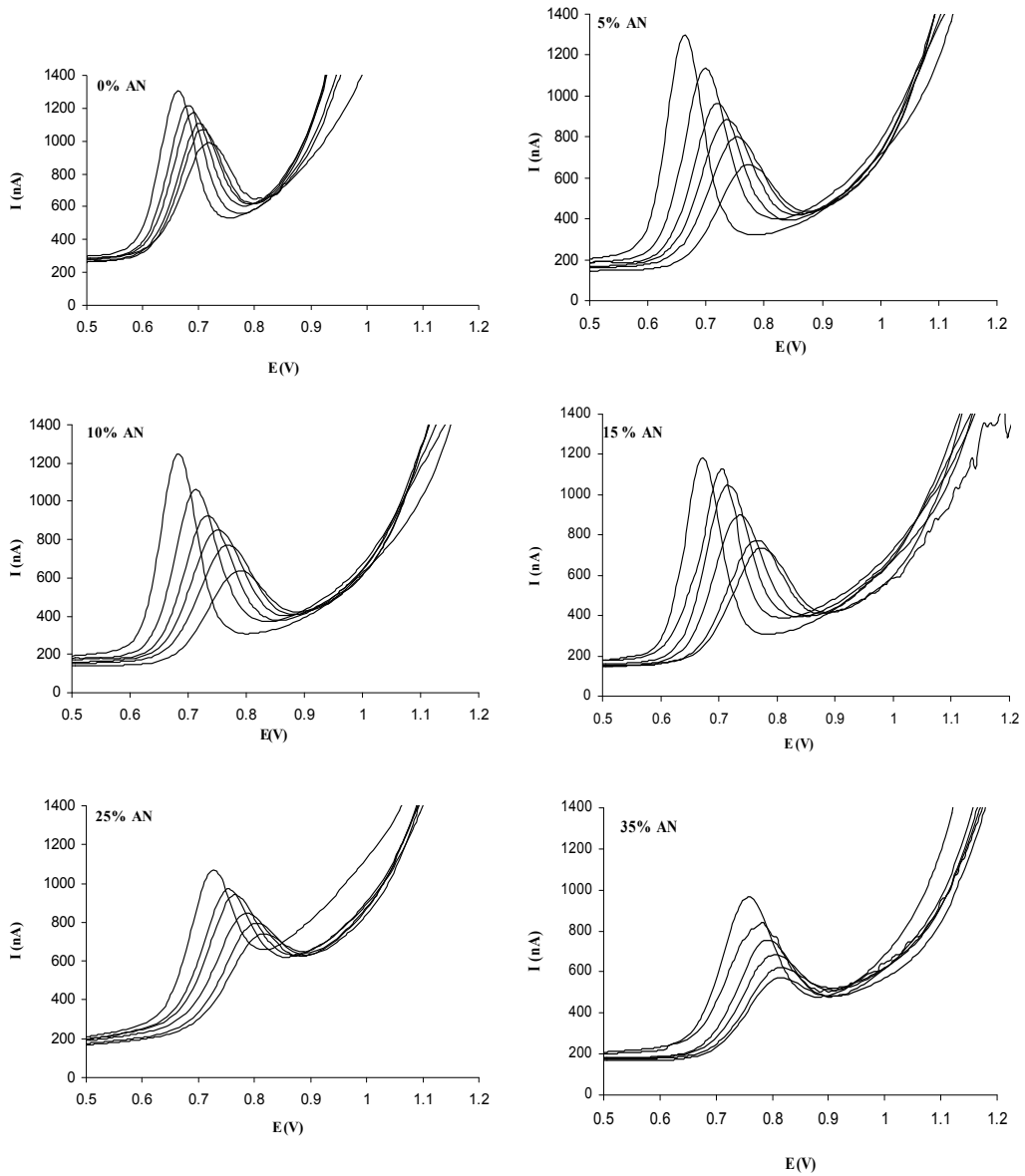


Fig. 23. Differential pulse voltammograms of 4×10^{-5} M of GU in the addition of different amount of Im (0.0, 2.0, 4.0, 6.0, 8.0, 10.0 × 10^{-5} M) in Tris-HCl buffer and binary mixture of AN- buffer with pH=7.2.

%AN	Linear Equation [58]	R^2	Log K_f
0	$\frac{1}{C_{Im}} = \frac{2.23\times10^3}{(1-\frac{i}{i_0})} - 7.48\times10^2$	0.9982	2.87
5	$\frac{1}{C_{Im}} = \frac{5.07\times10^3}{(1-\frac{i}{i_0})} - 1.27\times10^3$	0.9971	3.1
10	$\frac{1}{C_{Im}} = \frac{5.12\times10^3}{(1-\frac{i}{i_0})} - 2.24\times10^3$	0.9968	3.35
15	$\frac{1}{C_{Im}} = \frac{4.27\times10^3}{(1-\frac{i}{i_0})} - 3.13\times10^3$	0.9988	3.49
25	$\frac{1}{C_{Im}} = \frac{5.45\times10^3}{(1-\frac{i}{i_0})} - 6.92\times10^3$	0.9962	3.84
35	$\frac{1}{C_{Im}} = \frac{8.84\times10^3}{(1-\frac{i}{i_0})} - 7.33\times10^3$	0.9986	3.87

Table 2. The linear equation and log K_f of Im-GU complex calculated by DPV in different binary mixture of buffer-AN.

5. References

Abhilash, P.C. & Singh, N. (2009). Pesticide use and application: An Indian scenario. *Journal of Hazardous Materials*. 165, 1–12.

Ahmadi, F.; Alizadeh, A.A; Bakhshandeh-Saraskanrood, F.; Jafari, B. & Khodadadian, M. (2010). Experimental and computational approach to the rational monitoring of hydrogen-bonding interaction of 2-Imidazolidinethione with DNA and guanine. *Food and Chemical Toxicology* 48 (2010) 29–36.

Ahmadi, F. & Bakhshandeh, F. (2009). In Vitro Study of Damaging Effects of 2,4-Dichlorophenoxyacetic Acid on DNA Structure by Spectroscopic and Voltammetric Techniques. *DNA and Cell Biology* 28, 527–533.

Ahmadi, F.; Gholivand, M.B. & Ravan, H. (2008) Interaction of Diazinon with DNA and the Protective Role of Selenium in DNA Damage. *DNA and Cell Biology* 27, 325–332.

Ali, D.; Nagpure, N.S.; Kumar, S.; Kumar, R. & Kushwah, B. (2008). Genotoxicity assessment of acute exposure of chlorpyrifos to freshwater fish Channa punctatus (Bloch) using micronucleusassay and alkaline single-cell gel electrophoresis, *Chemosphere*, 71, 1823–1831.

Askari, S.; Ahmadi, F.; Omidfar, K.; Ghobadi, S. & F. Abasi Tarighat. (2008). In Vitro Study of DNA Interaction with Clodinafop-Propargyl Herbicide. *DNA and Cell Biology*, 27, (2008) 581–586.

Ateeq, B.; Farah, M.A. & Ahmad, W. (2005). Detection of DNA damage by alkaline single cell gel electrophoresis in 2,4-dichlorophenoxyacetic-acid- and butachlor-exposed erythrocytes of Clarias batrachus. *Ecotoxicology and Environmental Safety* 62, 348–354.

Bard A.J. & Faulkner, L. R. (2001). *Electrochemical Methodes, Fundamentals and Applications*. Sec Edit. 2001.

Blair, A. & Zahm, S.H. (1991). Cancer among farmers. *Occupational Medicine*, 6, 335–354.

Georgellis, A.; Kolmodin-Hedman, B. & Kouretas, D. (1999). Can traditional epidemiology detect cancer risks caused by occupational exposure to pesticides*? Journal Experimental Clinical Cancer Research*, 18, 159–166.

Bolognesi, C.; Bonatti, S.; Degan, P.; Gallerani, E.; Peluso, M.; Rabboni, R.; Roggieri, P. & Abbondandolo, A. (1997). Genotoxic Activity of Glyphosate and Its Technical Formulation Roundup *Journal Agriculture Food Chemistry* 45, 1957-1962.

Bolognesi, C. & Morasso, G. (2000). Genotoxicity of pesticides: potential risk for consumers. *Trends in Food Science & Technology*, 11, 182-187.

Carpy, S.A.; Kobel, W. & Doe, J. (2001). Health risk of low-dose pesticides mixtures: a review of the 1985–1998 literature on combination toxicology and health risk assessment. *Journal Toxicology Environmental Health B: Critical Review,* 3, 1–25.

Cooper, J. & Dobson, H. (2007). The benefits of pesticides to mankind and the environment. *Crop Protection*, 26, 1337–1348.

Cueto Jr., C. (1980). Consideration of the possible carcinogenicity of some pesticides. *Journal Environmental Science Health* B, 15, 949–975.

Daniels, J.L.; Olshan, A.F. & Savitz, D.A. (1997). Pesticides and childhood cancers. *Environmental Health Perspective* 105, 1068–1077.

FAO, 1998. http://www.fao.org/ag/AGS/agse/activity.htm#PesticideApplication.

Garaj-Vrhovac, V. & Zeljezic, D. (2000). Evaluation of DNA damage in workers occupationally exposed to pesticides using single-cell gel electrophoresis (SCGE) assay Pesticide genotoxicity revealed by comet assay. *Mutation Research* 469, 279–285.

Gealy, R.; Wright-Bourque, J. L.; Kraynak, A.R.; McKelvey, T.W.; Barnum, J.E.; Storer R.D. (2007). Validation of a high-throughput *in vitro* alkaline elution/rat hepatocyte assay for DNA damage. *Mutation Research* 629, 49–63.

González, M.; Soloneski, S.; Reigosa, M.A. & Larramendy, M.L. (2003). Effect of dithiocarbamate pesticide zineb and its commercial formulation, azzurro IV. DNA damage and repair kinetics assessed by single cell gel electrophoresis (SCGE) assay on Chinese hamster ovary (CHO) cells. *Mutation Research* 534, 145–154.

Hodgson, E. & Levi, P.E. (1996). Pesticides: an important but underused model for the environmental health sciences. *Environmental Health Perspective*, 104, 97–106.

International Agency for Research on Cancer (IARC). (1991). IARC Monographs on the Evaluation of the Carcinogenic Risks to Humans. *Occupational Exposures in Insecticide Application, and Some Pesticides*, 53, 115-175.

Köhne, J. M.; Köhne, S. & Simůnek, J. (2009). A review of model applications for structured soils: b) Pesticide transport. *Journal of Contaminant Hydrology*, 104, 36–60.

Kohn, K.W. & Ewig, R.A. (1973). Alkaline elution analysis, a newapproach to the study of DNA single-strand interruptions in cells. *Cancer Research* 33, 1849–1853.

Leary, E.S. O.; Vena, J.E.; Freudenheim, J.L. & Brasure, J. (2004). Pesticide exposure and risk of breast cancer: a nested case-control study of residentially stable women living on Long Island. *Environmental Research*, 94, 134–144.

Nasterlack, M. (2006). Do pesticides cause childhood cancer? *International Archive Occupational Environmental Health* 79, 536–544.

Nasterlack, M. (2007). Pesticides and childhood cancer: An update. International Journal of Hygiene and Environmental Health 210, 645–657.

Nwani, C.D.; Lakra, W.S.; Nagpure, N.S.; Kumar, R.; Kushwaha, B. & Srivastava, S.K. (2010). Mutagenic and genotoxic effects of carbosulfan in freshwater fish Channa punctatus (Bloch) using micronucleus assay and alkaline single-cell gel electrophoresis. *Food and Chemical Toxicology* 48, 202–208.

[a] Olive, P.L.; Banath, J.P. & Durand, P.E. (1990). Detection of Etoposide Resistance by Measuring DNA Damage in Individual Chinese Hamster Cells. *Journal of the National Cancer Institute* 82, 779-783.

[b] Olive, P.L.; Banath, J.P.& Durand, R.E. (1990). Radiat. Res. 122, 86.

Olive, P.L. & Durand, R.E. (1992). Detection of Hypoxic cells in a Murine Tumour With the Use of the Comet Assay. *Journal National Cancer Institute* 84, 707-711.

Pandey, S.; Nagpure, N.S.; Kumar, R.; Sharma, S.; Srivastava, S.K. & Verma, M.S. (2006). Genotoxicity evaluation of acute doses of endosulfan to freshwater teleost Channa punctatus (Bloch) by alkaline single-cell gel electrophoresis. *Ecotoxicology and Environmental Safety* 65, 56–61.

Phillips, D.H. & Arlt, V. M. (2007). The ^{32}P-postlabeling assay for DNA adducts. *Nature Protocols* 2, 2772- 2781.

Pino, A.; Maura, A. & Grillo, P. (1988). DNA damage in stomach, kidney, liver and lung of rats treated with atrazine. *Mutation Research* 209, 145–147.

Poli, P.; de Mello, M.A.; Buschini, A.; de Castro, V.L.S.S.; Restivo, F.M.; Rossi, C. & Zucchi, T.M.A.D. (2003). Evaluation of the genotoxicity induced by the fungicide fenarimol in mammalian and plant cells by use of the single-cell gel electrophoresis assay. *Mutation Research* 540, 57–66.

Prakash, A.S.; Rao, K.S. & Dameron, C.T. (1998). Cadmium Inhibits BPDE Alkylation of DNA in the Major Groove but Not in the Minor Groove. *Biochemical and Biophysical Research Communications*, 244, 198–203.

Rahman, M.F.; Mahboob, M.; Danadevi, K.; Saleha Banu, B. & Grover, P. (2002). Assessment of genotoxic effects of chloropyriphos and acephate by the comet assay in mice leucocytes. *Mutation Research* 516, 139–147.

Reus, G.Z.; Valvassori, S.S.; Nurenberg, H.; Comim, C.M.; Stringari, R. B.; Padilha, P.T.; Leffa, D.D.; Tavares, P.; Dagostim, G.; Paula, M.M.S.; Anderade, V.M. & Queved. J. (2008). DNA Damage after Acute and Chronic Treatment with Malathion in Rats. *Journal Agriculture Food Chemistry* 56, 7560–7565.

Rull, R.P.; Gunier, R.; Von Behren, J.; Hertz, A.; Crouse, V.; Buffler, P.A. & Reynolds, P. (2009). Residential proximity to agricultural pesticide applications and childhood acute lympho blastic leukemia. *Environmental Research* 109, 891–899.

Sasaki, Y.F.; Izumiyama, F.; Nishidate, E.; Matsusaka, N.; Tsuda, S. (1997). *Mutation Research* 391, 201.

Shah, R.G.; Lagueux, J.; Kapur, S.; Levallois, P.; Ayotte, P.; Tremblay, M. & Poirier, J.Z.G.G. (1997). Determination of genotoxicity of the metabolites of the pesticides Guthion, Sencor, Lorox, Reglone, Daconil and Admire by ^{32}P-postlabeling. M*olecular and Cellular Biochemistry*, 169, 177–184.

Singh, N.P.; McCoy, M.T.; Tice, R.R. & Schneider, L.E. (1988). A simple technique for quantization of low levels of DNA damage in individual cells. *Experimental Cell Research*, 175, 184-191.

Tennant, A.H.; Peng, B. & Kligerman, A.D. (2001). Genotoxicity studies of three triazine herbicides: in vivo studies using the alkaline single cell gel (SCG) assay. *Mutation Research* 493 1–10.

Vigreux, C.; Poul, J.M.; Deslandes, E.; Lebailly, P.; Godard, T.; Sichel, F.; Henry-Amar, M. & Gauduchon, P. (1998). DNA damaging effects of pesticides measured by the single cell gel electrophoresis assay comet assay and the chromosomal aberration test, in CHOK1 cells. *Mutation Research* 419, 79–90.

WHO, 1986. Informal consultation on planning strategy for the prevention of pesticide poisoning. WHO, Geneva. WHO/VBC/86.926.

Whysner, J.; Montandon, F.; Mc Clain, R. M.; Downing, J.; L. K. Verna, R. E. Steward, G. M. Williams, Absence of DNA Adduct Formation by Phenobarbital, Polychlorinated Biphenyls, and Chlordane in Mouse Liver Using the ^{32}P-Postlabeling Assay. Toxicol. Applied Pharmacol. 148 (1998) 14–23.

Yanez, L.; Borja-Aburto, V.H.; Rojas, E.; dela Fuente, H.; Gonzalez-Amaro, R.; Gomez, H.; Jongitud, A.A. & Dı´ azBarriga, F. (2004). DDT induces DNA damage in blood cells. Studies in vitro and in women chronically exposed to this insecticide. *Environmental Research* 94, 18–24.

Yin, X.H.; Li, S.N.; Ying, L.S.; Zhu, G.N. & Zhuang, H.S. (2008). Genotoxicity evaluation of low doses of clodinafop-propargyl to the silkworm *Bombyx mori* using alkaline single-cell gel electrophoresis. *Environmental Toxicology and Pharmacology* 26, 162–166.

Zahm, S.H. & Blair, A. (1992). Pesticides and non-Hodgkin's lymphoma. *Cancer Research* 52, 5485s–5488s.

Zahm, S.H. & Ward, M.H. (1998). Pesticides and childhood cancer. *Environmental Health Perspective* 106, (Suppl. 3), 893–908.

19

The Contribution of Molecular Modelling to the Knowledge of Pesticides

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1. Introduction

Evolution of computer hardware has made available massive amounts of computing power in practically all fields of chemistry. Personal computers currently have computational speed and storage capacities that exceed old supercomputers and are much cheaper. Computational Chemistry and Molecular Modelling are areas of Theoretica1 Chemistry with emphasis on application-oriented molecular problems being solved for a wide range of systems. So modelling calculations and computational chemistry have naturally entered applied sciences as for example soil science.

Most molecular modelling studies involve three stages (Leach, 2001). In the first stage a model is selected to describe the intra- and inter-molecular interactions in the system. The two most common models that are used in molecular modelling are quantum mechanics (QM) and molecular mechanics (MM). These models enable the energy of any arrangement of the atoms and molecules in the system to be calculated, and allow the modeller to determine how the energy of the system varies as the positions of the atoms and molecules change. The second stage of a molecular modelling study is the calculation itself, such as an energy minimisation, a molecular dynamics or Monte Carlo simulation, or a conformational search. Finally, the calculation must be analysed, not only to calculate properties but also to check that it has been performed properly.

The fundamental equation determining the properties of atomic and molecular systems is the Schrödinger equation. Its analytic solution is only possible for a few simple model cases, but computer programs have been developed for the quantum chemical computation of atoms and molecules. The QM approximation for the calculation of molecular properties are: ab initio and DFT (density functional theory) methods, and semiempirical methods (for an introductory level, see Atkins & de Paula, 2006). The first two ones are computationally most expensive, but they usually yield the most reliable results. Ab initio calculations use the complete and correct hamiltonian and do not use other experimental data except fundamental physical constants. The density functional method does not intend to calculate the molecular wavefunction, but calculates the electron probability density rho (ρ) and calculates the molecular electronic energy starting from rho. Semiempirical methods are computationally less expensive, they use a simpler hamiltonian in the Shrödinger equation but require parameter sets for successful application.

Force-field and interatomic potential methods are the cheapest ones. They are not quantum mechanics methods and do not use the hamiltonian operator nor a molecular wavefunction. They rely on the empirical adjustment of parameters. Molecules are usually visualized as an assembly of atoms connected by bonds, and molecular energy is expressed as a sum of terms which correspond to bond, angle, torsion, van der Waals and electrostatic interaction energies. The mathematical form of the energy terms varies from force field to force field. The potential function of the system depends on the relative positions of the atoms with respect to each other and it returns energy as a function of conformation.

Considering a single or a few molecules alone, physically corresponds to the gas phase or vacuum. Many chemical processes, however, occur in solution, on surfaces, in solid state or involve macromolecules which have many closely separated conformational energy minima and contain large numbers of atoms or molecules. Computer simulation methods enable the study and prediction of properties of such dynamic systems through the use of techniques that consider small replications of the macroscopic system with manageable numbers of atoms or molecules. The two most common simulation techniques used in molecular modelling are the molecular dynamics (MD) and the Monte Carlo (MC) methods. MD is a computer simulation technique where the time evolution of a set of interacting particles (atoms, molecules) is predicted by integrating their equations of motion, following the laws of classical mechanics (Jensen, 1999; Leach, 2001). Velocities are governed by the forces that the atoms of the system exert on each other. The force on an atom can be calculated from the change in energy between its current position and its position a small distance away. Knowledge of the atomic forces and masses can then be used to solve the equations to give the positions of each atom along a series of extremely small time steps of the order of femtoseconds (10^{-15} seconds). The resulting series of snapshots of structural changes over time is called a trajectory. Though deterministic, MD is a statistical mechanics method: it is a means of obtaining a set of configurations distributed according to some statistical distribution function, or statistical ensemble. A trajectory obtained by MD provides such a set of configurations and the value of a physical quantity is obtained as an arithmetic average of its instantaneous values assumed during the MD run.

MC method (Jensen, 1999; Leach, 2001) starts from a given geometry and a new configuration is generated by making random changes to the positions of one or more atoms. The new geometry is accepted as starting point for the next perturbing step if it is lower in energy than the current. If it is higher, it is evaluated whether it can exist in equilibrium with configurations of lower potential energy at a given temperature. This is done by calculating the ratio of populations of the two states involved (with energy separation ΔE), i.e. $\exp(-\Delta E / kT)$ where k is the Boltzmann constant. The value is compared to a random number between 0 and 1. If the factor is larger than this number, then the configuration is accepted; if the factor is not larger, the configuration is rejected and the next step is taken from the old geometry. A sequence of configurations is generated from which, for example, geometries may be selected for subsequent minimization.

MC calculations are somewhat similar to the MD calculations. But while MD simulations use an equation of motion as the basis for generating new configurations, MC simulations employ a statistical sampling technique to generate configurations that represent a trajectory. MD calculate ensemble averages by calculating averages over time, whereas MC calculations evaluate ensemble averages by sampling configurations from the statistical ensemble. In principle and in a long run, both should lead to the same average results for the same system.

This chapter is a review of selected theoretical reports that appeared during the last twelve years referred to molecular modelling applied to the field of pesticides and to some biological systems involved in their (bio)chemistry.

2. Nitrocompounds

Compounds containing one or more nitro groups are commonly used as explosives (Spain 1995), organic solvents (Scholz et al., 2005), herbicides and pesticides (Harrison et al., 2005), and drugs (Ahlner et al., 1991; Balbi, 2004). As a result, they appear as contaminants in water sources, such as surface water and industrial waste waters.

Natural adsorbents and catalysts which are based on the alumino–silica minerals and other types of soils occupy one of the most important places in the development of clean-up technologies. Clays are layer-type aluminosilicate minerals based on a two-dimensional stack of layers. These are made of either tetrahedral sheets of SiO2 motifs or octahedral sheets of metal oxide and hydroxide (where the metal can be Al, Fe, Mg) or both. Cohesion between the layers is maintained by weak electrostatic and van der Waals interactions mediated by interlayer cations and water molecules. Boulet et al. (Boulet et al., 2006) have contributed a review of the electronic structure computer simulation studies of clays, focusing on important case studies, where it was shown how ab initio calculations can help in the interpretation of experimental observation and the relationships between the structure and properties of clays. Here we offer two examples of how careful calculations and analyses of model clays interacting with nitrocompounds have been carried out.

Haderlein et al. (Haderlein, 1993; 1996; Weissmahr et al., 1997) proposed that the adsorption of nitroaromatic compounds (NACs) on clays under natural conditions was due to the NAC interaction with the siloxane sites of clays. The interaction was postulated to be electron donor-acceptor in nature: NACs (electron acceptors) interact via the electron deficient π-system with the oxygen atoms of the siloxane surface (electron donors). To further investigate this interaction, Pelmenschikov & Leszczynski (1999) performed analyses of the adsorption of 1,3,5-trinitrobenzene (TNB) with the basal siloxane surface of clay minerals by computations of molecular models at several ab initio and DFT levels of theory. Calculations were performed with the GAUSSIAN-94 package (Frisch et al., 1995), geometries were optimized and binding energies were computed. For the DFT calculations, the three parameter B3LYP functional (Becke, 1988; Lee et al., 1988) was used. The geometry of TNB and its position with respect to the surface was fully optimized within adopted symmetry constraints. The silicon-oxygen clusters mimicking the siloxane sites were constructed of SiO_4 tetrahedra with the Si-O bond length being equal to 1.61 Å, the average value for clay minerals (Bish, 1993). The border oxygen atoms of the silicon-oxygen clusters were saturated with H atoms located on the corresponding "broken" O-Si bonds, at 0.95 Å. The geometry of the surface clusters was not optimized, so the effect of the interaction on the surface geometry was neglected**.** Results suggested that the complexation of NACs with the siloxane sites of clays is mainly governed by dispersion interaction, accounted for by the planar structure of both the NACs and the siloxane surface. Due to the pair-additive character of the attractive dispersion interaction between the atoms of two interacting partners, the superimposition of plane NACs on the siloxane layer resulted in a strong stabilization effect. The averaged binding energy (38 kJ/mol) was in good agreement with the experimental data (ΔHads≈-40 kJ/mol). The energetically optimal arrangement of TNB with respect to the surface would be governed by the balance between favourable dispersion and electrostatic

forces, and repulsive exchange forces. Considering that dispersion interaction is short-range in nature, the nonplanarity of NACs with branched alkyl substituents would prevent the maximum closeness of the molecules to the surface, causing the binding to be weaker in such cases. According to the authors, the predominant role of the short-range dispersion interaction in the adsorption, justified the use of small molecular models of the siloxane surface in their study.

Leszczynski et al. (Gorb et al., 2006) introduced later a more realistic model of a clay surface, considering three ditrigonal cavities of clay montmorilonite and they calculated the components of the interaction energy of TNB with such surface. They modelled, at a quantum-chemical level, the interaction of TNB with the siloxane surface by applying the ONIOM methodology (Svensson et al., 1996). This is an n-layered integrated quantum mechanical and molecular mechanics (QM/MM) method designed to facilitate accurate ab initio calculations of large chemical species. QM/MM methods treat by different levels of theory different parts of a structure or system: usually, an important one is calculated by electronic structure methods (semiempirical, ab initio or DFT), while the less important part is calculated by a force field method. In ONIOM a typical calculation is constructed from a series of layers: e.g., an inner core may be treated with the density functional approach, the intermediate layer with a low level ab initio theory and the outer layer with a force field. So Gorb et al. represented the target cluster with the chemical formula $Al_{22}Si_{13}O_{81}H_{44}$,which was subdivided into three levels of theory, though all of them QM levels. For comparison, the interaction of TNB with a pure silicon–oxygen cluster with formula $Si_{13}O_{37}H_{22}$ was also considered, with calculations performed at the ab initio and DFT levels of theory using the GAUSSIAN 98 package (Frisch et al., 1998). The interaction energy of TNB was investigated by the authors using an energy decomposition scheme proposed by Sokalski et al. (1988): the interaction energy is partitioned into several terms, i.e. the first-order electrostatic ε'_{el} , first order exchange ε'_{ex} and high order deformation ε_{def} . The latter accounts for the charge transfer and polarization interactions. To take into account the correlation energy, an additional ε_{corr} term was also considered by the authors, aiming at the inclusion of all intermolecular and higher order correlation energy. As for the latter, ab initio calculations are based in first approximation on the one-electron model: each electron in a molecule moves in the *average* field created by the other electrons. Actually, electrons interact instantaneously and tend to avoid each other. This correlation results in a lower average interelectronic repulsion and thus a lower state energy. The difference between electronic energies calculated at lower (self consistent field, SCF) level and the exact nonrelativistic energies is the *correlation energy*. Gorb's et al. results suggest that the attractive term of the adsorption energy consists of two contributions. One is the electrostatic interaction, of specific character and responsible for the orientation of the adsorbed NAC. The other contribution, without a specific character, originates from dispersion energy. Only approximately half of the adsorption energy contributes to the specific interaction with the siloxane sites of clay minerals, which would explain the high mobility of adsorbed TNB as revealed by NMR studies (Weissmahr et al., 1997). The interaction energy decomposition was implemented in the GAMESS program (Schmidt et al., 1993). The calculations of electrostatic potential were performed and visualized by the MOLDEN program (Schaftenaar & Noordik, 2000).

Another study of inetractions with a solid surface was peformed by Wahab and Koutselos (2009), involving nitrobenzene (NB) as a model pollutant. The authors modelled the adsorption and primary oxidation step for the photodegradation of NB by using the

semiempirical MSINDO SCF MO method. This method has been documented for the first-, second-and third-row main group elements and first-row transition metal elements (Ahlswede & Jug, 1999a, b; Bredow et al., 2001). Molecular dynamics simulations of the cluster-substrate (TiO_2- $C_6H_5NO_2$) models were performed for 2000, 4000 and 6000 femtoseconds at 300 K. The adsorption geometries of NB onto TiO_2 surface were simulated focusing on the parallel and perpendicular conformations. The computed energies of both adsorption modes indicated that the adsorption process is exothermic. The authors found that the adsorption energy is influenced by the substrate geometry and that the perpendicular conformation is favoured through a preferential interaction between the oxygen atoms of NO_2 group and the surface of TiO_2. The quantum chemical calculations of the heterogeneous oxidation of NB required an extended saturated cluster for the modelling of the anatase TiO_2 (100) surface such as $Ti_{36}O_{90}H_{36}$. The authors also analysed the primary steps of the oxidation of NB by OH radical, in the homogeneous gas phase (photochemical) and on the anatase TiO_2 surface (photocatalytic). In order to identify the primary OH initiated photooxidation intermediates, they employed two different theoretical approaches which revealed that the *meta*-hydroxynitrocyclohexadienyl radical is energetically more favoured than the analogous *para*- and *ortho*- radicals for the photochemical photolysis, following the expected selectivity rules. As for photocatalysis, the OH radical attack appeared to be non-selective and all three possible isomers showed comparable stabilities.

Although nitro compounds are of general and biological significance, only a limited number of studies have focused on developing force field (FF) parameters for use in molecular simulations (Michael & Benjamin, 1998; Janssen et al., 1999; Price et al., 2001; 2005; Jorge et al., 2006). Price et al. (2001) developed nitro-parameters for the OPLS-AA FF (Jorgensen et al, 1988; 1996) that resulted in good agreement with experimental gas-phase and liquid properties, e.g., density, heats of vaporization, and free energies of salvation, from molecular simulations. A comparison of nitrobenzene FFs and experiment was discussed by Jorge et al. (2006), who found that OPLS-AA compares most favourably with observations.

For the CHARMM FF (Brooks et al., 1983), Klauda & Brooks (2008) developed nitro-parameters consistent with CHARMM optimization procedures (MacKerell, 2001; 2004; 2005). Their new parameter set, referred to as C27rn, was then tested on pure liquid and interfacial systems of nitroalkanes and nitrobenzene. Special focus in the FF development was on potential energy scans of two nitro torsional angles, i.e., C-C-N-O and C-C-C-N. Therefore, highly accurate ab initio methods were used to describe the conformational energies of nitroalkanes and nitrobenzene. The Gaussian03/Rev. B.03 suite of programs (Frisch et al., 2004) was used for the QM calculations and the FF was adjusted accordingly to best match them. MD simulations were performed with CHARMM using C27r for the alkane portion of the FF (Klauda et al., 2005a, b) and adjustments to the nitro FF, i.e. C27rn. Simulations yielded an increased population (74%) of *gauche* conformers compared to OPLS-AA, as the calculated *gauche* conformer of the C-C-C-N torsion resulted more stable than the *trans* one. Bulk and interfacial properties from the nitro simulations with C27rn, such as densities, heats of vaporization, and surface tensions, were in agreement with experiment. However, the calculated diffusion constant of liquid nitrobenzene with both OPLS-AA and C27rn was lower than in experiment. Since these new parameters accurately represent interaction energies between water and nitro compounds and pure component properties, C27rn was proposed to be used in simulations of biologically relevant compounds.

3. Organophosphorous compounds

There is an increasing interest in developing strategies for the detection and detoxification of organophosphorous compounds (phosphorus-containing organic chemicals). They are often used as pesticides and warfare agents. Because of their high toxicity, there is a serious demand for more-sophisticated and useful techniques for the detection and decomposition of these substances, as well as for information about their (thermo)chemical properties or behaviour.

In order to evaluate the ability of beta-cyclodextrin (β-CD) to discriminate between different enantiomers of pesticides, Manunza et al. (1998) carried out molecular dynamics (MD) experiments to investigate the mechanism of selective binding of β-CD with the R or S enantiomers of dichlorprop, 2- phenoxypropionic acid and dioxabenzofos. The dichlorprop and the 2-phenoxypropionic acid molecules are similar as both have a phenyl ring with a substituent bearing a carboxylic function. Molecular structures were constructed and six inclusion 1:1 complexes were formed, one for each enantiomer, after a docking process into the cavity was performed. These adducts were the starting structures in the MD experiments. The MD runs were performed employing the DLPOLY2 (Smith & Forester, 1994) program. The AMBER (Weiner et al., 1984; Cornell et al, 1995) plus GLYCAM (Woods et al., 1995) force field was used with the necessary adaptations, while the β-CD partial atomic charges were calculated by the Gasteiger method (Berendsen et al., 1981). The starting β-CD structure for the simulation was taken from the "BCDEX04" entry of the Cambridge Crystallographic Database. All the MD simulations were performed in the NVT ensemble (constant number of particles, volume and temperature) inside a 35 Å cubic cell at a temperature of 298 K. The system was allowed to equilibrate for 200ps and the trajectory was collected over 1000ps. Results account for the formation of adducts with the dichlorprop and the 2-phenoxypropionic acid molecules which are stable at room temperature, while neither of the dioxabenzofos enantiomers entered the β-CD cavity completely. Computational results agreed with the experimental evidence that β-CD forms stable complexes with both dichlorprop and 2-phenoxypropionic acid after 7 h at 70°C and after 24h at room temperature, whereas no experimental evidence was observed for dioxabenzofos complexation. The energetic data indicated that the β-CD molecule shows preference for S enantiomers. The plots of the radial distribution functions showed that the R enantiomers form hydrogen bonds mainly with the oxygen atoms of the secondary hydroxyl groups in the β-CD, while the S enantiomers formed H-bonds even with the oxygens in the primary hydroxyl groups of the cycle. Energy data revealed that complexes with the R enantiomers of dichlorprop and 2- phenoxypropionic acid exhibit a more advantageous H-bond network, and that adducts with the S enantiomers form by the release of strain energy mainly by the β-CD molecule.

The application of cyclodextrins to pesticide formulation was, until recent years, rather modest (Szejtli, 2004), but the situation has significantly changed since the cost of technical quality β-CD, entirely acceptable for the pesticide formulation industry, has declined. So potential use of β-CD could be of great interest to enhance biological activity of these chemicals (Nair et al., 2006). The alkaline hydrolysis of three organophosphorous pesticides, i.e. fenitrothion, parathion and methylparathion, appears to be inhibited by β-CD (Vico et al., 2002). This effect, ascribed to a shallower inclusion of the guest in the cavity, is lower in the case of fenitrothion (Kamiya et al., 1995). Induced circular dichroism analysis revealed that the inclusion of the pesticides occurs by the nitro group and the same happens with the

analogous carboxylic esters, whose hydrolysis is, contrarily, catalyzed by β-CD (Osa & Suzuki, 1996). Coscarello et al. (2009) analyzed the complexation process of both pesticides and their carboxylic ester analogues by β-CD. The authors applied molecular mechanics and the PM3 (Stewart, 1989a; b) semiempirical methods contained in the Hyperchem-7 (2002) program. The complexation of fenitrothion was further explored, since experiments proved that its hydrolysis is relatively less inhibited and progresses mainly through a different pathway. Results showed that complex structures involving the carboxylic esters enable effective interactions between the guest carbonyl and the rim of the host. Methylparathion and parathion, however, appeared deeply included in the cavity of β-CD, so conditions for a nucleophilic attack by the β-CD would not be favourable. As for fenitrothion, different complex geometries were yielded, none being apparently prone to an attack by the β-CD, but favouring instead the approach of an external OH^- group, according with experiment.

Dichlorvos (2,2-dichlorovinyl phosphate, DDVP) is a widely used organophosphorus insecticide. DDVP may be released into the atmosphere and can also be released into the environment as a major degradation product of other organophosphate pesticides, such as trichlorfon (Murphy et al., 1996)) and metrifonate (Hofer, 1981; Pettigrew et al., 1998). The reaction of DDVP with atmospheric OH radicals is considered to be a dominant removal process for gaseous DDVP. Knowledge of the OH-initiated DDVP oxidation mechanism and the major degradation products is limited. Feigenbrugel et al. (2006) detected phosgene (Cl_2CO) and carbon monoxide (CO) and proposed a possible reaction mechanism to explain the observed products, in which CO formation is initiated by H abstraction from the CH_3O group of DDVP, and then the product radical may further react with O_2/NO leading to HCO. The observed CO would be formed through the reaction of HCO with O_2. The formation of Cl_2CO would be initiated via OH addition to the carbon-carbon double bond. Zhang et. al (2007) carried out a theoretical study on the OH-initiated atmospheric photooxidation reaction of DDVP. High-level ab initio molecular orbital calculations were carried out for the OH-initiated atmospheric photooxidation of DDVP in the presence of O_2 and NO, and using the Gaussian 03 package (Frisch et al., 2004). The geometrical parameters of reactants, transition states, intermediates, and products were optimized at the TPSSh density functional level (Staroverov et al., 2003; Tao et al., 2003). The vibrational frequencies were also calculated to determine the nature of the stationary points, the zero-point energy (ZPE), and the thermal contributions to the free energy of activation. Each transition state was verified to connect the designated reactants with products by an intrinsic reaction coordinate (IRC) analysis (Fukui, 1981). The energies of various species were determined more accurately, for a more accurate evaluation of the energetic parameters. The profile of the potential energy surface was constructed and possible secondary reaction pathways were also studied to find the mechanism of formation of secondary pollutants from the OH-initiated atmospheric reaction of DDVP. Because of the absence of experimental information on the thermochemical parameters for the reaction system, the authors calculated the standard formation enthalpies, $\Delta_f H^0(298)$, of CCl_2O and PCl_3 . The calculated values were in agreement with the available experimental ones (Curtiss et al., 1997; David, 2007). On the basis of the product yields of CCl_2O and CO, Feigenbrugel et al. had suggested that H abstraction from the CH_3O group accounts for 43% of the overall reaction and that OH addition to the >C=C< bond accounts for 47% of the overall reaction. Zhang et al., however, found that four product pathways are energetically feasible for the degradation of DDVP initiated by atmospheric OH radicals. In addition to CCl_2O and CO, HO_2 and a closed-shell organophosphorous compound denoted P10 would be also easily produced from the

pathway initiated by H abstraction from the CH_3O group, and CCl_2CHO and $(CH_3O)_2P(O)OH$ are also energetically feasible products from the pathway of OH addition to the >C=C< bond. Thus, the authors inferred that calculation of the branching ratios of H abstraction from the CH_3O group and OH addition to the >C=C< bond only from the productivities of CCl_2O and CO, would not be reasonable.

Inorganic metal oxides are well-known for their use in chemical industry as adsorbents, sensors, catalyst, etc. Because of their unique morphological features and high surface area, nanocrystals of metal oxides were used as adsorbents for decomposition or detection of variety of pollutants and harmful substances, including organophosphorous compounds (Richards et al., 2000). Recently, zinc oxide (ZnO) nanoparticles have received much attention, because of applications such as ultraviolet absorption, decomposition, deodorization, and antibacterial treatment. It is a well-known catalyst, adsorbent, toxic gas sensor, etc. (Yun et al., 2005). Among the unique chemical properties of ZnO is that it lies on the borderline between ionic and covalent solids (Duke et al., 1977). Leszczynski et al. (Paukku et al., 2009) studied the adsorption of Tabun (TB) -a nerve agent- and of dimethyl methylphosphonate (DMMP) -an organophosphorous simulant- on polar and nonpolar ZnO surfaces, using the cluster model approach. Different cluster sizes and adsorption sites of ZnO were considered, and the influence of different computational methods on the nature of interactions of these molecules with the crystal surface of ZnO was investigated. Two types of surfaces, polar and nonpolar, were considered for ZnO, the latter being the most stable one for the solid. These ZnO surfaces have been studied experimentally (Duke et al., 1977; 1978). Calculations of adsorption of DMMP and TB on the model surfaces of ZnO were conducted at several ab initio levels of theory, and using the ONIOM method as implemented in the Gaussian 03/Rev.C02 program package (Frisch et al., 2004), in order to test the influence of the method on the intermolecular interactions. The geometries of the target molecules were fully optimized while the ZnO fragment was kept frozen. DMMP contains three different groups that can be involved in the intermolecular interactions with the active sites of the ZnO surface: P=O, O-CH3, and CH3. Therefore, several different initial orientations of DMMP toward the oxide cluster were tested. To test the effect of the surrounding Zn and O atoms and additional layers on the adsorption of the target molecule, the authors performed the ONIOM calculations: the molecular system was divided into two layers, which were treated at different levels of theory, DFT and PM3 methods. From the results, the authors concluded that on both ZnO surfaces, the molecular adsorption proceeds as chemisorption via the formation of a Zn····O chemical bond in the case of the DMMP adsorption complex, and a P····O covalent bond or a Zn····N chemical bond for TB adsorption complexes. The type of surface greatly affected the strength of the intermolecular interactions and the interaction energies. The results indicated that the adsorption of DMMP and TB is energetically more favourable on the nonpolar ZnO surface. TB was determined to be bound more tightly to the ZnO surface than DMMP, but the adsorption energies were approximately twice as low as the values revealed for the adsorption of TB and DMMP on the CaO surface, as reported previously by the same authors (Michalkova et al., 2007; Paukku et al. 2008). Therefore, conclusion was that the decomposition of these compounds would proceed easier on CaO, whereas ZnO should be an efficient sensor for their detection.

The experimental enthalpies of formation ($\Delta_f H°(298)$) for many important organophosphorous compounds are often unknown or known with relatively large uncertainties, due to incomplete combustion and formation of a mixture of the various phosphorous oxyacids that make difficult the precise definition of the final system (Pilcher,

1990). Because of the lack of accurate thermochemical data, alternative approaches, such as quantum chemical calculations should be used to predict the $\Delta_f H°(298)$ values for organophosphorous compounds. To predict accurately thermochemical properties for larger molecules at relatively low computational cost, the composite Gaussian-*n* [G2, G3, G3(MP2), G3X, G3X(MP2), etc.] methods were developed (Curtiss et al., 1998, 2000a, 2000b, 2001) (Baboul et al., 1999). In these methods, calculations from different levels of theory are combined in order to produce energy differences accurate to about 1 kcal/mol, as compared to experimental results. They have been calibrated on a reference set of atomic and molecular properties (atomization energies, ionization potentials, electron and proton affinities, etc.). The general principle is to perform a calculation at a high level of theory and then correct this value for deficiencies using less expensive, lower level theories.

The modification of the G3 theory, in particular, called Gaussian-3X (G3X), was designed to improve the agreement between theoretical and experimental $\Delta_f H°(298)$ for molecules, which contain second row atoms (Na-Ar). This method gives a good agreement with experiment for inorganic phosphorus compounds. Dorofeeva & Moiseeva (2006) calculated the $\Delta_f H°(298)$ of organic compounds containing phosphorus. Their purpose was: a) to assess generally accepted experimental data on organophosphorus(III) compounds using the G3X method; b) to recommend a set of consistent $\Delta_f H°(298)$ values based on best quality experimental values and G3X results; c) to calculate accurate $\Delta_f H°(298)$ values for organophosphorus(III) compounds with missing experimental thermochemical data; d) to derive a consistent and accurate set of group activitiy values (GAVs) needed to estimate the $\Delta_f H°(298)$ of larger organophosphorus(III) molecules, account taken that the empirical group additivity method of Benson (Benson et al., 1969) (Cohen & Benson, 1993) can predict the thermochemical properties of organic compounds with chemical accuracy, i.e. within 4 kJ/mol. So the $\Delta_f H°(298)$ of 55 organophosphorus(III) compounds were calculated at the G3X, G3X(MP2), and DFT levels of theory using the atomization energy procedure and the method of isodesmic reactions. This method is based on the principle that, for a given reaction, a particular computational approach can be expected to have similar deficiencies for both reactants and products that are chemically similar; thus, deficiencies are expected to cancel appreciably when the enthalpy of a reaction is calculated. Dorofeeva & Moiseeva calculated as well, the $\Delta_f H°(298)$ values for 50 moderate sized molecules with 2-10 non-hydrogen atoms directly from the G3X atomization energies. Examples of such group are $P(CH_3)_3$, $P(C_2H_5)_3$, $P(OCH_3)_3$, n-$C_4H_9OPCl_2$, $[(CH_3)_2N]_2PCl$, $(C_2H_5)_2$-$NPCl_2$, and $[(CH_3)_2N]_2PCN$. By comparison of the available experimental data with the G3X results, it was found that the G3X method succeeded in reproducing well established $\Delta_f H°(298)$ to an accuracy of ±10 kJ/ mol. A good agreement between the known experimental values and G3X results for 14 compounds provided support to the predictions for remaining species with unknown experimental $\Delta_f H°(298)$ or known with large uncertainties. The $\Delta_f H°(298)$ values obtained in this work provided a consistent set of reliable estimates for the thermodynamic modelling of processes involving phosphorus(III) containing species. The recommended $\Delta_f H°(298)$ values were used to derive the GAVs for 45 groups involving the phosphorus(III) atom and thus extending the applicability of Benson's group additivity method to estimate the $\Delta_f H°(298)$ of larger organophosphorus(III) compounds (for which high level quantum chemical calculations could appear impracticable).

Hemelsoet et al. (2010) performed a comprehensive ab initio study on phosphorus-containing species with three primary aims: a) to assess a broad variety of current computational methods to determine an appropriate level of theory for the calculation of

reliable bond dissociation properties of phosphorous compounds; b) to provide thermochemical data such as the enthalpy of formation, the heat capacity and the entropy for a set of phosphorus-containing species representing industrially important coke-inhibiting additives; c) to compute bond dissociation enthalpies (BDEs) of these compounds to establish the stability of the formed radicals and their reactivity trends. Standard ab initio molecular orbital theory and density functional theory calculations were carried out using the Gaussian03/Rev. D.01 (Frisch et al., 2004), Molpro 2002.6 (Werner et al., 2002), and NWChem5 (Bylaska et al., 2007) software packages. Geometries were optimized at the DFT level of theory. Harmonic vibrational frequencies were computed at the same level of theory and were used to provide zero-point vibrational energies and to confirm the nature of the stationary points. Subsequent single-point energy calculations were performed using a variety of levels of theories and several methods were tested for their performance. It was shown that the composite G3(MP2)-RAD (Hodgson & Coote, 2005) method generates heats of formation of small phosphorus-containing molecules that are in good agreement with experimental data. Hodgson & Coote investigated the relative stabilities of phosphoranyl radicals •P(CH3)3X and introduced a new measure of stability, i.e., the α-radical stabilization energy (α-RSE). As opposed to the standard RSE definition, the α-RSE measures the stability of the radical with respect to P(CH3)2X instead to H-P(CH3)X. This means that it assesses the stability of the radical on the basis of its susceptibility to α-scission of the methyl radical rather than to hydrogen abstraction. The study provided a large set of high-level calculated data that were taken as benchmark values by Hemelsoet et al.. The bond dissociation energies D(•P-C) and D(•P-X) of the phosphoranyl radicals •P(CH3)3X were calculated. The benchmark values of the D(•P-C) group could accurately be reproduced using various low-cost DFT methods, whereas this was much more difficult for the D(•P-X) values. In the case of D(•P-C), the ROB2PLYP (Grimme, 2006) and ROMPW2PLYP (Schwabe & Grimme, 2006) methods performed the best. The D(•P-X) values, on the other hand, were overall best reproduced using the SOS- (Jung, 2004) and SCS-ROMP2 (Grimme, 2003) methods. The RO prefix designs calculations on radicals that were performed with a restricted-open-shell reference wave function, as opposed to the unrestricted-open-shell computations. Bond dissociation enthalpies were calculated using the BMK (Boese & Martin, 2004), M05-2X (Coote & Henry, 2005) , and SCS-ROMP2 level of theory. The three methods give the same stability trend. No correlations between BDEs and geometrical parameters or atomic (spin) charges were obtained. The BDEs of the phosphorus(III) molecules were found to be lower than their phosphorus(V) counterparts. Overall, the authors report the following found ordering: BDE(P-OPh) < BDE(P-CH3) < BDE(P-Ph) < BDE(P-OCH3). Additionally, standard enthalpies of formation, entropies and heat capacities of a set of ten organophosphorous species, representing coke-inhibiting additives, were computed using the low-cost BMK functional. Results were consistent with available experimental data and could be used as input in single-event kinetic models.

4. Enzymatic activity

Among the chemical classes that have been developed as pesticides, organophosphorous compounds (together with carbamates), represent the most significant share of the world pesticide market (Casida & Quistad, 1998). Both classes owe their acute toxicity to the inhibition of acetylcholinesterase (AChE), enzyme that regulates the concentration of the neurotransmitter acetylcholine (ACh). The principal role of AChE is in the nervous system,

where it serves to terminate impulse transmission at cholinergic synapses by hydrolysis of the neurotransmitter acetylcholine at nearly diffusion-limited rates (Silman & Sussman, 2005). The enzyme phosphotriesterase (PTE) has the ability to hydrolyze a wide range of organophosphate triester compounds including pesticides and insecticides as well as chemical warfare agents, and it has a great potential for use in bioremediation of environmental contaminants.

Koča et al. (2001) examined theoretically the geometry and mobility of various PTE active site-substrate complexes, with paraoxon and sarin, as the chosen substrates. Observations indicated that the positioning of the substrate in the active site of the enzyme, as well as the flexibility of the active site, play important roles in the enzymatic process. As the enzymatic reaction is very fast, obtaining direct experimental data about the enzyme/substrate complex is difficult , so the authors recurred to molecular dynamics (MD) simulations on solvated PTE-substrate complexes, as well as quantum mechanical calculations on a simplified model of the active site complexed with the substrates, to address questions about the protein behaviour before and when interacting with the substrate. MD simulations were carried out on subunit of the PTE dimer, using AMBER 5.0 (Case et al., 1997) with the force field reported by Cornell et al (1995). In the current work, seven 500-picoseconds simulations were produced, five on the fully solvated protein and two on the gas-phase substrates, paraoxon and sarin. Partial atomic charges for paraoxon, sarin, and the carbamylated Lys-169 of the active site were determined by using the restrained electrostatic potential (RESP) procedure (Cornell et al., 1995). Quantum chemical calculations were carried out by using the Gaussian94 (Frisch et al., 1995) and Gaussian98 (Frisch et al., 1998) programs. Geometries of the PTE-substrate complex models were fully optimized by employing DFT theory, B3LYP functional (Becke, 1988; Lee et al., 1988). The corresponding vibrational frequencies were evaluated at the optimized geometries to verify their true stability. The results obtained from the molecular dynamics simulations showed that multiple orientations of paraoxon and sarin in the active site of zinc-substituted PTE are possible. The phosphoryl oxygen becomes strongly coordinated to the less buried zinc cation –there are two present in the active site-, which allows for strong polarization of the reaction center of the substrate. These results indicate that the enzymatic hydrolysis occurs as a multistep process, in which formation of the substrate-protein complex is the first step. There are conformational changes that occur throughout the active site region of PTE when the enzyme is immersed in the water bath and relaxed by MD. The most remarkable change is the opening of the gateway in a pocket where the location of the leaving group is expected. The enzyme's gateway opens with and without substrate being present in the active site. The size of the opening is dependent on the substrate and may range from 11 to 18 Å. Different conformational behaviors are observed for the same substrate substituents within different pockets, for different simulations, as well as in the gas phase. This shows that the active site pockets generally contribute to the substrate binding. Detailed analysis of all trajectories revealed that this contribution in not based on hydrogen bonding. The pockets, in which the substrate substituents are localized, thus exhibit different flexibility and interact with the substrate with coordinated conformational adjustments.

Wong & Gao (2007) also undertook the PTE hydrolysis of paraoxon to determine the potential of mean force (PMF), by using a dual-level QM/MM approach. The intrinsic (gas-phase) energies of the active site in the QM region were determined by using density functional theory (B3LYP) and second-order Møller-Plesset perturbation theory (MP2)

(Hehre et al.,1986); the molecular dynamics free energy simulations were performed by using semiempirical QM/MM interactions, i.e. the mixed AM1:CHARMM potential (Dewar et al., 1985; 1988; 1989) (MacKerell, 2001; 2004; 2005). As already mentioned, a key feature of the active site of PTE is the binuclear zinc center, in which each zinc ion is coordinated to five ligands in a distorted trigonal bipyramidal structure. The simulation results suggested that the reaction free energy profile is mirrored by structural motions of the binuclear metal center in the active site. A carbamylate group from Lys169 and the nucleophile hydroxide ion both form bridged coordinations to the two zinc ions in a compact conformation with an average zinc-zinc distance of 3.5 ± 0.1 Å. The P─O bond of the substrate paraoxon is activated by adopting a tight coordination to the more exposed Zn_{β}^{2+} ion, and releases the coordinate to the hydroxide ion, increasing its nucleophilicity. The result is a loose binuclear conformation, characterized by an average zinc-zinc distance of 5.3± 0.3 Å at the transition state and the product state. It was also found that a water molecule enters into the binding pocket of the loosely bound binuclear center, originally occupied by the nucleophilic hydroxide ion. It was suggested that the proton of this water molecule is taken up by residue His254 of the PTE at low pH or released to the solvent at high pH, resulting in a hydroxide ion that pulls the Zn_{β}^{2+} ion closer to form the compact configuration and restores the resting state of the enzyme.

Taking into account that the non-harmful transformation of hazardous chemicals, such as organophosphorous compounds, could also be attained by enzymatic biodegradation (Raushel, 2002), Leszczynski et al. (Dyguda-Kazimierowicz et al., 2008) focused on the comprehensive ab initio study of possible gas phase mechanisms of the alkaline hydrolysis of bacterial PTE substrates, regarding bonds such as:

a. P─O, phosphorus-oxygen (e.g., *O*,*O*-diethyl *p*-nitrophenyl phosphate (paraoxon), *O*,*O*-diethyl *p*-nitrophenyl thiophosphate (parathion)),
b. P─F, phosphorus-fluorine (e.g., *O*,*O*-diisopropyl phosphorofluoridate (DFP), *O*-isopropyl methyl phosphonofluoridate (sarin, SA)),
c. P─S, phosphorus-sulfur (e.g., *O*,*S*-dimethyl *N*-acetyl phosphoramidothioate (acephate), *O*,*O*-diethyl *S*-2-ethylthioethyl phosphorothioate (demeton-S)), and
d. P─CN, phosphorus-cyanide (e.g., *O*-ethyl *N*,*N*-dimethyl phosphoramidocyanidate (tabun, TB))

Given the chemical diversity of PTE substrates, the authors deemed it essential to elucidate common features, if any, in the hydrolysis process that allow all of these substrates to be accommodated in the PTE active site and subsequently be subjected to catalysis. Hydrolysis of a P─O bond can be interpreted as an S_N2-like concerted associative mechanism, which along with the apparent involvement of a hydroxide as a nucleophile, was used to rationalize the authors' approach to utilize gas phase results for alkaline hydrolysis as a reasonable starting point for the study of the reaction occurring in the PTE active site. As for the analysis of P─F bond breakdown, though DFP and SA were selected, *O*,*O*-dimethyl phoshorofluoridate was chosen as a model compound for a preliminary study of reaction pathways. The model of demeton-S was also simplified by truncation of the last methyl group belonging to the S-2-ethylthioethyl moiety. Finally, the influence of solvent on the relative stability of structures occurring along particular reaction coordinates was examined.

The gas phase reaction profiles were studied at the ab initio level. For all the first-order saddle points, intrinsic reaction coordinate (IRC) calculations were performed, revealing the geometries of the local minima associated with a given transition state. Thermodynamic

properties (enthalpies and Gibbs free energies) were determined from vibrational frequencies computed at the fully optimized structures of stationary points along a reaction coordinate. To account for the influence of aqueous solvation, the polarizable continuum model (PCM) was applied (Mennucci et al., 2002). All calculations were performed using the Gaussian 03/Rev. C02 program. (Frisch et al., 2004). Conclusions were: 1) While all base-catalyzed hydrolysis reactions that were studied appear to follow an associative mechanism, the cleavage of P─O and P─S bonds (except of acephate molecule) occurs according to a one-step direct-displacement mechanism involving the presence of a single S_N2-like transition state. The hydrolysis of P─F and P─CN bonds, however, is consistent with an addition-elimination scheme employing several trigonal bipyramidal intermediates. 2) Except for acephate, two alternative reaction pathways are possible for each of these mechanisms that differ in the position of the attacking hydroxide relative to the phosphoryl oxygen atom. Apparently, the most energetically favourable reaction coordinate involves the hydroxide proton being stabilized by a phosphoryl oxygen. Which mechanism will occur inside the PTE active site remains, according to the authors, undisclosed. 3) In the case of a multistep addition-elimination mechanism, relatively significant energy barriers are associated with the nucleophilic attack of a hydroxide (i.e., formation of the first intermediate) and the departure of a leaving group (i.e., decomposition of the final intermediate). Judging from the results of *O*,*O*-dimethyl phoshorofluoridate hydrolysis, the energy barriers for conformational transitions are of minor importance relative to the chemical steps encompassing the formation or breakage of a chemical bond. 4) The rate-limiting step of multistep mechanisms appears to be associated with an intermediate formation. 5) Since all the reaction pathways considered constitute variants of an associative mechanism of hydrolysis, they could presumably be accommodated by a common active site of PTE.

Sensory technology has enabled the development of recyclable enzyme-based biosensors, through the confinement of enzimes within nanomaterials (Lei et al., 2002; Cao, 2005). The bacterial enzyme organophosphorous hydrolase (OPH), another name of PTE, is a candidate to be immobilized because it detoxifies organophosphorous compounds catalytically. In OPH active site -with two divalent metal ions bridged by a water molecule and a carbamylated Lys169 residue- Zn^{2+} is the native metal, but activity is also achieved via substitution by Co^{2+} , Cd^{2+} , Mn^{2+}, or Ni^{2+} (Ombrugo et al., 1992; Rochu et al., 2004). OPH catalyzes the cleavage of P─O, P─F, and P─S bonds in a variety of organophosphate triesters and related phosphonates. The immobilization of OPH in functionalized mesoporous silica (FMS) was shown to enhance stability and increase enzyme catalytic activity by 200% as compared to OPH free in solution (Lei et al., 2002), but the effect of confinement on the catalytic activity of enzymes is not clearly understood. Gomes et al. (2008) developed models of confinement and carried out MD simulations for OPH free in solution (OPH_{free}), OPH confined through atom positional constraints (OPH_{fix}) and through the coarse-grain representation of the FMS pore (OPH_{fms}). The molecular model of the enzyme OPH was built from crystallographic coordinates. Atoms were represented by a van der Waals atomic model containing atom-centered point charges. The AMBER force field (Cornell et al., 1995) was used to treat bonded and non-bonded interactions. Zinc ions in the active site were treated using a non-bonded model, while partial atomic charges and parameters for the carbamylated Lys169 were calculated as described by Soares et al. (2007).

The interactions between OPH and the FMS have steric and electrostatic components. Steric interactions, due to the inert nature of the silica material, were approximated by a non-atomic model where the positions of N-atoms of lysine residues were harmonically constrained (except Lys169). Lysine side-chains are the linkage sites in covalently linked OPH-FMS complexes (Lei et al., 2007). Electrostatic interactions, due to functionalization of the mesopore, were modelled as a cylindrical, uniform array of atoms, each atom corresponding to a given functional group. As for the $COO^{-}(CH_2)_n$ group, experimentally used to functionalize the mesoporous silica, the pore surface was represented by -1 charged point particles and van der Waals parameters corresponding to a carboxylate anion derived from the AMBER force field. The OPH structure was docked to the FMS pore wall based on the complementarity of their electrostatic potential surfaces calculated with the program APBS (Baker et al., 2001). Within simulation times of 5 nanoseconds, several structural properties reached convergence. All simulations were performed with the NWChem program (Bylaska et al., 2006) and the analyses of molecular trajectories were carried out with the GROMACS program (Lindahl et al., 2001). The OPH_{fix} simulation seemed to better describe the pool of configurations around the X-ray structure. However, this positional constraint model suppressed the flexibility of the loop region located in the entrance of the enzyme active site with the carbamylated Lys169 that coordinates the Zn^{2+} cations required by OPH for full catalytic activity; and it also suppressed the conformational fluctuations of the whole enzyme in a non-selective fashion, which would translate into a decrease of catalytic efficiency (Boehr et al., 2006). The interaction between the coarse-grained FMS model and the all-atom OPH enzyme appeared not to affect any native state motions of the free enzyme. A coarse-grain representation of the functional groups would yield a more homogenous description of the charge distribution along the silica mesoporous material, but the resulting average potential of mean force should be equivalent to that of an atomistic model. According to the authors, a physical representation of the mesoporous material, instead of the positional restraint approach, will be crucial to determine diffusion coefficients or collision rates in the confined environment. So the multiscale approach appears as a viable model for more tangible simulations of confined proteins. Such an approach allows complex biological phenomena to be modelled at different scales, attaining simultaneously accuracy and economy. These range from the sub-atomic scales of quantum mechanics, to the atomistic level of molecular mechanics, molecular dynamics and Monte Carlo methods, and even mesoscale modelling.

Recently Kwasnieski et al. (2009) analysed directly the interaction of tabun with AchE. As mentioned above, AChE catalyzes hydrolysis of acetylcholine in choline and acetic acid and thus regenerates cholinergic neuron. The catalytic site is inside a gorge of about 20 Å; the catalytic cycle involves a catalytic triad composed of three residues Ser203, His447, and Glu334 in mouse AChE. The oxyanion hole composed of Ala204, Glu121, and Glu122 is very important as it activates the substrate via hydrogen bonds (Warshel et al. 1989; Fuxreiter & Warshel, 1998). Disfunctions of AChE due to organophosphorous compounds are a major threat because they inhibit AChE irreversibly leading to convulsions, and possibly death by asphyxiation. A covalent bond is formed between the oxygen of Ser203 and the phosphorus of the organophosphorous compound. It is commonly accepted that the leaving group is *anti* to the oxygen of Ser203. Tabun reactivity is particularly interesting as tabun-inhibited AChE is one of the more difficult complexes to reactivate. Also, tabun is produced as a

mixture of two enantiomers, and one of them is 6.3 times more potent. In order to understand whether the kinetics differ for the two enantiomers or it is a different binding mechanism, the authors studied theoretically tabun inhibition of AchE by examining four possibilities for tabun fixation modes: either the fixation of the (*S*) tabun enantiomer with the cyano group *anti* or *syn* to the oxygen atom of Ser203 (S-Syn and S-Anti); or the (*R*) tabun enantiomer with the cyano group *anti* or *syn* to the oxygen atom of Ser203 (R-Syn and R-Anti). The authors used a hybrid quantum mechanics/molecular mechanics (QM/MM) methodology. Calculations were performed using BP86 (Becke, 1988) functional. Single points were also done with B3LYP and PBE0 (Adamo, 1999) functionals. Four possible attacks of tabun on the oxygen of Ser203 were studied using two crystallographic structures (PDB codes 2C0P and 3DL7): (*S*) tabun with the cyano group *syn* to the oxygen of Ser203 and (*R*) tabun with the cyano group *anti*, corresponding to the experimental X-ray structure; (*S*) tabun with the cyano group *anti* to the oxygen of Ser203 and (*R*) tabun with the cyano group *syn*, leading to a different isomer than was experimentally seen. The two X-ray structures (PDB 2C0P and 3DL7) gave analogous results for the calculations previously described. Using the PDB 2C0P, the fixation of the two enantiomers lead to the experimental X-ray structure, namely, S-Syn and R-Anti with a mechanism going through an addition elimination pathway. The kinetically determinant step appeared to be the cyano group departure, as tabun fixation on Ser203 departure is almost barrierless. As for the PDB 3DL7 structure, four possible attacks of tabun on the oxygen of Ser203 were considered: S-Syn and R-Anti, which led to the experimental X-ray structure, and S-Anti and R-Syn, which led to the isomer which has opposite relative positions of the *N*-dimethyl group and the ethoxy group in the active site as compared to the experimental structure. It appeared that the most active enantiomer is S-Syn. Thus it seems that the cyano group does not leave *anti* to the oxygen of Ser203, as expected, due to repulsive polar interaction between cyanide and aromatic residues in the active site, in particular residues Phe295, Phe297, and Phe338.

From the preceding reports, two aspects are noticeable about theoretical studies of enzymatic mechanisms involved in pesticide metabolism and degradation: they contribute both to foresee and to modulate the toxicity of waste; and they promote the use of different strategies regarding the use of enzymes in the decontamination of the environment.

5. References

Adamo, C. & Barone, V. (1999). Toward reliable density functional methods without adjustable parameters: The PBE0 model. *J. Chem. Phys.*, 110, 6158-6170.

Ahlner, J.; Andersson, R. G. G.; Torfgard, K.; Axelsson, K. L. (1991). Organic Nitrate Esters-Clinical Use And Mechanisms Of Actions. *Pharmacol. Rev.*, 43, 351-423.

Ahlswede, B.; Jug, K. (1999a). Consistent modifications of SINDO1: I. Approximations and parameters. *J. Comput. Chem.*, 20, 563–571.

Ahlswede, B.; Jug, K. (1999b). Consistent modifications of SINDO1: II. Applications to first- and second-row elements. *J. Comput. Chem*. 20, 572–578.

Atkins, P. & Paula, J.d. (2006). *Physical Chemistry*, 8th Ed., Oxford University Press, ISBN 9780198700722 ISBN 0198700725, United States.

Baboul, A. G.; Curtiss, L. A.; Redfern, P. C.; Raghavachari, K. (1999). Gaussian-3 theory using density functional geometries and zero-point energies. *J. Chem. Phys*., 110 (16), 7650-7657.

Baker, N.A.; Sept, D.; Joseph, S.; Holst, M. J.; McCammon, J. A. (2001). Electrostatics of nanosystems: application to microtubules and the ribosome. *Proceedings of the National Academy of Sciences,* 98 (18), 10037–10041, August 2001, USA.

Balbi, H. J. (2004). Chloramphenicol: A review. *Pediatr. Rev*., 25, 284-288.

Becke, A.D.(1988), Density-functional exchange-energy approximation with correct asymptotic behavior, *Phys. Rev. A*, 38 (6), 3098-3100.

Benson, S. W.; Cruickshank, F. R.; Golden, D. M.; Haugen, G. R.; O'Neil, H. E.; Rodgers, A. S.; Shaw, R.; Walsh, R. (1969). Additivity Rules for the Estimation of Thermochemical Properties. *Chem. Rev*., 69 (3), 279-324.

Berendsen, H. J. C.; Postma, J. P. M.; van Gunsteren, W. F. & Hermans, J. (1981). Interaction models for water in relation to protein hydration. In: *Intermolecular Forces*, B. Pulmann (Ed.), pp 331-342. D. Reidel Publishing Company, Dordrecht.

Bish, D. L. (1993). Rietveld refinement of the kaolinite structure at 1.5K. *Clays and Clay Miner.*, 41, 738-744.

Boehr, D.D.; Dyson, H. J.; Wright, P. E. (2006). An NMR Perspective on Enzyme Dynamics. *Chem. Rev*. 106, 3055–3079.

Boese, A. D.; Martin, J. M. L. (2004). Development of density functionals for thermochemical kinetics. *J. Chem. Phys*., 121, 3405-3416.

Boulet, P.; Greenwell, H. C.; Stackhouse, S. & Coveney, P.V. (2006). Recent advances in understanding the structure and reactivity of clays using electronic structure calculations. *J. Molecular Structure: THEOCHEM*, 762, 33–48.

Bredow, T.; Geudtner, G.; Jug, K. (2001). MSINDO parameterization for third-row transition metals. *J. Comput. Chem*., 22, 861–887.

Brooks, B. R.; Bruccoleri, R. E.; Olafson, B. D.; States, D. J.; Swaminathan, S.; Karplus, M. (1983). CHARMM-a Program for Macromolecular Energy, Minimization, and Dynamics Calculations. *J. Comput. Chem.,* 4 (2), 187-217.

Bylaska, E. J.; Jong, W.A.d.; Kowalski, K.; Straatsma, T.P.; Valiev, M.; Wang, D.; Aprà, E.; Windus, T. L.; Hirata, S.; Hackler, M.T.; Zhao, Y.; Fan, P.-D.; Harrison, R.J.; Dupuis, M.; Smith, D. M. A.; Nieplocha, J.; Tipparaju, V.; Krishnan, M.; Auer, A. A.; Nooijen, M.; Brown, E.; Cisneros, G.; Fann, G. I.; Frücht, H.; Garza, J.; Hirao, K.; Kendall, R.; Nichols, J. A.; Tsemekhman, K.; Wolinsk, K.; Anchell, J.; Bernholdt, D.; Borowski, P.; Clark, T.; Clerc, D.; Dachsel, H.; Deegan, M.; Dyall, K.; Elwood, D.; Glendening, E.; Gutowski, M.; Hess, A.; Jaffe, J.; Johnson, B.; Ju, J.; Kobayashi, R.; Kutteh, R.; Lin, Z.; Littlefield, R.; Long, X.; Meng, B.; Nakajima, T.; Niu, S.; Pollack, L.; Rosing, M.; Sandrone, G.; Stave, M.; Taylor, H.; Thomas, G.; Lenthe, J.v.; Wong, A.; Zhang, Z. (2006). *NWChem, A Computational Chemistry Package for Parallel Computers,* Version 5.0. Pacific Northwest National Laboratory, Richland, Washington 99352-0999, USA (A modified version, 2006).

Bylaska, E. J.; de Jong, W. A.; Govind, N.; Kowalski, K.; Straatsma, T. P.; Valiev, M.; Wang, D.; Apra, E.; Windus, T. L.; Hammond, J.; Nichols, P.; Hirata, S.; Hackler, M. T.; Zhao, Y.; Fan, P.-D.; Harrison, R. J.; Dupuis, M.; Smith, D. M. A.; Nieplocha, J.;

Tipparaju, V.; Krishnan, M.; Wu, Q.; Van Voorhis, T.; Auer, A. A.; Nooijen, M.; Brown, E.; Cisneros, G.; Fann, G. I.; Fruchtl, H.; Garza, J.; Hirao, K.; Kendall, R.; Nichols, J. A.; Tsemekhman, K.; Wolinski, K.; Anchell, J.; Bernholdt, D.; Borowski, P.; Clark, T.; Clerc, C.; Dachsel, H.; Deegan, M.; Dyall, K.; Elwood, D.; Glendening, E.; Gutowski, M.; Hess, A.; Jaffe, J.; Johnson, B.; Ju, J.; Kobayashi, R.; Kutteh, R.; Lin, Z.; Littlefield, R.; Long, X.; Meng, B.; Nakajima, T.; Niu, S.; Pollack, L.; Rosing, M.; Sandrone, S.; Stave, M.; Taylor, H.; Thomas, G.; van Lenthe, J.; Wong, A.; Zhang, Z. *NWChem, A Computational Chemistry Package for Parallel Computers*, Version 5.1; Pacific Northwest National Laboratory: Richland, WA, 2007.

Cao, L. (2005). Immobilized Enzymes: science or art. *Current Opinion in Chemical Biology,* 9, 217–226.

Case, D. A.; Pearlman, D. A.; Caldwell, J. W.; T. E. Cheatham, I.; Ross, W. S.; Simmerling, C.; Darden, T.; Merz, K. M.; Stanton, R. V.; Cheng, A.; Vincent, J. J.; Crowley, M.; Ferguson, D. M.; Radmer, R.; Seibel, G. L.; Singh, U. S.; Weiner, P. K.; Kollman, P. A. (1997). *AMBER*, 5.0; University of California: San Francisco.

Casida, J. E.; Quistad, G. B. (1998). Golden age of insecticide research: past, present or future?. *Annu. Rev. Entomol*. 43, 1–16.

Cohen, N.; Benson, S. W. (1993). Estimation of heats of formation of organic compounds by additivity methods. *Chem. Rev.*, 93 (7), 2419-2438.

Coote, M. L.; Henry, D. J.(2005). Effect of substituents on radical stability in reversible addition fragmentation chain transfer polymerization: an ab initio study. *Macromolecules*, 38(4), 1415-1433.

Cornell, W. D.; Cieplak, P.; Bayly, C. I.; Gould, I. R.; Merz, K. M. Jr.; Ferguson, D. M.; Spellmeyer, D. C.; Fox, T.; Caldwell, J. W. & Kollman, P.A. (1995). A Second Generation Force Field for the Simulation of Proteins, Nucleic Acids and Organic Molecules, *J. Am. Chem. Soc.* 117, 5179-5197.

Coscarello, E. N.; Barbiric, D. A.; Castro, E. A.; Vico, R.V.; Bujan, E. I.; de Rossi, R. H. (2009). Comparative Analysis of Complexation of Pesticides (fenitrothion, methylparathion, parathion) and their Carboxylic Ester-Analogues by β-Cyclodextrin. Theoretical Semiempirical Calculations. *J. Structural Chemistry*, 50 (4), 705-713. ISSN: 0022-4766. Ed. Springer. J.N° 10947.

Curtiss, L. A.; Raghavachari, K.; Redfern, P. C.; Pople, J. A. (1997). Assessment of Gaussian-2 and density functional theories for the computation of enthalpies of formation. *J. Chem. Phys.*, 106 (3), 1063-1079.

Curtiss, L. A.; Raghavachari, K.; Redfern, P. C.; Rassolov, V.; Pople, J. A. (1998). Gaussian-3 (G3) theory for molecules containing first and second-row atoms. *J. Chem. Phys.*, 109, 7764-7776.

Curtiss, L. A.; Raghavachari, K.; Redfern, P. C.; Pople, J. A. (2000a). Gaussian-3 theory using scaled energies. *J. Chem. Phys.*, 112 (3), 1125-1132.

Curtiss, L. A.; Raghavachari, K.; Redfern, P. C.; Pople, J. A. (2000b). Assessment of Gaussian-3 and density functional theories for a larger experimental test set. *J. Chem. Phys.*, 112, 7374-7383.

Curtiss, L. A.; Redfern, P. C.; Raghavachari, K.; Pople, J. A. (2001). Gaussian-3X (G3X) theory: Use of improved geometries, zero-point energies, and Hartree–Fock basis sets. *J. Chem. Phys.*, 114, 108-117.

David, R. L., Ed. (2007). CRC *Handbook of Chemistry and Physics*, 87th ed.; Taylor and Francis: Boca Raton, FL, ; http:/www.hbcpnetbase. com.

Dewar, M. J. S.; Zoebisch, E. G.; Healy, E. F. & Stewart, J. P. (1985) AM1: A new general purpose quantum mechanical molecular model, *J. Am. Chem. Soc.* 107, 3902-3909.

Dewar, M. J. S. & Merz, K. M.,Jr. (1988). AM1 parameters for zinc, *Organometallics* 7, 522-524.

Dewar, M. J. S., and Jie, C. (1989). AM1 parameters for phosphorus, *THEOCHEM* 187, 1-13.

Dorofeeva, O. V.; Moiseeva, N. F. (2006). Computational Study of the Thermochemistry of Organophosphorus(III) Compounds. *J. Phys. Chem. A* , 110, 8925-8932.

Duke, C. R.; Lubinsky, A. R.; Chang, S. C.; Lee, B. W.; Mark, P. (1977). Low-energy-electron-diffraction analysis of the atomic geometry of ZnO (10$\bar{1}$0). *Phys. Rev. B* , 15, 4865-4873.

Duke, C. B.; Meyer, R. J.; Paton, A.; Mark, P. (1978). Calculation of low-energy-electron-diffraction intensities from ZnO(10$\bar{1}$0). II. Influence of calculational procedure, model potential, and second-layer structural distortions. *Phys. Rev. B* , 18, 4225-4240.

Dyguda-Kazimierowicz E.; Sokalski, W.A. & Leszczynski, J. (2008). Gas-Phase Mechanisms of Degradation of Hazardous Organophosphorus Compounds: Do They Follow a Common Pattern of Alkaline Hydrolysis Reaction As in Phosphotriesterase? *J. Phys. Chem. B*, 112, 9982–9991.

Feigenbrugel, V.; Person, Calve, A. L.; Mellouki, S. L.; A.; Munoz, A.; Wirtz, K. (2006). Atmospheric fate of dichlorvos: Photolysis and OH-initiated oxidation studies. *Environ. Sci. Technol.*, 40 (3), 850-857.

Frisch, M. J.; Trucks, G. W.; Schlegel, H. B.; Gill, P. M. W.; Johnson, B. G.; Robb, M. A.; Cheeseman, J. R.; Keith, G. A.; Petersson, D. A.; Montgomery, J. A.; Al-Laham, M. A.; Zakrzewski, V. G.; Ortiz, J. V.; Foresman, J. B.; Cioslowski, J.; Stefanov, B. B.; Nanayakkara, A.; Challacombe, M.; Peng, C. Y.; Ayala, P. Y.; Chen, W.; Wong, M. W.; Andres, J. L.; Replogle, E. S.; Gomperts, R.; Martin, R. L.; Fox, D. J.; Binkley, J. S.; Defrees, G. J.; Baker, J.; Stewart, J. P.; Head-Gordon, M.; Gonzalez, C.; Pople, J. A. (1995); *Gaussian 94 (Revision A.1); Gaussian, Inc.: Pittsburgh, PA*.

Frisch, M. J.; Trucks, G. W.; Schlegel, H. B.; Scuseria G. E.; Robb, M. A.; Cheeseman, J. R.; Zakrzewski, V. G. Jr.; Montgomery, J. A.; Stratmann, R. E.; Burant J. C.; Dapprich, S.; Millam, J. M.; Daniels, A. D.; Kudin, K. N.; Strain, M. C.; Farkas, O.; Tomasi, J.; Barone, V.; Cossi, M.; Cammi, R.; Mennucci, B.; Pomelli, C.; Adamo, C.; Clifford, S.; Ochterski, J.; Petersson, G. A.; Ayala, P.Y.; Cui, Q.; Morokuma, K.; Malick, D. K.; Rabuck, A. D.; Raghavachari, K.; Foresman, J. B.; Cioslowski, J.; Ortiz, J. V.; Stefanov, B. B.; Liu, G.; Liashenko, A.; Piskorz, P.; Komaromi, I.; Gomperts, R.; Martin, R. L.; Fox, D. J.; Keith, T.; Al-Laham, M. A.; Peng, C. Y.; Nanayakkara, A. ; Gonzalez, C.; Challacombe, M.; Gill, P. M. W.; Johnson, B.; Chen, W.; Wong, M. W.; Andres, J. L.; Gonzalez, C.; Head-Gordon, M.; Replogle, E. S.; Pople, J. A. (1998); *Gaussian 98 (Revision A.6); Gaussian, Inc.: Pittsburgh, PA*.

Frisch, M. J.; Trucks, G. W.; Schlegel, H. B.; Scuseria, G. E.; Robb, M. A.; Cheeseman, J. R.; Montgomery J. A., Jr.; Vreven, T.; Kudin, K. N.; Burant, J. C.; Millam, J. M.; Iyengar, S. S.; Tomasi, J.; Barone, V.; Mennucci, B.; Cossi, M.; Scalmani, G.; Rega, N.; Petersson, G. A.; Nakatsuji, H.; Hada, M.; Ehara, M.; Toyota, K.; Fukuda, R.;

Hasegawa, J.; Ishida, M.; Nakajima, T.; Honda, Y.; Kitao, O.; Nakai, H.; Klene, M.; Li, X.; Knox, J. E.; Hratchian, H. P.; Cross, J. B.; Bakken, V.; Adamo, C.; Jaramillo, J.; Gomperts, R.; Stratmann, R. E.; Yazyev, O.; Austin, A. J.; Cammi, R.; Pomelli, C.; Ochterski, J. W.; Ayala, P. Y.; Morokuma, K.; Voth, G. A.; Salvador, P.; Dannenberg, J. J.; Zakrzewski, V. G.; Dapprich, S.; Daniels, A. D.; Strain, M. C.; Farkas, O.; Malick, D. K.; Rabuck, A. D.; Raghavachari, K.; Foresman, J. B.; Ortiz, J. V.; Cui, Q.; Baboul, A. G.; Clifford, S.; Cioslowski, J.; Stefanov, B. B.; Liu, G.; Liashenko, A.; Piskorz, P.; Komaromi, I.; Martin, R. L.; Fox, D. J.; Keith, T.; Al.Laham, M. A.; Peng, C. Y.; Nanayakkara, A.; Challacombe, M.; Gill, P. M. W.; Johnson, B.; Chen, W.; Wong, M. W.; Gonzalez, C.; Pople, J. A. (2004). *Gaussian 03, Revision X.0X. Gaussian, Inc., Wallingford CT, 2004.*

Fuxreiter, M.; Warshel, A. (1998). Origin of the Catalytic Power of Acetylcholinesterase: Computer Simulation Studies. *J. Am. Chem. Soc.*, 120, 183-194.

Fukui, K. (1981). The path of chemical reactions: The IRC approach. *Acc. Chem. Res.*, 14 (12), 363-368.

Gomes, D. E. B.; Lins, R. D.; Pascutti, P. G.; Straatsma, T. P.; Soares, T. A. (2008). Molecular Models to Emulate Confinement Effects on the Internal Dynamics of Organophosphorous Hydrolase. *Lecture Notes in Bioinformatics*, 5167, 68-78. A.L.C. Bazzan, M. Craven, and N.F. Martins (Eds.). Springer-Verlag Berlin Heidelberg.

Gorb, L.; Lutchyn, R.; Zub, Yu.; Leszczynska, D.; Leszczynski, J. (2006). The origin of the interaction of 1,3,5-trinitrobenzene with siloxane surface of clay minerals. *J. Molecular Structure: THEOCHEM*, 766, 151–157.

Grimme, S. (2003). Improved second-order Møller–Plesset perturbation theory by separate scaling of parallel- and antiparallel-spin pair correlation energies. *J. Chem. Phys.*, 118, 9095-9102.

Grimme, S. (2006). Semiempirical hybrid density functional with perturbative second-order correlation. *J. Chem. Phys.*, 124, 034108-034123.

Haderlein, S. B.; Schwarzenbach, R. P. (1993). Adsorption of substituted nitrobenzenes and nitrophenols to mineral surfaces. *Environ. Sci. Technol.*, 27(2), 316-26.

Haderlein, S. B.; Weissmahr, K. W.; Schwarzenbach, R. P. (1996). Specific adsorption of nitroaromatic explosives and pesticides to clay minerals. *Environ. Sci. Technol.,* 30 (2), 612-622.

Harrison, M. A. J.; Barra, S.; Borghesi, D.; Vione, D.; Arsene, C.; Olariu, R. L. (2005). Nitrated phenols in the atmosphere: a review. *Atmos. Environ.*, 39, 231-248.

Hehre, W. J.; Radom, L.; Schleyer, P. v. R. & Pople, J. A. (1986). *Ab initio molecular orbital theory*, Wiley, New York.

Hemelsoet, K.; Van Durme, F.; Van Speybroeck, V.; Reyniers, M-F & Waroquier, M. (2010). Bond Dissociation Energies of Organophosphorus Compounds: an Assessment of Contemporary Ab Initio Procedures. *J. Phys. Chem. A*, *114,* 2864–2873

Hodgson, J. L.; Coote, M. L. (2005). Effects of Substituents on the Stability of Phosphoranyl Radicals. *J. Phys. Chem. A*, 109, 10013- 10021.

Hofer, W. (1981). Chemistry of metrifonate and dichlorvos. *Acta Pharmacol. Toxicol.*, 49 (Suppl. 5), 7-14.

HyperChem 7 for Windows, Hypercube Inc., 2002.

Janssen, R. H. C.; Theodorou, D. N.; Raptis, S.; Papadopoulos, M. G. (1999). Molecular simulation of static hyper-Rayleigh scattering: A calculation of the depolarization ratio and the local fields for liquid nitrobenzene. *J. Chem. Phys.*, 111, 9711-9719.

Jensen, F. (1999*): Introduction into computational chemistry*. John Wiley, ISBN 0471984256, England.

Jorge, M.; Gulaboski, R.; Pereira, C. M.; Cordeiro, M. (2006). Molecular dynamics study of nitrobenzene and 2-nitrophenyloctyl ether saturated with water. *Mol. Phys.*, 104, 3627-3634.

Jorgensen, W. L.; Tirado-Rives, J. (1988). The OPLS Force Field for Proteins. Energy Minimizations for Crystals of Cyclic Peptides and Crambin. *J. Am. Chem. Soc.,* 110, 1657–1666.

Jorgensen, W.L.; Maxwell. D.S.; Tirado-Rives, J. (1996). Development and Testing of the OPLS All-Atom Force Field on Conformational Energetics and Properties of Organic Liquids. *J. Am. Chem. Soc.,* 118 (45), 11225–11236.

Jung, Y.; Lochan, R. C.; Dutoi, A. D.; Head-Gordon, M. (2004). Scaled opposite-spin second order Møller–Plesset correlation energy: An economical electronic structure method. *J. Chem. Phys.*, 121 (20), 9793-9803.

Kamiya, M.; Nakamura, K.; Sasaki, C.(1995). Inclusion effects of β-cyclodextrins on the hydrolysis of organophosphorus pesticides. *Chemosphere,* 30 (4), 653-660.

Klauda, J. B.; Pastor, R. W.; Brooks, B. R. (2005a). Adjacent gauche stabilization in linear alkanes: Implications for polymer models and conformational analysis. *J. Phys. Chem. B*, 109 (33), 15684–15686.

Klauda, J. B.; Brooks, B. R.; MacKerell, A. D. Jr.; Venable, R. M.; Pastor, R. W. (2005b). An Ab Initio Study on the Torsional Surface of Alkanes and its Effect on Molecular Simulations of Alkanes and a DPPC Bilayer. *J. Phys. Chem. B* , 109, 5300-5311.

Klauda, J. B. & Brooks, B. R. (2008). CHARMM Force Field Parameters for Nitroalkanes and Nitroarenes. *J. Chem. Theory Comput*., 4, 107-115.

Koča, J.; Zhan, C.-G.; Rittenhouse, R.C. & Ornstein, R.L. (2001). Mobility of the Active Site Bound Paraoxon and Sarin in Zinc-Phosphotriesterase by Molecular Dynamics Simulation and Quantum Chemical Calculation. *J. Am. Chem. Soc*., 123, 817-826.

Kwasnieski, O.; Verdier, L.; Malacria, M. & Derat, E. (2009). Fixation of the Two Tabun Isomers in Acetylcholinesterase: A QM/MM Study. *J. Phys. Chem. B*, 113, 10001–10007.

Leach, A. R. (2001). *Molecular Modelling, principles and applications*, 2nd Ed. 2001, Pearson Education Limited, ISBN 0-582-38210-6, England.

Lee, C.; Yang, W.; Parr, R. G. (1988). Development of the Colle-Salvetti correlation-energy formula into a functional of the electron density. *Phys. Rev. B*, 37, 785-789.

Lei, C.; Shin, Y.; Liu, J.; Ackerman, E. J. (2002). Entrapping enzyme in a functionalized nanoporous support. *J. Am. Chem. Soc.,* 124, 11242–11243.

Lei, C.; Shin, Y.; Liu, J.; Ackerman, E. J. (2007). Synergetic effects of nanoporous support and urea on enzyme activity. *Nano Letters,* 7, 1050–1053.

Lindahl, E.; Hess, B.; Spoel, D.v.d. (2001). GROMACS 3.0: A package for molecular simulation and trajectory analysis. *J. Molecular Modeling*, 7, 306–317.

MacKerell, A. D., Jr. (2001). Atomistic Models and Force Fields. In: *Computational Biochemistry and Biophysics*; Becker, O. M., MacKerell, A. D., Jr., Roux, B., Watanabe, M., Eds., p 7-38, Marcel Dekker: New York.

MacKerell, A. D. Jr., (2004). Empirical force fields for biological macromolecules: Overview and issues. *J. Comput. Chem.*, 25, 1584-1604.

MacKerell, A. D. Jr. (2005). Interatomic Potentials: Molecules. In: *Handbooks of Material* Modeling; Yip, S., Ed. p 509-525; Springer: The Netherlands.

Manunza, B.; Deiana, S.; Pintore, M..; Delogu, G.; Gessa, C. (1998). A Molecular Dynamics Investigation on the Inclusion of Chiral Agrochemical Molecules in β-Cyclodextrin. Complexes with Dichlorprop, 2-Phenoxypropionic Acid and Dioxabenzofos. *Pestic. Sci.*, 54, 68-74.

Mennucci, B.;Tomasi, J.; Cammi, R.; Cheeseman, J.R.; Frisch, M.J.; Devlin, F.J.; Gabriel, S.; Stephens, P.J. (2002). Polarizable Continuum Model (PCM) Calculations of Solvent Effects on Optical Rotations of Chiral Molecules, *J. Phys. Chem. A.*, 106, 6102-6113.

Michael, D.; Benjamin, I. (1998). Molecular dynamics simulation of the water-nitrobenzene interface. *J. Electroanal. Chem.*, 450 (2), 335-345.

Michalkova, A.; Paukku, Y.; Majumdar, D.; Leszczynski, J. (2007). Theoretical study of adsorption of tabun on calcium oxide clusters. *Chem. Phys. Lett.*, 438, 72-77.

Murphy, K. C.; Cooper, R. J.; Clark, J. M. (1996). Volatile and dislodgeable residues following trichlorfon and isazofos application to turfgrass and implications for human exposure. *Crop Sci.*, 36 (6), 1446-1454.

Nair, R. K.; Kadam, M. M.; Sawant, M. R.(2006). Effect on the Solubility, Wettability, and Dispersibility of the β-CD-Deltamethrin Inclusion Complex in Presence of Mixed Surfactant (C_{14}MEA/AOS) Blend. *J. Disper. Sci. Tech.* 27 (7) , 1015 – 1019. Taylor & Francis Eds. ISSN: 1532-2351.

Omburo, G. A.; Kuo, J. M.; Mullins, L. S.; Raushel, F. M. (1992). Characterization of the zinc binding site of bacterial phosphotriesterase. *J. Biological Chemistry,* 267, 13278–13283.

Osa, T.; Suzuki, I. (1996). In: *Comprehensive Supramolecular Chemistry (Vol. 3) Cyclodextrins*. Eds. J. Szejtli and T. Osa., pp 367 – 400. Pergamon, Oxford.

Paukku, Y.; Michalkova, A. & Leszczynski, J. (2008). Adsorption of dimethyl methylphosphonate and trimethyl phosphate on calcium oxide: an ab initio study. *J. Struct. Chem.*, 19 (2), 307-320.

Paukku, Y.; Michalkova, A. & Leszczynski, J. (2009). Quantum-Chemical Comprehensive Study of the Organophosphorus Compounds Adsorption on Zinc Oxide Surfaces. *J. Phys. Chem. C* 2009, 113, 1474–1485

Pelmenschikov, A. & Leszczynski, J. (1999). Adsorption of 1,3,5-Trinitrobenzene on the Siloxane Sites of Clay Minerals: Ab Initio Calculations of Molecular Models. *J. Phys. Chem. B,* 103, 6886-6890.

Pettigrew, L. C.; Bieber, F.; Lettiere, J.; Wermeling, D. P.; Schmitt, F. A.; Tikhtrman, A. J.; Ashford, J. W.; Smith, C. D.; Wekstein, D. R.; Markesbery, W. R.; Orazem, J.; Ruzicka, B. B.; Mas, J.; Gulanski, B. (1998). Pharmacokinetics, pharmacodynamics, and safety of metrifonate in patients with Alzheimer's disease. *J. Clin. Pharmacol.*, 38 (3), 236-245.

Pilcher, G. (1990). Thermochemistry of phosphorus(III) compounds. In: *The Chemistry of Organophosphorus Compounds*; Hartley, F. R., Ed.; Vol. 1, p 127-136. J. Wiley & Sons, New York.

Price, M. L. P.; Ostrovsky, D.; Jorgensen, W. L. (2001). Gas-phase and liquid-state properties of esters, nitriles, and nitro compounds with the OPLS-AA force field. *J. Comput. Chem*., 22, 1340-1352.

Price, D. J.; Brooks, C. L. (2005). Detailed considerations for a balanced and broadly applicable force field: A study of substituted benzenes modeled with OPLS-AA. *J. Comput. Chem*., 26 (14), 1529-1541.

Raushel, F. M. (2002). Bacterial detoxification of organophosphate nerve agents. *Curr. Opin. Microbiol*., 5, 288–295.

Richards, R.; Li, W.; Decker, S.; Davidson, C.; Koper, O.; Zaikovski, V.; Volodin, A.; Rieker, T.; Klabunde, K. J. (2000). Consolidation of Metal Oxide Nanocrystals. Reactive Pellets with Controllable Pore Structure That Represent a New Family of Porous, Inorganic Materials. *J. Am. Chem. Soc.*, 122 (20), 4921-4925.

Rochu, D.; Renault, F.; Viguille, N.; Crouzier, D.; Froment, M. T.; Masson, P. (2004). Contribution of the active-site metal cation to the catalytic activity and to the conformational stability of phosphotriesterase: temperature- and pH-dependence. *Biochemical Journal,* 380, 627–633.

Schaftenaar, G.; Noordik, J. H. (2000). MOLDEN: a pre- and post-processing program for molecular and electronic structures. *J. Comput.-Aided Mol. Des.* 14, 123-134.

Schmidt, M.W.; Baldridge, K. K.; Boatz, J. A.; Elbert, S. T.; Gordon, M. S.; Jensen, J. J.; Koseki, S.; Matsunaga, N.; Nguyen, K. A.; Su, S.; Windus, T. L.; Dupuis, M.; Montgomery, J. A. (1993). GAMESS Version 21 Nov. 1995, *J. Comput. Chem*. 14 , 1347-1363.

Scholz, F.; Schroder, U.; Gulaboski, R. (2005). *Electrochemistry of Immobilized Particles and Droplets*; Springer: Berlin, Heidelberg, New York. ISBN 3-540-22005-4.

Schwabe, T.; Grimme, S. (2006). Towards chemical accuracy for the thermodynamics of large molecules: new hybrid density functionals including non-local correlation effects. *Phys. Chem. Chem. Phys.*, 8 (38), 4398-4401.

Silman, I. & Sussman, J. L. (2005). Acetylcholinesterase: "Classical" and "non-classical" functions and pharmacology. *Curr Opin. Pharmacol.,* 5, 293-302.

Smith, W. & Forester, T. R. (1994). *DLPOLY2, package of molecular simulation routines*, copyright the Council for the Central Laboratory of the Research Councils, Daresbury Laboratory, Warrington U.K. (1994-6).

Soares, T. A.; Osman, M.; Straatsma, T. P. (2007). Molecular dynamics of organophosphorous hydrolases bound to the nerve agent soman. *J. Chemical Theory and Computation,* 3, 1569–1579.

Sokalski, W.A.; Roszak, S.; Pecul, K. (1988). An efficient procedure for decomposition of the SCF interaction energy into components with reduced basis set dependence. *Chem. Phys. Lett*., 153, 153-159.

Spain, J. C. (1995). Biodegradation of Nitroaromatic Compounds. *Annu. Rev. Microbiol*., 49, 523-555.

Staroverov, V. N.; Scuseria, G. E.; Tao, J.- M.; Perdew, J. P. (2003). Comparative assessment of a new nonempirical density functional: Molecules and hydrogen-bonded complexes. *J. Chem. Phys*., 119 (23), 12129-12137.

Stewart, J. J. P. (1989 a). Optimization of Parameters for Semi-Empirical Methods. I-Method. *J. Comput. Chem*., 10, 209-220.

Stewart, J. J. P. (1989b). Optimization of Parameters for Semiempirical Methods. II. Applications. *J. Comput. Chem*., 10, 221-264.

Svensson, M.; Humbel, S.; Froese, R.D.J.; Matsubara, T.; Sieber, S. & Morokuma, K. (1996). ONIOM. A Multilayered Integrated MO + MM Method for Geometry Optimisations and Smgle Point Energy Predictions. A Test for Diels-Alder Reactions and $Pt(P(t\text{-}Bu)_3)_2+H_2$ Oxidative Addition. *J. Phys. Chem*.,100, 19357-19363.

Szejtli, J.(2004). Past, present, and future of cyclodextrin research. *Pure Appl. Chem*. 76 (10), 1825–1845.

Tao, J. -M.; Perdew, J. P.; Staroverov, V. N.; Scuseria, G. E. (2003). Climbing the Density Functional Ladder: Nonempirical Meta- Generalized Gradient Approximation Designed for Molecules and Solids. *Phys. Rev. Lett.*, 91(14), 146401/1-4.

Vico R.V., Buján E.I., de Rossi R.H. (2002). Effect of cyclodextrin on the hydrolysis of the pesticide fenitrothion [*O,O*-dimethyl *O*-(3-methyl-4-nitrophenyl)phosphorothioate]. *J. Phys. Org. Chem*.15 (12), 858– 862.

Wahab, H.S.; Koutselos A. D. (2009). Computational modeling of the adsorption and •OH initiated photochemical and photocatalytic primary oxidation of nitrobenzene. *J. Mol. Model*., 15, 1237–1244.

Warshel, A.; Naray-Szabo, G.; Sussman, F.; Hwang, J. K. (1989). How do serine proteases really work?. *Biochemistry*, 28, 3629-3637.

Weiner, S. J.; Kollman, P. A.; Case, D.A.; Singh, U. C.; Ghio, C.; Alagona, G.; Profeta, S. & Weiner, P. (1984). A New Force Field for Molecular Mechanical Simulation of Nucleic Acids and Proteins. *J. Am. Chem. Soc*., 106, 765-784.

Weissmahr, K. W.; Haderlein, S. B.; Schwarzenbach, R. P. (1997). In situ spectroscopic investigations of adsorption mechanisms of nitroaromatic compounds at clay minerals. *Environ. Sci. Technol*.,31(1), 240-247.

Werner, H.-J.; Knowles, P. J.; Amos, R. D.; Bernhardsson, A.; Berning, A.; Celani, P.; Cooper, D. L.; Deegan, M. J. O.; Dobbyn, A. J.; Ecker, F.; Hampel, C.; Hetzer, G.; Knowles, P. J.; Korona, T.; Lindh, R.; Lloyd, A. W.; McNicholas, S. J.; Manby, F. R.; Meyer. W.; Mura, M. E.;Nicklass, A.; Palmieri, P.; Pitzer, R.; Rauhut, G.; Schutz, M.; Schumann, U.; Stoll, H.; Stone, A. J.; Tarroni, R.; Thorsteinsson, T. (2002). *MOLPRO 2002.6*; University of Birmingham: Birmingham, U.K..

Wong, K-.Y. & Gao, J. (2007). The Reaction Mechanism of Paraoxon Hydrolysis by Phosphotriesterase from Combined QM/MM Simulations. *Biochemistry*, 46, 13352-13369

Woods, R. J.; Dwek, R. A.; Edge, C. J. & Fraser-Reid, B. (1995). Molecular mechanical and molecular dynamical simulations of glycoproteins and oligosaccharides. 1. GLYCAM- 93 parameter development, *J. Phys. Chem*., 99, 3832-3846.

Yun, K.-H.; Yun, K.-Y.; Cha, G.-Y.; Lee, B.-H.; Kim, J.-C.; Lee, D.-D.; Huh, J.-S. (2005). Gas Sensing Characteristics of ZnO-doped SnO_2 Sensors for Simulants of the Chemical

Agents. *Mater. Sci. Forum* , Vols. *486-487*, pp.9-12 in Eco-Materials Processing & Design VI. DOI: 10.4028/www.scientific.net/MSF.486-487.9

Zhang, Q.; Qu, X. ; Wang, W.(2007). Mechanism of OH-Initiated Atmospheric Photooxidation of Dichlorvos: A Quantum Mechanical Study. *Environ. Sci. Technol*., 41, 6109-6116.

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