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Pesticides

Recent Trends in Pesticide Residue Assay

Edited by R.P. Soundararajan

PESTICIDES – RECENT TRENDS IN PESTICIDE RESIDUE ASSAY

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Notice

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Meet the editor

Dr R.P. Soundararajan is currently working as Assistant Professor at National Pulses Research Centre, Tamil Nadu Agricultural University, Vamban, Tamil Nadu, India. He completed his Ph.D. in Agricultural Entomology at Tamil Nadu Agricultural University, India in 2000. He is specialized in viz., Host Plant Resistance to Insect pests, molecular marker assisted breeding for insect resistance in plants and Insecticide Resistance Management. He has published 27 research articles and 7 book chapters. He has also co-authored the book on 'Pest Management in Field Crops – Principles and Practices'.

Contents

Preface

In recent times agricultural and health workers have been compelled to handle more and more pesticides in the form of insecticides, fungicides, herbicides and other form of chemical compounds. Though alternative methods of controlling insects, rodents, insect pests, pathogens, microbes and weeds have been developed, pesticides could not be replaced. The prosperous role of pesticides in the management of insect pests and diseases is indisputable. However, its nature of non selective toxicity towards other organisms and deposition in the environment warrants the legislation of usage. Pesticide use raises a number of environmental concerns. Over 98 per cent of sprayed insecticides and 95 per cent of herbicides reach a destination other than their target species, including non-target species, air, water and soil. Pesticide drift occurs when pesticides suspended in the air as particles are carried by wind to other areas, potentially contaminating them. Pesticides are one of the causes of water pollution and some pesticides are persistent organic pollutants and contribute to soil contamination. Detection of pesticide molecules in the food items from plant and animal origin become imperative for safe consumption of food. Pesticide residues are referred as quantities of persistent pesticide molecules that pollute the environment and food stuffs. Many pesticides are being highly stable and continue to kill the target and the host long after the application which is both advantageous and disadvantageous. Thus, the detection and assays of pesticide residues have significant role to know level of residual pesticide molecules. The present edition of volume attempted to consolidate significant advancement of research at worldwide on pesticide residues. The status of pesticide usage pattern and residue level in food from plant and animal origin at different regions are described in-depth in the chapters.

The book comprises of twelve chapters in two different sections. The first section of the book described on pesticide residue in nine chapters. Apart from methods of analysis of pesticide residues in the food and soil, the chapters with latest research on removal of residual pesticides and residue control by different methods are included. The other aspect of pesticide residue is sample preparation for residue analysis. Though the advance instruments chromatography to mass spectrometry are available, the precision and fast analysis for detection of molecules is depending on the sample preparation methods. The chapters dealing with advance methods of sample preparation is also included in the volume.

Metabolism of pesticides denotes that any foreign substance that enters in the living system and how it will be changed into non-toxic and thrown out due to the enzymatic activity and other mode of action. When chemical toxins applied to non-living substance (soil and water) that will lose the toxic principles by degradation process due to the action of biotic and abiotic factors. The time to nullify the toxicity is of prime importance and it vary depending upon the chemical molecule and environmental conditions. Thus the study on metabolites, degradation and accumulation of pesticides become more important for pesticide assays. The second section of this book volume described about these aspects in three chapters.

I hope that this volume will provide an update on the state of pesticide science with relevance to pesticides residues and metabolism and will stimulate innovative and significant impact on researchers and students on the subject and go further in the line of research. I am grateful to all the authors who contributed their expertise towards the production of a precious volume.

I am indebted to Professor K.Gunathilagaraj, Tamil Nadu Agricultural University, India for his inspiration and eminent guidance to hone my skills in editing. I acknowledge Dr. N. Chitra my wife, for her support and encouragement during the book chapters review process.

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Pesticide Residue

Chapter 1

Exposure to Pesticides in Tomato Crop Farmers in Merced, Colombia: Effects on Health and the Environment

Marcela Varona Uribe, Sonia Mireya Díaz, Andrés Monroy, Edwin Barbosa, Martha Isabel Páez and René A. Castro

Additional information is available at the end of the chapter

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1. Introduction

The advance of chemicals in industry during the XX century gave rise to a number of highly aggressive compounds to human beings, and that altered the ecosystems balancing. Human population is inevitably exposed to environmental pollution through air-degraded products, water, the soil and food and their introduction into the food chain (Gomez et al, 2011).

The use of pesticides has been recognized and accepted as an essential ingredient in the modern agriculture for the control of pests, which damage crops and as a result, they produce a severe loss in food production. However, the extended use of pesticides, together with the inadequate behaviors of prevention and use of basic protection requisites will increase the probability of accidental intoxication in a notorious manner (Ntow et al, 2009), (Páez et al, 2011).

The estimated worldwide pesticides application is about 4 million tons (Elersek and Filipic, 2011) and according to Instituto Colombiano Agropecuario (ICA) Colombia produce 16.999.216 litters of herbicides, 6.392.387 litters of insecticides, and 19.690.293 kilograms of fungicides (ICA, 2010) during 2010.

Approximately 1.8 billion people worldwide are engaged in agriculture and it has been estimated that up to 25 million agriculture laborers have suffered non-intentional intoxications every year (Alavanja, 2008). In developing countries, pesticides are the cause of up to one million cases of intoxication and up to 20.000 deaths a year (Duran-Nah and Colli-Quintal, 2000).

Among the different pesticides used, 85% are used for agriculture applications and the remaining 15% are used in homes, gardens, business applications, public health and veterinary (Idrovo, 2000) (CEPIS/PAHO, 2005).

Certain works like agriculture or pests killing represent the biggest risks of acute intoxication, while there is a latent danger for the population at large in their food chain (Ospina et al, 2009) (Thundiyil et al, 2008).

The agricultural development model in Colombia is mainly based upon the use of agro chemicals and according data reported by the Public Health Surveillance System of Colombia (SIVIGILA), there were in 2008 6.650 intoxication cases for the use of pesticides followed by 7.405 cases in 2009 and 8.016 cases in 2010, being the organophosporic and carbamate pesticides the principal reasons for intoxications (SIVIGILA, 2010). Such pesticides are widely used agricultural inputs, and they are esters of the phosphoric acid and the derivates thereof, and they share in common as a pharmacological characteristic, the action of inhibiting enzymes having esteracic activities, and more specifically, the inhibition of the acetylcholinesterase. They are easily hydrolyze and they have a low capacity of remaining in the environment (Palacios and Moreno, 2004), (Chakraborty, 2009), (Ntow et al, 2009).

Other pesticides under study are the organochlorated, which are persistent, lypophilic and very steady. They can be accumulated in ecosystems, causing many toxic effects on reproduction, development and immunological functions of animals (Waliszewski et al, 2005). They have been universally reported in the adipose tissue and human serum (Rivas et al, 2007), (Côte et al, 2006).

This study determined the biomarkers the inner dosages, exposure and effect caused by the use of organophosphoric (OF), carbamates (C) and organochlorated (OC) pesticides. The levels for these pesticides were established in a sample of tomato and the good agriculture practices were implemented for the crops of tomato, which afforded to assure the crop sustainable management and the perception of hazards on the pesticides adequate usage and management.

2. Materials and methods

A descriptive cross section study was done including 132 laborers of the tomato crop in the location of la Merced – Caldas, during 2009 and 2010. This study considered three phases: the first was the diagnosis to determine the biomarkers for the chosen pesticides. An analysis was made on pesticides residues in tomatoes as well as the characterization of the present productive systems of the crop through a participating rural diagnosis. The second phase was intervention to guide the demonstration plots implementation wherein the good agricultural practices (GAPs) were shown which were compared to other plots managed under a traditional production system. The process of intervention was assessed during the last phase.

A questionnaire was applied including variables social, demographic, occupational, clinical, toxicological and tomatoes consumption habits. The pilot study was carried out on 10% of the total of the sample, although they did not make part of the research.

Following the criteria of inclusion, all laborers engaged in tomato planting entered into the study, provided that they were permanent residents in the community, who had used OF, C and OC pesticides at least during the six months previous to the study, and also who volunteered to participate in the study.

For the analysis of biological samples two blood samples were taken, one with 5 ml heparin for the determination of acetyl cholinesterase (AChE) and pseudo cholinesterase (PChE) by the technique of Michel and Aldrige (Vorhaus and Kark, 1953) and another of 5 ml without anticoagulant for the analysis of OC pesticides in serum. For this group 12 different pesticides (α-BHC, β-BHC, HCB, heptachlor, oxychlordane, α-chlordane, i-chlordane, α - endosulfan, β-endosulfan, 4,4-DDE, endosulfan and 2,4-DDT) were considered, which were determined by gas chromatography with electron micro capture (EPA, 1995) reporting the levels found.

With respect to the sampling of tomato it occurred at the beginning, on the highest peak and at the end of the production stage of tomato. For each sampling unit a zigzag path by the crop was followed, harvesting a tomato every three places along the zig-zag, and then based on a quartering system, obtaining a sample of 1 kg per plot. For determination of pesticide residues OF and OC the internal method for extraction AR-NE-03 was used, based on the multiresidue S-19 extraction method of the German Convention (DFG, 1987), followed by the gas chromatographic analysis with flame photometric detector FPD and ECD electron capture. Meanwhile, for the determination of residues of N-methyl carbamate an internal method based on W. Blass and C. Philipowsky (Blass and Philipowsky, 1992) was used. The levels found for these pesticides were considered as contamination.

A descriptive analysis by frequency counting, central trends measures and dispersion was made for those continuous type variables, as some of the variables inherent to laborers, and environmental and biological measurements. Continuous variables were transformed to normalize them. We also explored possible relationships between some variables and they were crossed by constructing contingency tables. We used the Student's t tests and chi square tests for the comparison of quantitative and categorical variables. Subsequently, we then performed a bivariate, stratified and logistic regression analysis. To compare results among laborers who worked in plots with GAP and traditional applications, the paired data test was used of Wilcoxon and Fisher for quantitative variables and the MacNemar test for qualitative variables. This study took into account Resolution 8430 of 1993 by the Ministry of Health, which classified this research as a minimal risk work. This study was approved by the Technical Committee of Research and by the Ethics Committee of the National Institute of Health.

3. Results analysis of the total population included in the study

3.1. Social and demographic variables

A total of 132 agricultural laborers were registered for the study, which were occupationally exposed to pesticides in the location of La Merced, belonging to the urban area 12,1% (16) and to the rural area 87,9% (116). Some general characteristics of the population are shown in Table 1.

With respect to gender, 90,9% (120) were men and 9,1% (12) women. We found a statistically significant difference between ages by sex $p < 0,05$. As for affiliation to the social security system, 99,2% (131) of individuals in the sample had some form of social health security.

3.2. Occupational history

At the time of the interview, 100,0% (132) of individuals reported to be engaged in agriculture, of them, five laborers were enrolled in a GAP program, which abolished the pesticide use as compared with other five who followed the traditional practices.

The time of exposure to pesticides ranged from three months to 35 years (Table 1), we found a statistically significant difference in the time of exposure to pesticides among men and women (p = 0,006).

Regarding the frequency of spraying 78,1% (104) of laborers reported applying at least once a week and 21,9% (28) used to apply pesticides every 15 days or more. Other variables related to the pesticide use are shown in Table 1.

Characteristics	Participants	Standard deviation	Range		Intervals of confidence
Age (years)					
Mean (SD) for both sexes	40,0	10,8	13	74	35,9-49,3
Men	39,7	10,8	18	69	37,7-41,6
Women	42,6	10,5	13	74	35,9-49,3
Time using pesticides (months)					
Men	109,2	88,1	3	420	94,8 - 127,2
Women	70,8	73,1	4	240	55,0 – 147,9
Hours a day of application					
Mean (SD)	5	2,5	------	------	--------
Smoking while using pesticides, N (%)					
Yes	10 (7,8%)	----	----	----	----
No	118 (92,2%)	----	----	----	----

Table 1. Population characteristics of location La Merced-Caldas, 2010.

In dealing about the storing of pesticides 95,5% (126) of laborers reported having an exclusive area and 19,5% (25) keep them indoors. On the use of personal protective equipment (PPE) a high percentage of laborers 96,2% (127) reported using some type of PPE, only 3,8% (5) did not use them, being the most frequent the use of the high heel boot (86,3%), and it is important to clarify that an employee may report using more than one element.

Laborers reported the greatest use of PPE related to protection of the body (120,5%), followed by protection of the lower limbs (93,9%), while the high boot the most common.

82,3% (105) of laborers said they were changing their work clothes at the end of the workday. The highest percentage of laborers 99,2% (131) washed their clothes at home and of these, 36 (27,3%) reported washing work clothes together with the rest of the family's clothes.

Types and trade names of pesticides used,	Active ingredient	Control group	Toxicological category	Quantity of application per harvest	Application number of applications per harvest	Standard deviation	Median	Maximum	Minimo
Organophosphoric Compounds									
Lorsban	Chlorpyrifos	Insecticide	III	25.414 L	63	101,55	2	500.000	0,12
Tamaron	Methamidophos	Insecticide	I	2,81 L	36	4,91	2	30	0,5
Monitor	Methamidophos	Insecticide	I	8.334 L	30	45,64	1	250.000	0,12
Roxion	Dimethoate	Insecticide	II	1,54 L	29	1,02	1	4	0,12
Sistemin	Dimethoate	Insecticide	II	9.093 L	22	42,63	1.125	200.000	0,1
Carbamate compounds									
Furadan	Carbofuran	Insecticide	I	17.648 L	51	73,36	2	400.000	0,2
Roundup	Glyphosate	Herbicide	IV	3.850 L	13	13,86	3	50.000	1

Table 2. Use and amount of pesticides studied and applied by agricultural laborers, in La Merced-Caldas, 2010.

An inquire was made on training on the safe handling of pesticides, being established that 74,2% (98) had never been trained, therefore, they had no knowledge about the use and handling of pesticides.

In relation to pesticide exposure, 74 (61,2%) reported having presented some symptoms at the time they were using them and of these 85,2% (63) did nothing with respect to this issue, or they self-medicated and only 14,8% (11) consulted a doctor. It was determined that Furadan was the pesticide causing most of intoxications to the population under study, being this toxicity category I (extremely toxic).

3.3. Use of pesticides

Pesticides reported by individuals in the sample evidenced that the most commonly used were the insecticides in OF group, of these Lorsban (chlorpyrifos) was the most widely used for a crop of tomatoes. The most frequently used toxicological category was II (highly toxic), followed by I (extremely toxic) (Table 2).

3.4. Clinical manifestations

The most frequently ailment reported was headache with 43,9%, followed by dizziness with 38,6%, weakness 36,4%, ocular burning 34,8% and redness of eyes with 31,8%. Grouped symptoms by systems most frequently found were in the central nervous system (95,5%) (Figure 1).

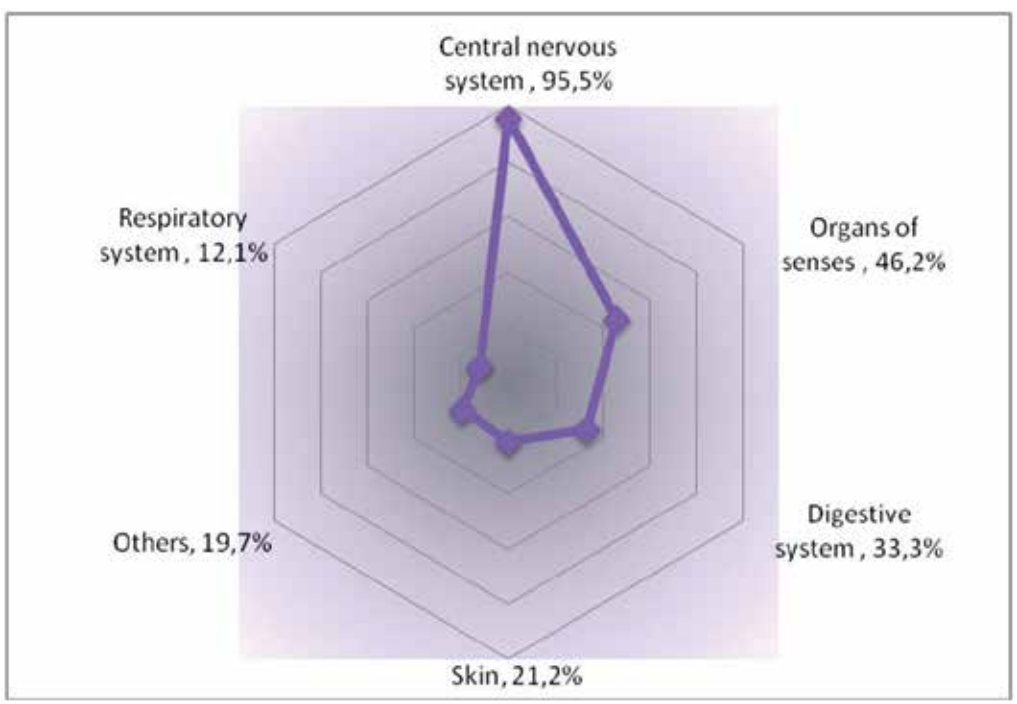

Figure 1. Distribution of systems, location of La Merced-Caldas 2010.

3.5. Biomarkers of internal dose, exposure and effect

Determinations were made for OC in 132 farm laborers, who showed an average level of 1,3 μg/L, with the highest values 4,4 DDE (mean = 3,4 μg/L, SD = 2,8, Minimum = 0,3, maximum = 13,5, IC = 2,9 to 4,0) and hepta chlorine (mean = 2,3 μg/L, SD = 1,0, = 6,1 Minimum, Maximum = 0,6, CI = 2,1 to 2,5). The average was obtained from the results of twelve OC pesticides, which perform the same mechanism of action.

Out of the total of workers, 45 (34,1%) showed inhibition of AChE enzyme in erythrocytes (mean = 0,84, SD = 0,020, IC = 0,80 to 0,88) and only one (0,8%) in plasma level (mean = 1,69, SD = 0,025, IC = 1,64 to 1,74). Of those with inhibition of the enzyme in erythrocytes, 14 (10,6%) individuals were below 25% with respect to the reference value.

4. Analysis of agricultural laborers included in the project demonstration plots

As specified in the methodology, 10 laborers were taken, 5 of which voluntarily agreed to participate in planting the tomato crop using the GAP, that is, pesticides were not used however a biological control was made. The remaining 5 laborers planted tomatoes as they were used to, using the pesticides frequently applied. The results shown below correspond to these 10 laborers who are involved in the project within the intervention phase.

It was seen that laborers of the parcels with GAP used more protection than those of conventional plots.

Plots were compared with GAP and traditional regarding the change of clothes at the completion of the workday and it was seen that laborers in both plots after receiving training in the proper use and handling of pesticides, successfully performed this activity, which became a protective factor.

4.1. Use of pesticides

Laborers who worked in traditional plots reported the use of seven pesticides, all of them being insecticides, of which six belong to the chemical group of OF and one belongs to C. The most frequently used pesticides were Lorsban and Latigo with 28,6% (4) each. Two of the seven employees, (28,6%) belonged to the toxicity category I, three (42,8%) to the toxicity classes II and two (28,6%) to the toxicity category III.

4.2. Clinical manifestations

With respect to manifestations grouped by systems, laborers who worked in the plots using GAP, showed more symptoms corresponding to organs of senses, while laborers in the traditional plot the referred symptomatology belonged to the central nervous system. In general, traditional plots laborers, had clinical manifestations more frequently in all systems, although there were no significant differences.

4.3. Biomarkers of internal dose, exposure and effect

With regards to biomarkers of exposure the presence of OC pesticide levels was found in serum, in laborers of both plots.

For OF and C the determination of the AChE enzyme showed some inhibition after the exposure in one of the five cases (20,0%) in conventional plots, while laborers belonging to GAP plots showed no inhibition. For plasma no inhibition of enzyme was seen in any worker.

4.4. Environmental samples

With regards to the samples of tomato, in crops were traditional practices were implemented, pesticide residues were found belonging to the chemical groups of OF (chlorpyrifos and phenthoate) and n-methyl carbamates (carbofuran and 3-hydroxycarbofuran), presenting the highest concentration of residues in OF. For plots with GAP the presence of residues of same pesticides n-methyl carbamates and chlorpyrifos was seen, being the only difference the finding of dimethoate. These active molecules were not in the formulation of products recommended by the agronomist for the control of pests and diseases.

Tomato production, had a statistically significant difference between plots with GAP and the traditional plots (p = 0,020) (Figure 2).

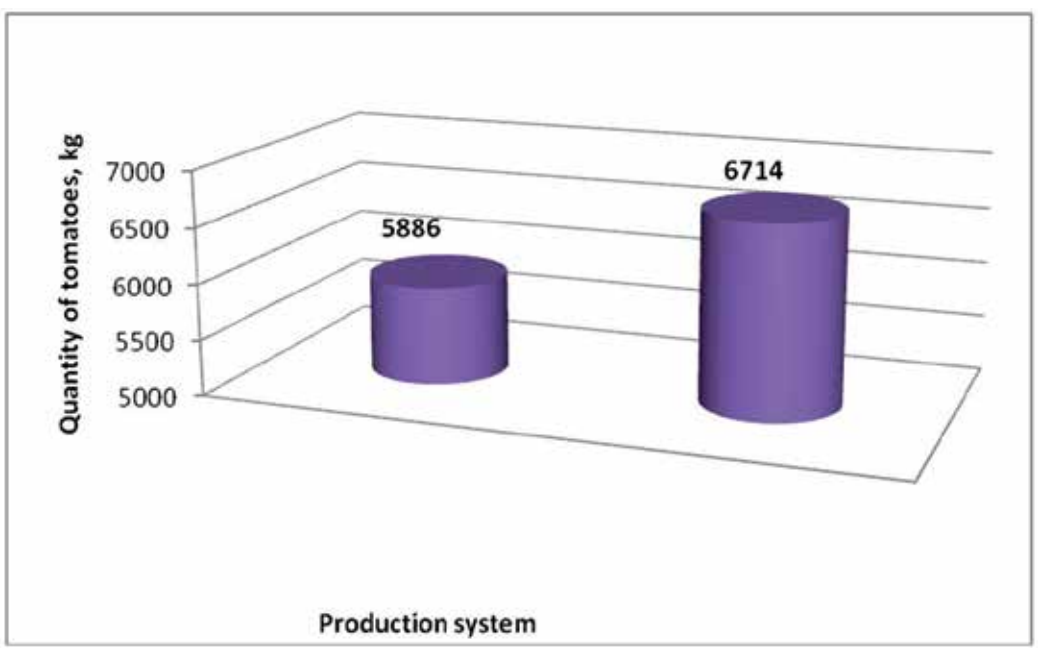

Figure 2. Comparison of production (kg of tomato) between production systems, in the location of La Merced-Caldas, 2010.

5. Discussion

Pesticides have been of great help to developing countries in their efforts to eradicate insects, endemic diseases and to produce adequate food (Alavanja, 2009), (Ecobichon, 2001). There is a controversy about the world's dependence on these agents, due to their excessive use, volatility, long-range transport and eventual contamination of the environment (Ecobichon, 2001).

In Colombia, pesticide exposure has become a public health problem (Ministry of Social Protection, 2003) due to the higher demand in the use thereof and to the impact on the population health and the environment.

According to this study, all laborers who were hired, used to work in the agricultural sector and they were occupationally exposed to pesticides, most of which laborers came from the rural area, and 90,9% belonged were males, with a wide age range from 18 to 69, indicating that this is a working population and that young adults are the ones most commonly hired to carry out agricultural activities. Although laborers who qualified for the study, used to be informal workers, and many of them had no working contract, a high percentage (87,1%) of individuals in the sample belonged to the subsidized system of health, in other words, they had health coverage.

Concerning the time of exposure to pesticides, this exposure was considered as chronic, as workers had been exposed for an extended period of time, having an average of 9 years of exposure, which can result in long-term harmful effects, being men who have longer exposure times because they are mostly engaged to farming. This data is also supported by the spraying operations frequency, since about 70% of laborers applied the products at least once a week at an average of 5 hours a day.

In dealing with the storage of pesticides, a high percentage of laborers (95,0%) reported having an exclusive area, which reduces exposure to both the worker and his family.

For EPP, (personal protection elements) laborers used to perform agricultural activities wearing clothes for work exclusively, being the high-leg boot the most commonly used item, as well as the disposable mouth masks. It is Important to point out that EPPs used by laborers were not commensurate with the risk they were exposed to, as for example, wearing face masks is not a proper practice for their protection as they have to handle these harmful chemicals, as they allow the entry via inhalation of pesticides, and it was also found that only a minority of laborers use gloves, allowing the entry of these substances by the dermal route, especially of those products having the characteristic of being lipo soluble pesticides such as OF and C. The parts of the body that were mostly protected were the trunk and lower limbs, being the upper limbs the ocular and respiratory regions the least frequently protected.

Although laborers reported that they changed their work clothes at the end of the workday, they washed their clothes at home mixing it with the clothes of the rest of their family, exposing their family members to intoxication risk by such substances.

We inquired about the training courses that laborers have received at some point in their working lives on topics like the safe handling of pesticides and it was found that 74,2% had never been trained, so they did not have the necessary skills to handle such substances.

Only a small percentage reported to see a doctor when they showed some kind of symptoms when they used pesticides, while others took home remedies or they medicated themselves which results in an underreporting of cases of poisoning by such substances, as the intoxication cases are not reported to the system of public health surveillance in Colombia (SIVIGILA).

A high number of pesticides is used in Colombia primarily in categories I and II toxicological categories, and by chemical group the OF and C. This information is confirmed by other studies conducted in Colombia (Varona et al, 2007, 2009), (SIVIGILA, 2010), thus increasing the chances of triggering effects on health. Among the clinical manifestations reported by laborers, most of them are related to neurological and sensory organs disorders. Neurological disorders, may be related to pesticides OF and C, while manifestations of sense organs can be triggered by the use of multiple chemicals, including pesticides that are the subject of this study. The same occurs with the manifestations of the digestive system, which can onset by the ingestion of different chemicals, although they can also have a bacterial and viral origin, among other causes.

This study used biomarkers of internal dose, exposure and effect, which allowed setting the pesticides levels in 132 biological samples. OC pesticides which were most frequently found in biological samples were 4,4-DDE and endosulfan. It is important to point out that laborers did not report the use of these pesticides in the tomato crop, so the presence of these is explained by the environmental pollution and toxicokinetics inherent to this group of pesticides. Although OC were banned in the country since 1993 due to their high persistence, their ability of bio-magnification and their neurotoxic effects, they were still used for about 40 years.

The determination of enzyme AChE continues to be widely used to measure the exposure to OF and C, however, interpretations of results are highly variable, since there are genetic and physiological causes as well as associated pathologies, which can decrease the levels of this enzyme (Varona et al, 2007).

In addition, there is a significant variation within the same individual, therefore, the medical surveillance of laborers continuously exposed to these two groups of pesticides must also include not only the medical examination, but also the determination of enzyme AChE pre-exposure (baseline) and quarterly for the duration of the exposure (Varona et al, 2007).

This study reported that 34,1% of all laborers showed inhibition of this enzyme in erythrocytes, which confirms the fact that OF and C compounds are the most used by the laborers included in the study.

Within the research project and as stated earlier in the methodology, a second phase known as intervention was conducted. During this stage 10 individuals were included, who had some schooling, which allowed them to gain a better understanding of the concepts used in training on GAP and thus the implementation thereof was facilitated.

It was found that laborers used pesticides in plots with GAP, but his recommendation was not given by the agronomist, while in conventional plots they reported the use of pesticides inhibiting the AChE, which are classified as extremely and highly toxic causing a large exposure to this chemical group of pesticides, which in turn affect the central nervous system, a situation that is related to the clinical manifestations reported by laborers in the study of these plots (Idrovo, 2000), (Cassaret and Doull, 2005) (Goldfrank et al, 2006). Regarding the use of EPP, laborers of parcels with GAP used more protection, especially respiratory and eye protection than the conventional plots.

The analysis of biomarkers of exposure and effect for laborers who participated in the intervention phase showed the presence of OC pesticide levels for both traditional plots for GAP. None of the laborers reported the use of these pesticides in the tomato crop, as stated in the diagnostic phase, and their presence is due to their high persistence and their ability of bio magnification. Despite of the fact that their use is prohibited in Colombia, it is not uncommon to identify patients with acute and chronic effects resulting from the exposure to this type of pesticides (Varona et al, 2010), (Córdoba, 2006).

The determination of enzyme AChE showed the after exposure inhibition in a worker of conventional plots, however, this reduction did not require any treatment but a medical surveillance. It is noteworthy to say that there was not a greater number of laborers with inhibition of AChE enzyme considering that in conventional plots the use of OF and C pesticides was reported. This can be explained because this group of pesticides are easily hydrolyzed and excreted by renal way, and there is no bioaccumulation or bio magnification, but this is also due to the fact, that the specimen taken for the determination of the enzyme AChE, should take place within 24 hours after the exposure as a maximum. Since this enzyme subsides to the exposure, it starts to regenerate and therefore, it cannot show the true percentage of inhibition.

When comparing the production of tomato obtained by the two crop systems, it is seen that the range of average production of crops following the traditional practices were very similar to the crops following the GAP. However, when comparing the average tomato production, crops with GAP showed a statistically significant difference higher than the results attained by the traditional system. This behavior can be explained because the fertilization plan in plots using GAP satisfactorily met the needs of the soil.

Regarding the pesticides residues that followed traditional practices, we have found that OF showed the highest levels of residues in the initial stage of crops, where chlorpyrifos was the highly concentrated pesticide but it did not exceed the Codex maximum residue limits (MRLs). For crops established with GAP, we found that five crops presented residues of pesticides the active molecules of which were not recommended by the agronomist for the control of pests and diseases. It was also found that the highest concentration of residuals detected were due to the use of OF.

Although there was no evidence of commitment to health in terms of the effects assessed in this study, we detected significant correlation with respect to one of the traditional plots. It

was reported for that plot that one of the individuals presented cholinesterase inhibition and coincidentally it is the plot where the residues in tomato reported the presence of chlorpyrifos. This is a situation of high concern, because this means that farmers in this area are significantly applying pesticides at this time.

The above implies that there is no risk perception by farmers, which makes it necessary to carry out educational campaigns to warn them about the need of at least meeting the rules and procedures laid down for each product. It is recommended to continue with the follow-up and support to this type of population through training and sensitization of laborers in an attempt to reduce the pesticide use and as a result to reduce the effects on their health from exposure to such substances.

It is necessary to strengthen farmers in the implementation of GAP and the advantages of this production system.

The use of chemical pesticides should be performed following the manufacturer's technical recommendations such as presence of pests, application dose, frequency of application and exhaust period in the context of an integrated pest management program which reduces the risk of finding concentrations exceeding the MRL which implies some weakness of competitiveness of crops.

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Chapter 2

Residue of DDT and HCH in Fish from Lakes and Rivers in the World

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Additional information is available at the end of the chapter

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1. Introduction

Organochlorine pesticides such as DDT and HCH had been abundantly produced and used since the around twentieth century in all over the world and environmental pollution, food contamination and human body pollution had developed into social problem because of their high bioconcentration potential and high persistency. The use and manufacturing of DDT and HCH were forbidden in many countries including Japan in all over the world in the first half in 1970's.

In Japan, the use of organochlorine pesticides registered in persistent organic pollutants (POPs) was prohibited, for example, in 1971 for DDT and HCH and in 1986 for chlordane. Levels of DDT and HCH have been monitored annually in wildlife such as fish, shellfish and bird from a freshwater lake, 17 sea areas and 2 land areas in Japan since 1979 [1-31]. Lake Biwa, the largest in Japan, was selected as a freshwater lake and a freshwater fish, Japanese dace from the lake was selected as a wildlife sample.

In the world, a few reports have been published for long-term monitoring of POPs in aquatic biota from lakes only in USA and Sweden. In USA, the use of DDT was prohibited in 1972. Levels of POPs such as DDT, chlordanes, Mirex, Dieldrin have been monitored annually in fish from the Grate Lakes since 1970 [32, 33]. In Sweden, the use of DDT was prohibited in 1970. Levels of DDT, HCB and HCH have been monitored annually in fish from Lake Storvindeln since 1968 [34].

On the other hand, many field data have been published for temporal monitoring of DDT and HCH in fish from lakes [35-70] and rivers [40, 51, 68, 71-101].

This study was performed for the accumulation of fundamental data on DDT and HCH contamination of fish in lakes and rivers in the world to evaluate their concentration changes by POPs Regulation. The data were collected from the published reports in which

the accuracy in the chemical analyses of the pesticides was over the standard level. This chapter consisted of (1) Residue of T-DDT and T-HCH in fish from lakes and rivers in the world, (2) Long-term trends of T-DDT and T-HCH in fish from lakes in the world, (3) Composition of T-DDT and T-HCH in fish from lakes and rivers in the world.

1. Each of the T-DDT and T-HCH concentration data in fish from lakes and rivers was compared for the 38 lakes surveyed in 8 countries of Europe and America and 8 countries of Asia and Africa from 1995 to 2008 and for the 28 rivers surveyed in 8 countries of Europe and America, 4 countries of Asia, Africa and Oceania from 2000 to 2009.
2. Long-term trends of T-DDT and T-HCH in Japanese dace from Lake Biwa were shown from 1979 to 2009 and half-lives ($t_{1/2}$) were calculated for T-DDT and T-HCH. The $t_{1/2}$ values were 9 years for T-DDT and 4 years for T-HCH. Similarly, long-term trends of T-DDT in fish from Lake Biwa, Lake Ontario, Lake Michigan and Lake Storvindeln were shown and the $t_{1/2}$ values of T-DDT were calculated. The $t_{1/2}$ values were 9, 11, 8 and 7 years, respectively, in Lake Biwa, Lake Ontario, Lake Michigan and Lake Storvindeln. There were no wide differences in the $t_{1/2}$ values between the four lakes.
3. Composition of T-DDT in fish from lakes and rivers in the world was compared for the 25 lakes in 15 countries of Europe, America, Asia and Africa from 1996 to 2008 and for the 16 rivers in 8 countries of Europe, America, Asia, Africa and Oceania from 2000 to 2009. Similarly, composition of T-HCH in fish from lakes and rivers in the world was compared for the 16 lakes in 8 countries of Europe, America, Asia and Africa from 1996 to 2008 and for the 11 rivers in 5 countries of Europe and Asia from 2001 to 2006.

2. Residue of T-DDT and T-HCH in fish from lakes and rivers in the world

Residue of T-DDT and T-HCH in fish from lakes and rivers in the world (Survey years: 1995~2009) was reviewed from literatures in the past. The residue data were summarized in Table 1 for the lakes [22-31, 35-70] and in Table 2 for the rivers [40, 51, 68, 71-101].

No.	Species	n[a]	Analyte	Lake	Year	Country	T-HCH [b]	T-DDT [c]	References
L-1	Japanese dace	5	Muscle	Lake Biwa	2000	Japan	3 ng/g wet wt	13 ng/g wet wt	Ministry of the Environment, Japan (2002)
L-2	Japanese dace	5	Muscle	Lake Biwa	2001	Japan	2 ng/g wet wt	10 ng/g wet wt	Ministry of the Environment, Japan (2003)
L-3	Japanese dace	5	Muscle	Lake Biwa	2002	Japan	1.79 ng/g wet wt	6.5 ng/g wet wt	Ministry of the Environment, Japan (2004)
L-4	Japanese dace	5	Muscle	Lake Biwa	2003	Japan	0.97 ng/g wet wt	8.0 ng/g wet wt	Ministry of the Environment, Japan (2005)
L-5	Japanese dace	5	Muscle	Lake Biwa	2004	Japan	0.55 ng/g wet wt	8.4 ng/g wet wt	Ministry of the Environment, Japan (2006)
L-6	Japanese dace	5	Muscle	Lake Biwa	2005	Japan	0.29 ng/g wet wt	9.3 ng/g wet wt	Ministry of the Environment, Japan (2007)

No.	Species	n[a]	Analyte	Lake	Year	Country	T-HCH [b]	T-DDT [c]	References
L-7	Japanese dace	5	Muscle	Lake Biwa	2006	Japan	0.90 ng/g wet wt	8.3 ng/g wet wt	Ministry of the Environment, Japan (2008)
L-8	Raibow trout (male)	4	Muscle	Lake Mashu	2003	Japan	2.98 ng/g wet wt	1.49 ng/g wet wt	Takazawa et al. (2005)
L-9	Raibow trout (female)	6	Muscle	Lake Mashu	2003	Japan	2.71 ng/g wet wt	1.72 ng/g wet wt	Takazawa et al. (2005)
L-10	Japanese dace (male)	3	Muscle	Lake Mashu	2003	Japan	1.75 ng/g wet wt	0.66 ng/g wet wt	Takazawa et al. (2005)
L-11	Japanese dace (female)	6	Muscle	Lake Mashu	2003	Japan	2.45 ng/g wet wt	0.56 ng/g wet wt	Takazawa et al. (2005)
L-12	Raibow trout (male)	3	Muscle	Lake Mashu	2002	Japan	3.23 ng/g wet wt	3.50 ng/g wet wt	Takazawa et al. (2005)
L-13	Raibow trout (female)	9	Muscle	Lake Mashu	2002	Japan	2.19 ng/g wet wt	1.27 ng/g wet wt	Takazawa et al. (2005)
L-14	Unkown	6	Whole	Taihu Lake Region	1999~2000	China	46 ng/g wet wt	12 ng/g wet wt	Feng et al. (2003)
L-15	Carp	3	Whole	Lake Tai	2000	China	64 ng/g fat wt	980 ng/g fat wt	Nakata et al. (2005)
L-16	Topmouth culter	3	Whole	Lake Tai	2000	China	67 ng/g fat wt	750 ng/g fat wt	Nakata et al. (2005)
L-17	Spotted steed	3	Whole	Lake Tai	2000	China	75 ng/g fat wt	700 ng/g fat wt	Nakata et al. (2005)
L-18	Catfish	3	Whole	Lake Tai	2000	China	68 ng/g fat wt	1000 ng/g fat wt	Nakata et al. (2005)
L-19	*Gymoncypris namensis*	4	Muscle	Nam Co Lake	2005	China	2.57 ng/g wet wt	17.2 ng/g wet wt	Yang et al. (2007)
L-20	*Gymoncypris waddellii*	4	Muscle	Yamdro Lake	2005	China	1.56 ng/g wet wt	2.76 ng/g wet wt	Yang et al. (2007)
L-21	*C. auratus*	8	Edible part	Gaobeidian Lake (Beijing)	2006	China	6.41 ng/g wet wt	21.96 ng/g wet wt	Li et al. (2008)
L-22	*M. anguillicaudatus*	5	Edible part	Gaobeidian Lake (Beijing)	2006	China	2.61 ng/g wet wt	14.08 ng/g wet wt	Li et al. (2008)
L-23	*H. leuciscultures*	8	Edible part	Gaobeidian Lake (Beijing)	2006	China	11.14 ng/g wet wt	84.4 ng/g wet wt	Li et al. (2008)
L-24	Herbivorous	9	Muscle	Songkhla Lake (Thale Luang)	1997	Thailand		170 ng/g fat wt	Kumblad et al. (2001)
L-25	Herbivorous	10	Muscle	Songkhla Lake (Thale Sap)	1997	Thailand		36 ng/g fat wt	Kumblad et al. (2001)
L-26	Herbivorous	8	Muscle	Songkhla Lake (Thale Sap Songkhla)	1997	Thailand		35 ng/g fat wt	Kumblad et al. (2001)
L-27	*Channa striata*	64	Muscle	Kolleru Lake	Unkown	India			Amaraneri & Pillala (2001)
L-28	*Channa striata*	56	Liver	Kolleru Lake	Unkown	India			Amaraneri & Pillala (2001)
L-29	*Catla catla*	58	Muscle	Kolleru Lake	Unkown	India			Amaraneri & Pillala (2001)
L-30	*Catla catla*	38	Liver	Kolleru Lake	Unkown	India			Amaraneri & Pillala (2001)
L-31	*P. phuturio*	2	Whole	Haleji Lake	1999	Pakistan		4.55 ng/g wet wt	Sanpera et al. (2002)
L-32	*C. lalia*	3	Whole	Haleji Lake	1999	Pakistan		5.58 ng/g wet wt	Sanpera et al. (2002)
L-33	*G. giuris*	1	Whole	Haleji Lake	1999	Pakistan		5.94 ng/g wet wt	Sanpera et al. (2002)

No.	Species	n[a]	Analyte	Lake	Year	Country	T-HCH [b]	T-DDT [c]	References
L-34	Several kinds	81	Edible part	Lake Jarun	2000	Croatia	0.40 ng/g wet wt*	0.80 ng/g wet wt*	Bosnir et al. (2007)
L-35	Arctic char	25	Muscle	Lake Ellasjøen	1996	Norway		60 ng/g wet wt	Evenset et al. (2004)
L-36	Arctic char	12	Muscle	Lake Øyangen	1996	Norway		4.3 ng/g wet wt	Evenset et al. (2004)
L-37	Whitefish (Female) 100-200g	13	Muscle	Lake Stuorajavri	2005	Norway	ND	0.35 ng/g wet wt	Christensen et al. (2007)
L-38	Whitefish (Male) 100-200g	10	Muscle	Lake Stuorajavri	2005	Norway	ND	0.41 ng/g wet wt	Christensen et al. (2007)
L-39	Pike	5	Muscle	Lake Stuorajavri	2005	Norway	ND	0.24 ng/g wet wt	Christensen et al. (2007)
L-40	Brown trout	1	Muscle	L. Tuma (remote alpine lake)	2003	Switzerland		1100 ng/g fat wt (+op')	Schmid et al. (2007)
L-41	Brown trout	1	Muscle	L. Moesola (remote alpine lake)	2003	Switzerland		680 ng/g fat wt(+op')	Schmid et al. (2007)
L-42	Lake trout	1	Muscle	L. Diavolezza (remote alpine lake)	2003	Switzerland		130 ng/g fat wt(+op')	Schmid et al. (2007)
L-43	Catfish	8	Muscle	Lake Trasimeno	1998	Italy	14.3 ng/g fat wt	216 ng/g fat wt	Elia et al. (2006)
L-44	Mullet (*Lisa aurata*)	13	Muscle	Lake Ganzirri	2001	Italy		3.8 ng/g wet wt (Max.)	Licata et al. (2003)
L-45	Landlocked	5	Whole	Lake Maggiore	2002-2004	Italy		2500 ng/g fat wt	Bettinetti et al. (2006)
L-46	Whitefish	5	Whole	Lake Maggiore	2002-2004	Italy		1370 ng/g fat wt	Bettinetti et al. (2006)
L-47	Perch	5	Whole	Lake Maggiore	2002-2004	Italy		1860 ng/g fat wt	Bettinetti et al. (2006)
L-48	Chub	5	Whole	Lake Maggiore	2002-2004	Italy		1190 ng/g fat wt	Bettinetti et al. (2006)
L-49	Rudd	5	Whole	Lake Maggiore	2002-2004	Italy		2770 ng/g fat wt	Bettinetti et al. (2006)
L-50	Tench	5	Whole	Lake Maggiore	2002-2004	Italy		2720 ng/g fat wt	Bettinetti et al. (2006)
L-51	Perch	1	Muscle	Bolsena Lake	2002	Italy	0.02 ng/g wet wt	2.26 ng/g wet wt	Orban et al. (2007)
L-52	Perch	1	Muscle	Bracciano Lake	2002	Italy	0.09 ng/g wet wt	0.38 ng/g wet wt	Orban et al. (2007)
L-53	Perch	1	Muscle	Salto Lake	2002	Italy	0.03 ng/g wet wt	0.61 ng/g wet wt	Orban et al. (2007)
L-54	Pelagic landlocked shad	1	Muscle	Lake Como (Como branch)	2006	Italy		1010 ng/g fat wt	Bettinetti et al. (2008)
L-55	Pelagic landlocked shad	1	Muscle	Lake Como (Como branch)	2007	Italy		840 ng/g fat wt	Bettinetti et al. (2008)
L-56	Pelagic landlocked shad	1	Muscle	Lake Como (Lecco branch)	2007	Italy		610 ng/g fat wt	Bettinetti et al. (2008)
L-57	Pelagic landlocked shad	1	Muscle	Lake Iseo	2007	Italy		570 ng/g fat wt	Bettinetti et al. (2008)

No.	Species	n[a]	Analyte	Lake	Year	Country	T-HCH [b]	T-DDT [c]	References
L-58	Pikeperch	4	Muscle	Beysehir Lake	Unkown	Turkey	70 ng/g wet wt	27 ng/g wet wt	Aktumsek et al. (2002)
L-59	Carp	17	Muscle	Sir Dam Lake	2003	Turkey	0.21 ng/g wet wt*	14.4 ng/g wet wt*	Erdogrul et al. (2005)
L-60	*Oresochromus niloticus*	4	Muscle	Lake Burullus	2006	Egypt	1.88 ng/g wet wt	5.13 ng/g wet wt	Said et al. (2008)
L-61	*Clarries sp.*	4	Muscle	Lake Burullus	2006	Egypt	9.83 ng/g wet wt	12.54 ng/g wet wt	Said et al. (2008)
L-62	*Bagrus meridionalis*	4	Muscle	Lake Malawi	1996, 1997	East Africa		13.1 ng/g wet wt	Kidd et al. (2001)
L-63	*Buccochromis nototaenia*	2	Muscle	Lake Malawi	1996, 1997	East Africa		3.4 ng/g wet wt	Kidd et al. (2001)
L-64	*Clarius sp.*	1	Muscle	Lake Malawi	1996, 1997	East Africa		1.4 ng/g wet wt	Kidd et al. (2001)
L-65	*Engraulicyprus sardella*	6	Whole	Lake Malawi	1996, 1997	East Africa		4.5 ng/g wet wt	Kidd et al. (2001)
L-66	*Genyochromis mento*	5	Whole	Lake Malawi	1996, 1997	East Africa		1.0 ng/g wet wt	Kidd et al. (2001)
L-67	*Labeotropheus fuelleborni*	6	Whole	Lake Malawi	1996, 1997	East Africa		1.1 ng/g wet wt	Kidd et al. (2001)
L-68	*Boulengerochromis microlepis*	1	Whole	Lake Tanganyika (North end)	1999	Burundi	288.2 ng/g fat wt	794.7 ng/g fat wt	Manirakiza et al. (2002)
L-69	*Chrysichthys sianenna*	1	Whole	Lake Tanganyika (North end)	1999	Burundi	90.6 ng/g fat wt	339.3 ng/g fat wt	Manirakiza et al. (2002)
L-70	*Oreochromis niloticus*	1	Whole	Lake Tanganyika (North end)	1999	Burundi	66.2 ng/g fat wt	393.1 ng/g fat wt	Manirakiza et al. (2002)
L-71	*Limnothrissa miodon*	1	Whole	Lake Tanganyika (North end)	1999	Burundi	21.2 ng/g fat wt	60.7 ng/g fat wt	Manirakiza et al. (2002)
L-72	*Stolothrissa tanganyikae*	1	Whole	Lake Tanganyika (North end)	1999	Burundi	55.1 ng/g fat wt	95.7 ng/g fat wt	Manirakiza et al. (2002)
L-73	Nile tilapia	43	Edible part	Lake Victoria (Napoleon Gulf)	1998	Uganda		1.39 ng/g wet wt	Kasozi et al. (2006)
L-74	Nile perch	37	Edible part	Lake Victoria (Napoleon Gulf)	1998	Uganda		1.67 ng/g wet wt	Kasozi et al. (2006)
L-75	Nile tilapia and Nile perch		Muscle	Lake Victoria (Kome Island)	1999	Tanzania		20 ng/g wet wt	Henry & Kishimba (2006)
L-76	Nile tilapia and Nile perch		Muscle	Lake Victoria (Katunguru)	1999	Tanzania		15 ng/g wet wt	Henry & Kishimba (2006)

No.	Species	n[a]	Analyte	Lake	Year	Country	T-HCH [b]	T-DDT [c]	References
L-77	Nile tilapia	10	Muscle	Lake Taabo	Unkown	Cote d'Ivoire	225.8 ng/g dry wt	124.1 ng/g dry wt	Roche et al. (2007)
L-78	African catfish	7	Muscle	Lake Taabo	Unkown	Cote d'Ivoire	130.0 ng/g dry wt	94.6 ng/g dry wt	Roche et al. (2007)
L-79	Raibow trout	3	Muscle	Rae Lake (Sierra Nevada)	1995, 1996	USA		1.24 ng/g wet wt	Ohyama et al. (2004)
L-80	Raibow trout	6	Muscle	Blue Lake (Sierra Nevada)	1995, 1996	USA		2.13 ng/g wet wt	Ohyama et al. (2004)
L-81	Raibow trout	5	Muscle	Pear Lake (Sierra Nevada)	1995, 1996	USA		9.99 ng/g wet wt	Ohyama et al. (2004)
L-82	Raibow trout	2	Muscle	Donner Lake (Sierra Nevada)	1995, 1996	USA		3.71 ng/g wet wt	Ohyama et al. (2004)
L-83	Raibow trout	4	Muscle	Huntingto n Lake (Sierra Nevada)	1995, 1996	USA		30.55 ng/g wet wt	Ohyama et al. (2004)
L-84	Raibow trout	5	Muscle	Lake Berryessa (Sierra Nevada)	1995, 1996	USA		13.01 ng/g wet wt	Ohyama et al. (2004)
L-85	Lake trout	10	Muscle	Lake Chelan (Wapato Basin)	2003	USA		943 ng/g wet wt	Washington State Department of Ecology (2005)
L-86	Burbot	7	Muscle	Lake Chelan (Wapato Basin)	2003	USA		315 ng/g wet wt	
L-87	Kokanee	7	Muscle	Lake Chelan (Wapato Basin)	2003	USA		57 ng/g wet wt	
L-88	Raibow trout	3	Muscle	Lake Chelan (Wapato Basin)	2003	USA		14 ng/g wet wt	
L-89	Burbot	3	Muscle	Lake Chelan (Lucerne Basin)	2003	USA		22 ng/g wet wt	
L-90	Raibow trout	1	Muscle	Roses Lake	2003	USA		96 ng/g wet wt	
L-91	Black Crappie	1	Muscle	Roses Lake	2003	USA		32 ng/g wet wt	
L-92	Lg Scale Sucker	15	Muscle	Vancouver Lake	2005-2006	USA	ND	23 ng/g wet wt	Washington State Department of Ecology (2007)
L-93	Common Carp	15	Muscle	Vancouver Lake	2006	USA	ND	65 ng/g wet wt	
L-94	Lg Mouth Bass	5	Muscle	Vancouver Lake	2006	USA	ND	42 ng/g wet wt	
L-95	N. pikeminnow (Large size)	10	Whole	Lake Washington	2001-2003	USA		258 ng/g wet wt	McIntyre & Beauchamp (2007)

No.	Species	n[a]	Analyte	Lake	Year	Country	T-HCH [b]	T-DDT [c]	References
L-96	Cutthroat trout (Large size)	10	Whole	Lake Washington	2001-2003	USA		168 ng/g wet wt	McIntyre & Beauchamp (2007)
L-97	Yellow perch (Large size)	9	Whole	Lake Washington	2001-2003	USA		59 ng/g wet wt	McIntyre & Beauchamp (2007)
L-98	Smallmouth bass (Large size)	3	Whole	Lake Washington	2001-2003	USA		63 ng/g wet wt	McIntyre & Beauchamp (2007)
L-99	Lake trout	10	Muscle	Kusawa Lake	1993	Canada	1.21 ng/g wet wt	40.85 ng/g wet wt	Ryan et al. (2005)
L-100	Lake trout	14	Muscle	Kusawa Lake	1999	Canada	1.68 ng/g wet wt	122.43 ng/g wet wt	Ryan et al. (2005)
L-101	Lake trout	9	Muscle	Kusawa Lake	2001	Canada	0.91 ng/g wet wt	49.71 ng/g wet wt	Ryan et al. (2005)
L-102	Lake trout	10	Muscle	Kusawa Lake	2002	Canada	0.62 ng/g wet wt	23.51 ng/g wet wt	Ryan et al. (2005)
L-103	Lake trout	24	Muscle	Lake Laberge	1993	Canada	4.69 ng/g wet wt	360.87 ng/g wet wt	Ryan et al. (2005)
L-104	Lake trout	13	Muscle	Lake Laberge	1996	Canada	6.50 ng/g wet wt	205.54 ng/g wet wt	Ryan et al. (2005)
L-105	Lake trout	5	Muscle	Lake Laberge	2000	Canada	2.30 ng/g wet wt	82.96 ng/g wet wt	Ryan et al. (2005)
L-106	Lake trout	16	Muscle	Lake Laberge	2001	Canada	0.80 ng/g wet wt	75.09 ng/g wet wt	Ryan et al. (2005)
L-107	Lake trout	5	Muscle	Lake Laberge	2002	Canada	1.58 ng/g wet wt	43.56 ng/g wet wt	Ryan et al. (2005)
L-108	Lake trout	8	Muscle	Lake Laberge	2003	Canada	0.54 ng/g wet wt	55.81 ng/g wet wt	Ryan et al. (2005)
L-109	Trahira (*Hoplias malabaricus*)	10	Muscle	Ponta Grossa Lake	2005	Brazil		92.3 ng/g dry wt	Miranda et al. (2008)
L-110	Trahira (*Hoplias malabaricus*)	10	Liver	Ponta Grossa Lake	2005	Brazil		54.68 ng/g dry wt	Miranda et al. (2008)
L-111	Japanese dace	5	Muscle	Lake Biwa	2007	Japan	0.51 ng/g wet wt	6.9 ng/g wet wt	Ministry of the Environment, Japan (2009)
L-112	Japanese dace	5	Muscle	Lake Biwa	2008	Japan	0.68 ng/g wet wt	8.0 ng/g wet wt	Ministry of the Environment, Japan (2010)
L-113	Japanese dace	5	Muscle	Lake Biwa	2009	Japan	0.41 ng/g wet wt	8.0 ng/g wet wt	Ministry of the Environment, Japan (2011)
L-114	Semutundu (*Bagrus docmac*)		Muscle	Lake Edward	Unkown	Uganda		33 ng/g wet wt	Ssebugere et al. (2009)
L-115	Mamba (*Protopterus aethiopinus*)		Muscle	Lake Edward	Unkown	Uganda		29 ng/g wet wt	Ssebugere et al. (2009)
L-116	Enjunguri (*Haprochromis nigripinnis*)		Muscle	Lake Edward	Unkown	Uganda		ND	Ssebugere et al. (2009)
L-117	Nile tilapia (*Oreochromis niloticus*)		Muscle	Lake Edward	Unkown	Uganda		33 ng/g wet wt	Ssebugere et al. (2009)

No.	Species	n[a]	Analyte	Lake	Year	Country	T-HCH [b]	T-DDT [c]	References
L-118	Male (*Clarias gariepinus*)		Muscle	Lake Edward	Unkown	Uganda		ND	Ssebugere et al. (2009)
L-119	Tilapia and Catfish	13	Edible part	Lake Volta, Lake Bosumtwi, Weija Lake	2008	Ghana	0.72 ng/g fat wt	329.4 ng/g fat wt	Adu-Kumi et al. (2010)
L-120	6 kinds of fish	60	Muscle	Lakes (n=8) in Tibetan Plateau	2006-2007	China	0.55 ng/g wet wt	4.0 ng/g wet wt (+op')	Yang et al. (2010)
L-121	Common carp	23	Unknown	Baiyang-dian Lake	2008	China	0.38 ng/g wet wt	1.28 ng/g wet wt	Dai et al. (2011)
L-122	Crucian carp	25	Unknown	Baiyan-gdian Lake	2008	China	0.47 ng/g wet wt	1.03 ng/g wet wt	Dai et al. (2011)
L-123	Crucian carp	1	Muscle	Lake Como	2007	Italy	1.0 ng/g dry wt	1.03 ng/g dry wt (+op')	Villa et al. (2011)
L-124	White fish	1	Muscle	Lake Como	2007	Italy	7.35 ng/g dry wt	12.4 ng/g dry wt (+op')	Villa et al. (2011)
L-125	Pike	1	Muscle	Lake Como	2007	Italy	0.4 ng/g dry wt	4.89 ng/g dry wt (+op')	Villa et al. (2011)
L-126	Chub	1	Muscle	Lake Como	2007	Italy	ND	5.89 ng/g dry wt (+op')	Villa et al. (2011)
L-127	Perch	1	Muscle	Lake Como	2007	Italy	0.7 ng/g dry wt	7.75 ng/g dry wt (+op')	Villa et al. (2011)
L-128	Pikeperch	1	Muscle	Lake Como	2007	Italy	0.57 ng/g dry wt	10.4 ng/g dry wt (+op')	Villa et al. (2011)

[a] No. of analyzed samples; Mean (n≥2) or single determination values (n=1) are listed for PCB, T-HCH and T-DDT (* Median value)
[b] T-HCH = α-HCH + β-HCH + γ-HCH
[c] T-DDT = pp'-DDE + pp'-DDD + PP'-DDT

Table 1. Concentrations of T-HCH and T-DDT in fish from lakes in the world

No.	Species	n[a]	Analyte	River	Year	Country	T-HCH [b]	T-DDT [c]	References
R-1	Color gudgeon	10	Whole	Guanting Reservoir	2002	China	7.15 ng/g wet wt	9.23 ng/g wet wt	Sun et al. (2005)
R-2	Feral carp	10	Whole	Guanting Reservoir	2002	China	0.72 ng/g wet wt	5.04 ng/g wet wt	Sun et al. (2005)
R-3	White fish	1	Unknown	Qiantang River (Downstream)	2005	China	3.96 ng/g wet wt	13.51 ng/g wet wt	Zhou et al. (2007)
R-4	Crucian carp	1	Unknown	Qiantang River (Downstream)	2005	China	3.84 ng/g wet wt	5.64 ng/g wet wt	Zhou et al. (2007)
R-5	Perch	1	Unknown	Qiantang River (Downstream)	2005	China	2.62 ng/g wet wt	8.34 ng/g wet wt	Zhou et al. (2007)
R-6	Snake head mullet	1	Unknown	Qiantang River (Upstream)	2005	China	3.18 ng/g wet wt	5.01 ng/g wet wt	Zhou et al. (2007)
R-7	Bulltrout	1	Unknown	Qiantang River (Upstream)	2005	China	2.85 ng/g wet wt	2.30 ng/g wet wt	Zhou et al. (2007)
R-8	*Ptychobarbus dipogon*	3	Muscle	Lhasa River	2005	China	0.286 ng/g wet wt	2.07 ng/g wet wt	Yang et al. (2007)
R-9	*Schizopygopsis younhusbandi*	3	Muscle	Lhasa River	2005	China	0.75 ng/g wet wt	2.99 ng/g wet wt	Yang et al. (2007)
R-10	*C. auratus*	5	Edible part	Huairou Reservoir (Beijing)	2006	China	0.34 ng/g wet wt	7.53 ng/g wet wt	Li et al. (2008)
R-11	*M. anguillicauda-tus*	6	Edible part	Huairou Reservoir (Beijing)	2006	China	5.42 ng/g wet wt	44.17 ng/g wet wt	Li et al. (2008)

No.	Species	n[a]	Analyte	River	Year	Country	T-HCH [b]	T-DDT [c]	References
R-12	*P. fulvidraco*	4	Edible part	Huairou Reservoir (Beijing)	2006	China	1.93 ng/g wet wt	34.5 ng/g wet wt	Li et al. (2008)
R-13	Crucian carp	1	Edible part	Qiantang River (Jinhua)	2006	China	253 ng/g fat wt	514 ng/g fat wt	Zhou et al. (2008)
R-14	White fish	1	Edible part	Qiantang River (Jinhua)	2006	China	222 ng/g fat wt	315 ng/g fat wt	Zhou et al. (2008)
R-15	Crucian carp	1	Edible part	Qiantang River (Fuchunjiang)	2006	China	1054 ng/g fat wt	1408 ng/g fat wt	Zhou et al. (2008)
R-16	White fish	1	Edible part	Qiantang River (Fuchunjiang)	2006	China	152 ng/g fat wt	1330 ng/g fat wt	Zhou et al. (2008)
R-17	Crucian carp	1	Edible part	Qiantang River (Fuyang)	2006	China	577 ng/g fat wt	155 ng/g fat wt	Zhou et al. (2008)
R-18	White fish	1	Edible part	Qiantang River (Fuyang)	2006	China	467 ng/g fat wt	715 ng/g fat wt	Zhou et al. (2008)
R-19	Crucian carp	1	Edible part	Qiantang River (Hangzhou)	2006	China	936 ng/g fat wt	1199 ng/g fat wt	Zhou et al. (2008)
R-20	White fish	1	Edible part	Qiantang River (Hangzhou)	2006	China	844 ng/g fat wt	3008 ng/g fat wt	Zhou et al. (2008)
R-21	Many kinds of fish	19	Whole	Pearl River Estuary	2004	China	0.20 ng/g wet wt*	77 ng/g wet wt*	Guo et al. (2008)
R-22	Kissing gourami	2	Whole	Ciliwung River	2003	Indonesia	24 ng/g fat wt	800 ng/g fat wt	Sudaryanto et al. (2007)
R-23	Common carp	1	Whole	Ciliwung River	2003	Indonesia	3.4 ng/g fat wt	37 ng/g fat wt	Sudaryanto et al. (2007)
R-24	Tilapia mossambique	1	Whole	Ciliwung River	2003	Indonesia	6.0 ng/g fat wt	1100 ng/g fat wt	Sudaryanto et al. (2007)
R-25	Walking catfish	4	Whole	Ciliwung River	2003	Indonesia	11 ng/g fat wt	610 ng/g fat wt	Sudaryanto et al. (2007)
R-26	Unknown (n=10)		Unknown	Rivers and streams in Kumaun Himalayas	1999	India	1 ng/g wet wt	13 ng/g wet wt	Sarkar et al. (2003)
R-27	*Scatophagus argus*	3	Muscle	Ferok River	2003	India	0.72 ng/g wet wt	3.11 ng/g wet wt	Sarkar et al. (2006)
R-28	*Platicephalus sp.*	3	Muscle	Korappuzha River	2003	India	5.4 ng/g wet wt	ND	Sarkar et al. (2006)
R-29	*Etroplus suratensis*	3	Muscle	Pukatri River	2003	India	0.06 ng/g wet wt	0.44 ng/g wet wt	Sarkar et al. (2006)
R-30	Burbot	3	Liver	Pechora River	1988	Russia	11 ng/g wet wt	57 ng/g wet wt	Zhulidov et al. (2002)
R-31	Burbot	3	Liver	Pechora River	1994	Russia	4 ng/g wet wt	9 ng/g wet wt	Zhulidov et al. (2002)
R-32	Several kinds	103	Edible part	Sava River	2000	Croatia	0.70 ng/g wet wt*	1.80 ng/g wet wt*	Bosnir et al. (2007)
R-33	4 kinds of fish	4	Muscle	Dniester River (Upstream)	2001	Moldova		17.3 ng/g wet wt	Sapozhnikova et al. (2005)
R-34	5 kinds of fish	5	Muscle	Dniester River (Downstream)	2001	Moldova		10.2 ng/g wet wt	Sapozhnikova et al. (2005)
R-35	European river lamprey	31	Whole	Rivers flowing (n=8) to Bothnian Bay	Unkown	Finland	3.03 ng/g wet wt	46 ng/g wet wt	Merivirta et al. (2006)
R-36	European river lamprey	19	Whole	Rivers (n=5) flowing to Bothnian Sea	Unkown	Finland	4.20 ng/g wet wt	68 ng/g wet wt	Merivirta et al. (2006)
R-37	Chub	3	Muscle	River Nestos (Paranesti)	2004	Greece	0.27 ng/g wet wt	0.40 ng/g wet wt	Christoforidis et al. (2008)

No.	Species	n[a]	Analyte	River	Year	Country	T-HCH [b]	T-DDT [c]	References
R-38	Chub	3	Muscle	River Nestos (Komnina)	2004	Greece	0.10 ng/g wet wt	ND	Christoforidis et al. (2008)
R-39	Barbel	3	Muscle	River Nestos (Paranesti)	2004	Greece	0.91 ng/g wet wt	0.47 ng/g wet wt	Christoforidis et al. (2008)
R-40	Barbel	3	Muscle	River Nestos (Komnina)	2004	Greece	0.15 ng/g wet wt	0.25 ng/g wet wt	Christoforidis et al. (2008)
R-41	Common trout	5	Muscle	River Turia	2000	Spain		4.3 ng/g wet wt	Bordajandi et al. (2003)
R-42	European eel	11	Muscle	River Turia	2000	Spain		45.3 ng/g wet wt	Bordajandi et al. (2003)
R-43	Brown trout	28	Whole	Two rivers in Cantabria	2001	Spain	0.55 ng/g dry wt	20.2 ng/g dry wt	Guitart et al. (2005)
R-44	Eurasian minnow	17	Whole	Two rivers in Cantabria	2001	Spain	1.04 ng/g dry wt	23.0 ng/g dry wt	Guitart et al. (2005)
R-45	European eel	16	Whole	Two rivers in Cantabria	2001	Spain	0.66 ng/g dry wt	39.4 ng/g dry wt	Guitart et al. (2005)
R-46	Barbel	3	Whole	Ebro River Basin (Presa de Pina)	2003	Spain		35.9 ng/g dry wt	Lacorte et al. (2006)
R-47	Bleak	6	Whole	Ebro River Basin (Presa de Pina)	2003	Spain		71.9 ng/g dry wt	Lacorte et al. (2006)
R-48	Common carp	1	Whole	Ebro River Basin (Flix)	2003	Spain		983 ng/g dry wt	Lacorte et al. (2006)
R-49	Bleak	3	Whole	Ebro River Basin (Flix)	2003	Spain		487 ng/g dry wt	Lacorte et al. (2006)
R-50	Barbel	2	Muscle	Cinca River (Upstream)	2002	Spain		31 ng/g wet wt (+op')	De la Cal et al. (2008)
R-51	Barbel	2	Muscle	Cinca River (Downstream)	2002	Spain		780 ng/g wet wt (+op')	De la Cal et al. (2008)
R-52	Bleak	1	Whole	Cinca River (Upstream)	2002	Spain		5 ng/g wet wt (+op')	De la Cal et al. (2008)
R-53	Bleak	2	Whole	Cinca River (Downstream)	2002	Spain		508 ng/g wet wt (+op')	De la Cal et al. (2008)
R-54	Tilapia zilli	2	Unknown	Ogba River	Unkown	Nigeria		56 ng/g wet wt	Ize-Iyamu et al. (2007)
R-55	Catfish	2	Unknown	Ogba River	Unkown	Nigeria		106 ng/g wet wt	Ize-Iyamu et al. (2007)
R-56	Tilapia zilli	2	Unknown	Ovia River	Unkown	Nigeria		61 ng/g wet wt	Ize-Iyamu et al. (2007)
R-57	Catfish	2	Unknown	Ovia River	Unkown	Nigeria		115 ng/g wet wt	Ize-Iyamu et al. (2007)
R-58	Tilapia zilli	2	Unknown	Ikoro Riber	Unkown	Nigeria		20 ng/g wet wt	Ize-Iyamu et al. (2007)
R-59	Catfish	2	Unknown	Ikoro Riber	Unkown	Nigeria		34 ng/g wet wt	Ize-Iyamu et al. (2007)
R-60	Smallmouth bass	3	Whole	Willamette River (Lower Superfund)	2000	USA (Oregon)	< 8 ng/g wet wt	320 ng/g wet wt	Sethajintanin et al. (2004)
R-61	Common carp	3	Whole	Willamette River (Lower Superfund)	2000	USA (Oregon)	< 8 ng/g wet wt	97 ng/g wet wt	Sethajintanin et al. (2004)
R-62	Carp	3	Muscle	Okanogan River (Oroville)	2001	USA		336 ng/g wet wt	Washington State Department of Ecology (2003)
R-63	Mountain whitefish	3	Muscle	Okanogan River (Oroville)	2001	USA		350 ng/g wet wt	
R-64	Smallmouth bass	3	Muscle	Okanogan River (Oroville)	2001	USA		157 ng/g wet wt	

No.	Species	n[a]	Analyte	River	Year	Country	T-HCH [b]	T-DDT [c]	References
R-65	Akupa sleeper fish	1	Unknown	Hanalei River	2001	USA (Hawai'i)	< 1 ng/g wet wt	< 2 ng/g wet wt	Orazio et al. (2007)
R-66	Chinook salmon		whole	Lower Columbia Estuary	2001-2002	USA		1800-27000 ng/g fat wt (+op')	Johnson et al. (2007)
R-67	Largemouth bass	10	whole	Mobile River basin (Lavaca)	2004	USA (Alabama)		24.9 ng/g wet wt	Hinck et al. (2009)
R-68	Largemouth bass	10	whole	Mobile River basin (Mcintosh)	2004	USA (Alabama)		6946 ng/g wet wt	Hinck et al. (2009)
R-69	Largemouth bass	10	whole	Mobile River basin (Bucks)	2004	USA (Alabama)		92.8 ng/g wet wt	Hinck et al. (2009)
R-70	Largemouth bass	8	whole	Mobile River basin	2004	USA		53.84 ng/g wet wt	Hinck et al. (2008)
R-71	Largemouth bass	6	whole	Apalachicola-Chattahoochee-Flint River Basin	2004	USA		87.45 ng/g wet wt	Hinck et al. (2008)
R-72	Largemouth bass	6	whole	Savannah River Basin	2004	USA		18.98 ng/g wet wt	Hinck et al. (2008)
R-73	Largemouth bass	6	whole	Pee Dee River Basin	2004	USA		37.84 ng/g wet wt	Hinck et al. (2008)
R-74	Carp	8	whole	Mobile River basin	2004	USA		41.58 ng/g wet wt	Hinck et al. (2008)
R-75	Carp	6	whole	Apalachicola-Chattahoochee-Flint River Basin	2004	USA		90.64 ng/g wet wt	Hinck et al. (2008)
R-76	Carp	6	whole	Savannah River Basin	2004	USA		16.42 ng/g wet wt	Hinck et al. (2008)
R-77	Carp	6	whole	Pee Dee River Basin	2004	USA		20.42 ng/g wet wt	Hinck et al. (2008)
R-78	Chub	10	Muscle	River Elbe (Downstream Pardubice)	2004	Czech Republic	24 ng/g fat wt	2850 ng/g fat wt (+op')	Randak et al. (2009)
R-79	Chub	8	Muscle	River Elbe (Downstream Neratovice)	2004	Czech Republic	486 ng/g fat wt	4830 ng/g fat wt (+op')	Randak et al. (2009)
R-80	Chub	4	Muscle	River Elbe (Downstream Usti nad Labem)	2004	Czech Republic	53 ng/g fat wt	6480 ng/g fat wt (+op')	Randak et al. (2009)
R-81	Chub (*Leuciscus cephalus*)	10	Muscle	Svratka River (Modřice)	Apr.-2007	Czech Republic	1.0 ng/g wet wt	34.9 ng/g wet wt (+op')	Lána et al. (2010)
R-82	Chub (*Leuciscus cephalus*)	10	Muscle	Svratka River (Modřice)	Oct.-2007	Czech Republic	0.7 ng/g wet wt	29.4 ng/g wet wt (+op')	Lána et al. (2010)
R-83	Chub (*Leuciscus cephalus*)	9	Muscle	Svratka River (Rajhradice)	Apr.-2007	Czech Republic	0.9 ng/g wet wt	40.0 ng/g wet wt (+op')	Lána et al. (2010)
R-84	Chub (*Leuciscus cephalus*)	11	Muscle	Svratka River (Rajhradice)	Oct.-2007	Czech Republic	2.6 ng/g wet wt	28.8 ng/g wet wt (+op')	Lána et al. (2010)
R-85	Fish (Large size)		whole	Mississippi River (Upper)	2004-2005	USA		11.16 ng/g wet wt	Blocksom et al. (2010)
R-86	Fish (Large size)		whole	Missouri River	2004-2005	USA		8.18 ng/g wet wt	Blocksom et al. (2010)
R-87	Fish (Large size)		whole	Ohio River	2004-2005	USA		18.32 ng/g wet wt	Blocksom et al. (2010)
R-88	Fish (Small size)		whole	Mississippi River (Upper)	2004-2005	USA		6.57 ng/g wet wt	Blocksom et al. (2010)

No.	Species	n[a]	Analyte	River	Year	Country	T-HCH [b]	T-DDT [c]	References
R-89	Fish (Small size)		whole	Missouri River	2004-2005	USA		5.47 ng/g wet wt	Blocksom et al. (2010)
R-90	Fish (Small size)		whole	Ohio River	2004-2005	USA		15.60 ng/g wet wt	Blocksom et al. (2010)
R-91	European eel	30	Muscle	Garigliano River (Campania region)	2005-2006	Italy		52.91 ng/g wet wt	Ferrante et al. (2010)
R-92	Brown trout (*Salmo trutta*)	9	Muscle	Quemquentreu river	2006	Argentina		1.7 ng/g wet wt	Ondarza et al. (2011)
R-93	Brown trout (*Salmo trutta*)	9	Liver	Quemquentreu river	2006	Argentina		7.4 ng/g wet wt	Ondarza et al. (2011)
R-94	Sábalo fish (*Prochilodus lineatus*)	7	Muscle	Río de la Plata basin	2003-2004	Argentina	9 ng/g wet wt	340 ng/g wet wt (+op')	Colombo et al. (2011)
R-95	Eel	10	Muscle	Rivers in South Canterbury	2009	New Zealand		33.5 ng/g wet wt *	Stewart et al. (2011)
R-96	Brown trout	5	Muscle	Rivers in South Canterbury	2009	New Zealand		16.8 ng/g wet wt *	Stewart et al. (2011)
R-97	Largemouth bass		Muscle	Blackwater River (Lower)	2004	USA (Florida)		ND	Karouna-Renier et al. (2011)
R-98	Largemouth bass		Muscle	Perdido River (Lower)	2004	USA (Florida)		0.51 ng/g wet wt	Karouna-Renier et al. (2011)
R-99	Largemouth bass		Muscle	Yellow River (Lower)	2004	USA (Florida)		ND	Karouna-Renier et al. (2011)
R-100	Tilapia	8	Muscle	Noha River (Okinawa-Manko)	2006	Japan	24 ng/g fat wt	3800 ng/g fat wt	Malarvannan et al. (2011)
R-101	Tilapia	8	Muscle	Hija River (Okinawa-Kadena)	2006	Japan	4.7 ng/g fat wt	1100 ng/g fat wt	Malarvannan et al. (2011)
R-102	Tilapia	8	Muscle	Shikaza River (Okinawa-Onna village)	2005	Japan	10 ng/g fat wt	680 ng/g fat wt	Malarvannan et al. (2011)

[a] No. of analyzed samples; Mean (n≥2) or single determination values (n=1) are listed for PCB, T-HCH and T-DDT (* Median value)
[b] T-HCH = α-HCH + β-HCH + γ-HCH
[c] T-DDT = pp'-DDE + pp'-DDD +pp'-DDT

Table 2. Concentarations of T-HCH and T-DDT in fish from rivers in the world

Residue of T-DDT and T-HCH in fish from lakes in the world is shown in Figures 1 and 2, respectively, for the concentration data (ng/g wet wt.) and (ng/g fat wt. and ng/g dry wt. possible to be calculated as ng/g wet wt.). Each of the T-DDT and T-HCH concentration data was compared for the 38 lakes surveyed in 8 countries of Europe and America and 8 countries of Asia and Africa from 1995 to 2008. Each data is shown as single determination value (n=1) and mean (n≧2) or mean and range values (n≧2) for the surveys in plural fish species, sampling sites and survey years.

T-DDT concentrations in the fish from the lakes of America were relatively high and the higher concentrations were detected in USA like the previous report [102]. Those of Europe were relatively low except for two lakes in Italy. Those of Asia and Africa including Japan were relatively low as a whole, although relatively high concentrations were detected in a

part of lakes in China. T-HCH concentrations in the fish from the lakes in the world were relatively low. The highest T-HCH concentration was 46 ng/g wet wt. in Taihu Lake of China [46] and relatively low concentration of 1.4 ng/g wet wt. (average, n=7) was detected in Lake Biwa of Japan [22-28].

Residue of T-DDT and T-HCH in fish from rivers in the world is shown in Figures 3 and 4, respectively, for the concentration data (ng/g wet wt.) and (ng/g fat wt. and ng/g dry wt. possible to be calculated as ng/g wet wt.). Each of the T-DDT and T-HCH concentration data was compared for the 28 rivers surveyed in 8 countries of Europe and America, 4 countries of Asia, Africa and Oceania from 2000 to 2009. Each data is shown as single determination value (n=1) and mean ($n \geqq 2$) or mean and range values ($n \geqq 2$) for the surveys in plural fish species, sampling sites and survey years.

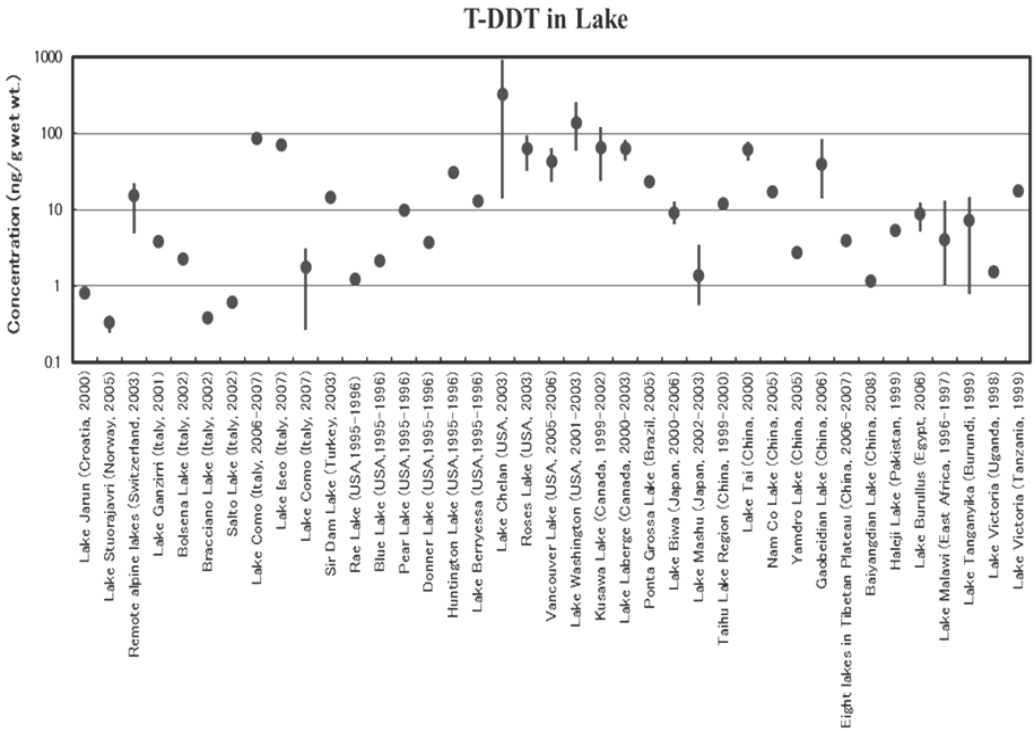

Figure 1. Residue of T-DDT in fish from lakes in the worl

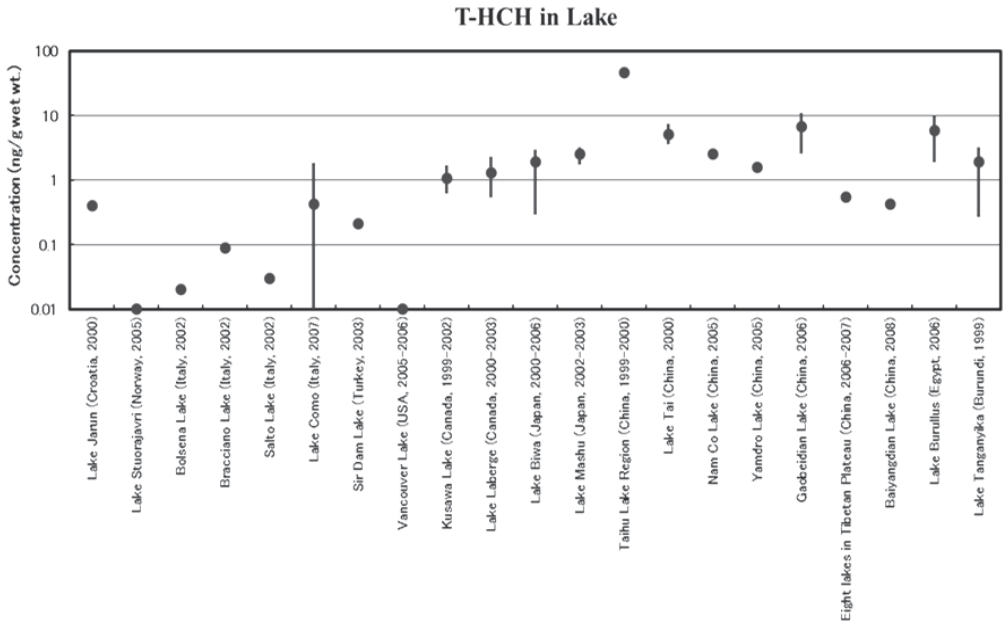

Figure 2. Residue of T-HCH in fish from lakes in the world

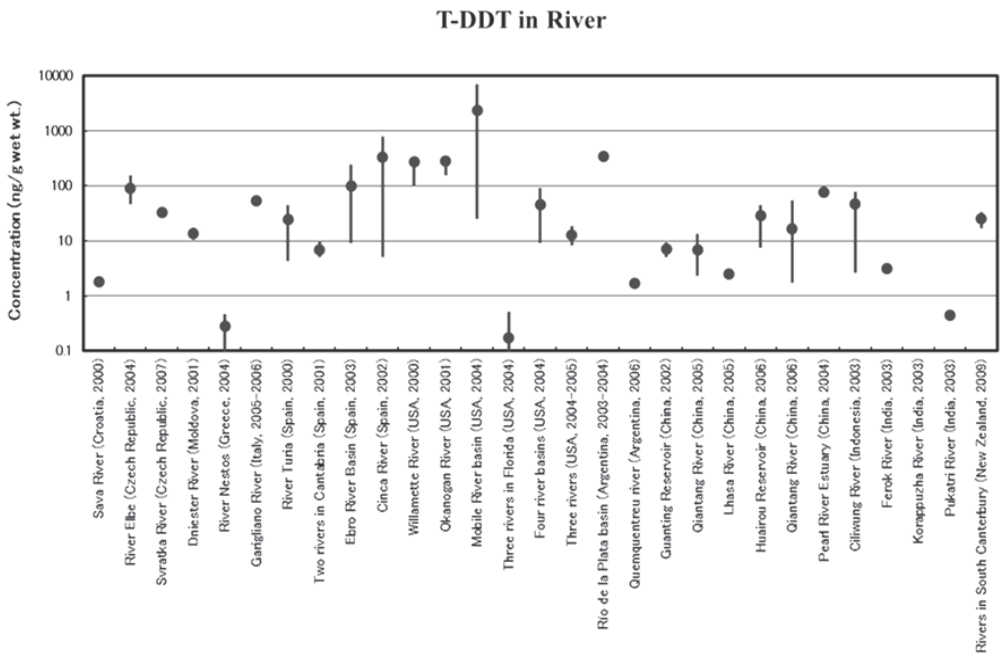

Figure 3. Residue of T-DDT in fish from rivers in the world

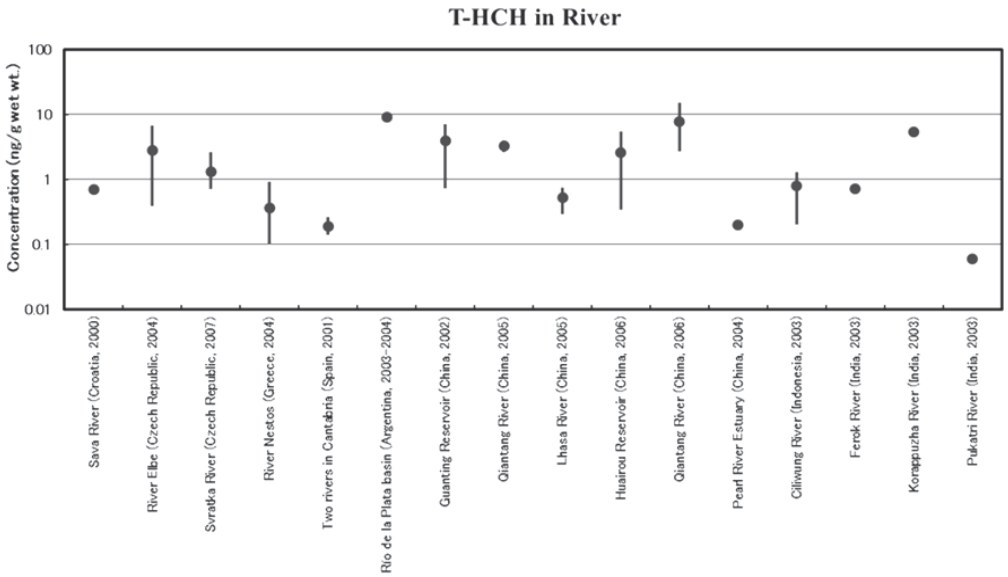

Figure 4. Residue of T-HCH in fish from rivers in the world

T-DDT concentrations in the fish from the rivers of USA were relatively high like the previous report [102]. Those of Europe were relatively low except for a part of lakes in Czech Republic and Spain. Those of Asia and Africa were relatively low as a whole, although relatively high concentrations were detected in a part of rivers of China.

T-HCH concentrations in the fish from the rivers in the world were relatively low like the lakes in the world and the highest T-HCH concentration was 7.7 ng/g wet wt. (average, n=8) in Qiantang River of China [101].

3. Long-term trends of T-DDT and T-HCH in fish from lakes in the world

T-HCH and T-DDT concentrations in fish were calculated as the simple sum of the constituents. T-HCH is the sum of α-HCH, β-HCH and γ-HCH and T-DDT is the sum of pp'-DDT, pp'-DDD and pp'-DDE. For Japan, T-DDT and T-HCH concentration data in Japanese dace from Lake Biwa were cited from reports of Ministry of the Environment, Japan (1980 – 2011) [1-31]. For Canada and USA, T-DDT concentration data in lake trout from Lake Ontario and Lake Michigan were cited from a report of Environment Canada and U.S. Environmental Protection Agency (2007) [32] and a source figure of U.S. Environmental Protection Agency (2009) [33], respectively. For Sweden, T-DDT concentration data in pike from Lake Storvindeln were cited from a source figure of Swedish EPA (2002) [34].

All data were analyzed using Microsoft Excel graph wizard. For each location and analyte, nonlinear procedure was used to fit the exponential model: $y=ae^{-kx}$, where y is the concentration in each composite sample, x is the sampling date, and a and k are model parameters estimated by nonlinear procedure to obtain a specific model that best fits the data.

Long-term trends of T-DDT and T-HCH in Japanese dace from Lake Biwa are shown from 1979 to 2009 in Figure 5 along with long-term trends estimated using the first-order model. Both insecticides were found to decline in a consistent pattern. Model parameters (a, k) and R^2 values were calculated for each of the data sets and are shown in Figure 5. Half-lives ($t_{1/2}$) were calculated for T-DDT and T-HCH from a parameter (k). The $t_{1/2}$ values were 9 years for T-DDT and 4 years for T-HCH.

Long-term trends of T-DDT in fish from Lake Biwa, Lake Ontario, Lake Michigan and Lake Storvindeln are similarly shown in Figure 6. The $t_{1/2}$ values of T-DDT were 9, 11, 8 and 7 years, respectively, in Lake Biwa, Lake Ontario, Lake Michigan and Lake Storvindeln. There were no wide differences in the $t_{1/2}$ values of T-DDT in the fish among the four lakes. The same extent of the decline rate in the T-DDT inflow into the four lakes was presumed from the same extent of the decline rate of T-DDT in the fish of the four lakes.

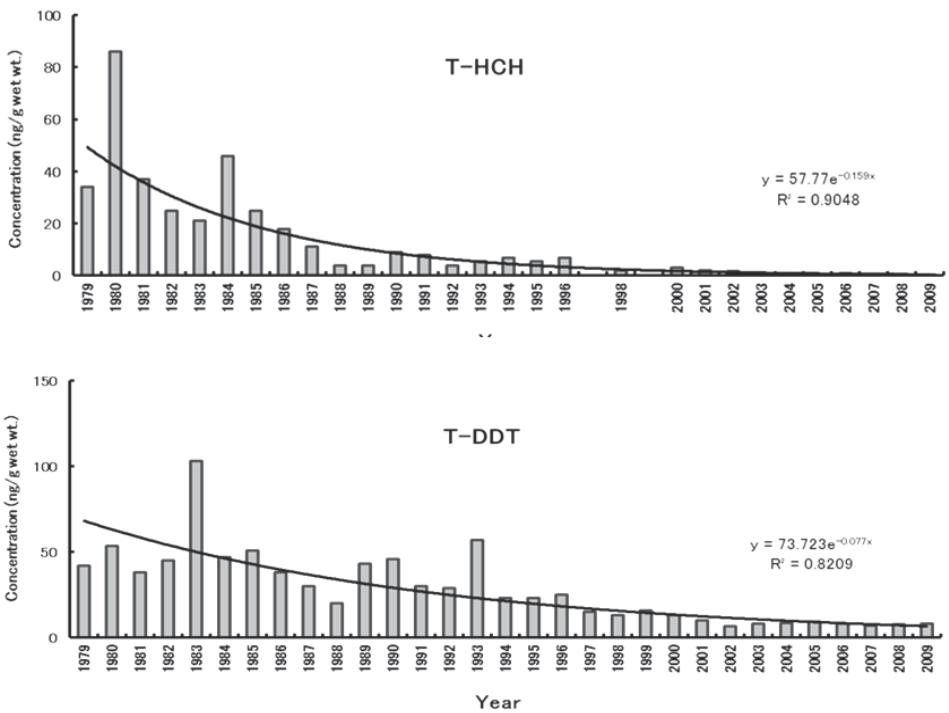

Figure 5. Long-term trends of T-DDT and T-HCH in Japanese dace from Lake Biwa

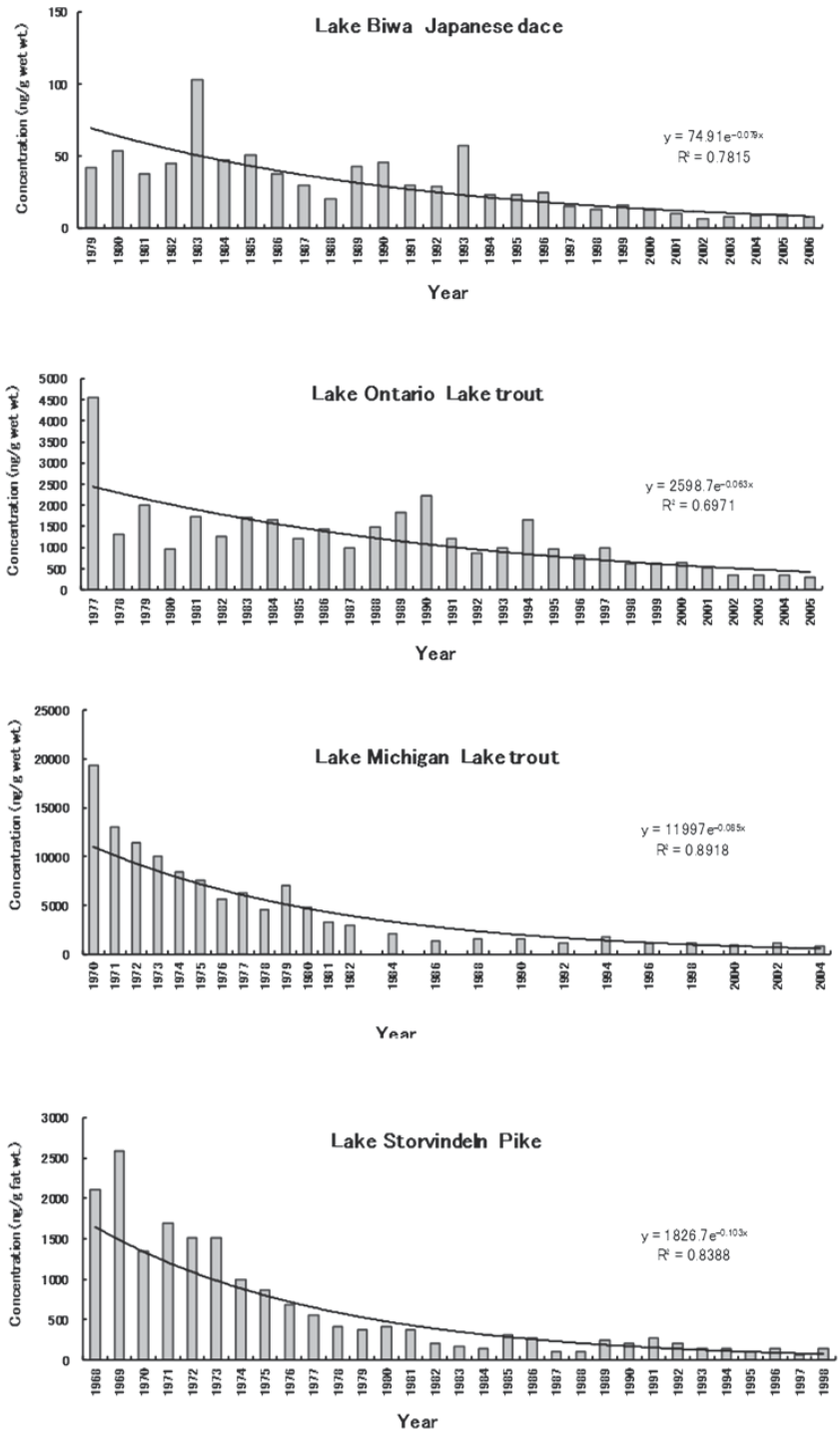

Figure 6. Long-term trends of T-DDT in fish from Lake Biwa, Lake Ontario, Lake Michigan and Lake Storvindeln

4. Composition of T-DDT and T-HCH in fish from lakes and rivers in the world

Composition of T-DDT in fish from lakes in the world is shown in Figure 7 for the survey data in the 25 lakes in 15 countries of Europe, America, Asia and Africa from 1996 to 2008 [22-28, 35, 39, 41, 42, 44, 47, 49-53, 55, 56, 58, 60, 61, 64, 65, 67, 68, 70]. Few (ND~0.6 %) or low (1~6 %) percentage of *PP'*-DDT was detected in Lake Biwa and Lake Mashu of Japan, Lake Tai and three lakes of China, Lake Stuorajavri of Norway, Lake Ganzirri of Italy and Sir Dam Lake of Turkey. Metabolites of *PP'*-DDT (*pp'*-DDE and *pp'*-DDD) were detected at high percentage and long-term no use of DDT was presumed in the countries. On the other hand, high percentage (44~88 %) of *PP'*-DDT was detected in Lake Edward of Uganda, three lakes of Ghana, Lake Burullus of Egypt, Lake Victoria of Tanzania and Ponta Grossa Lake of Brazil. DDT was presumed to be used in the countries in recent years or in the sampling date. The use of organochlorine pesticids such as DDT was prohibited or restricted in the 1970's for Japan and 1980's for Europe and in 1983 for China. This corresponded well to the survey data described above. Low percentage of *PP'*-DDT in the fish from Lake Michigan and Lake Superior of USA was reported in the previous report [102]. The percentage of *PP'*-DDT was similarly low (5 %) in the fish from Vancouver Lake in the present report. In Africa, the use of DDT was restricted in in the 1980's and low percentage of *PP'*-DDT was reported in the fish from Manzara Lake or Idku Lake in the previous report [102]. The present result in Lake Burullus was different from the previous report and the use of DDT was presumed in recent years or in the sampling date. Details of the restriction on the use of DDT in Egypt were obscure.

Composition of T-DDT in fish from rivers in the world is shown in Figure 8 for the survey data in the 16 rivers in 8 countries of Europe, America, Asia, Africa and Oceania from 2000 to 2009 [51, 68, 73, 77, 79, 80, 83, 86, 88, 91, 94, 95, 98, 100, 101].

In China, relatively high percentage (38 %) of *PP'*-DDT was detected in Qiantang River of China (2005). However, low percentage (2~10 %) of *PP'*-DDT was detected in Lhasa River, Huairou Reservoir and Qiantang River (2006). In Japan, India, Greece, Spain and USA, low percentage (ND~12 %) of *PP'*-DDT was similarly detected. No use of DDT was presumed in recent years or in the sampling date in all rivers except for Qiantang River of China surveyed in 2005.

Composition of T-HCH in fish from lakes in the world is shown in Figure 9 for the survey data in the 16 lakes in 8 countries of Europe, America, Asia and Africa from 1996 to 2008 [22-28, 35, 42, 44, 51, 53, 56, 58, 60, 61, 65, 68]. Composition of T-HC in Lake Biwa of Japan (α-HCH 7 %, β-HCH 91 % and γ-HCH 3 %) was similar to that in Lake Tai of China (α-HCH 10 %, β-HCH 84 % and γ-HCH 6 %). It was known that technical HCH (α-HCH 65~70 %, β-HCH 6~14 %, γ-HCH 10~13 % and δ-HCH 5~8 %) had been used in China, India and former Soviet Union since 1979 [103]. In Japan, technical HCH was also used without purification until 1971. This is the reason for the similarity of HCH composition in the fish between Japan and China (Lake Biwa and Lake Tai). However, the percentage of β-HCH in Yamdro Lake and Gaobeidian Lake was relatively low and the percentage of β-HCH in Lake

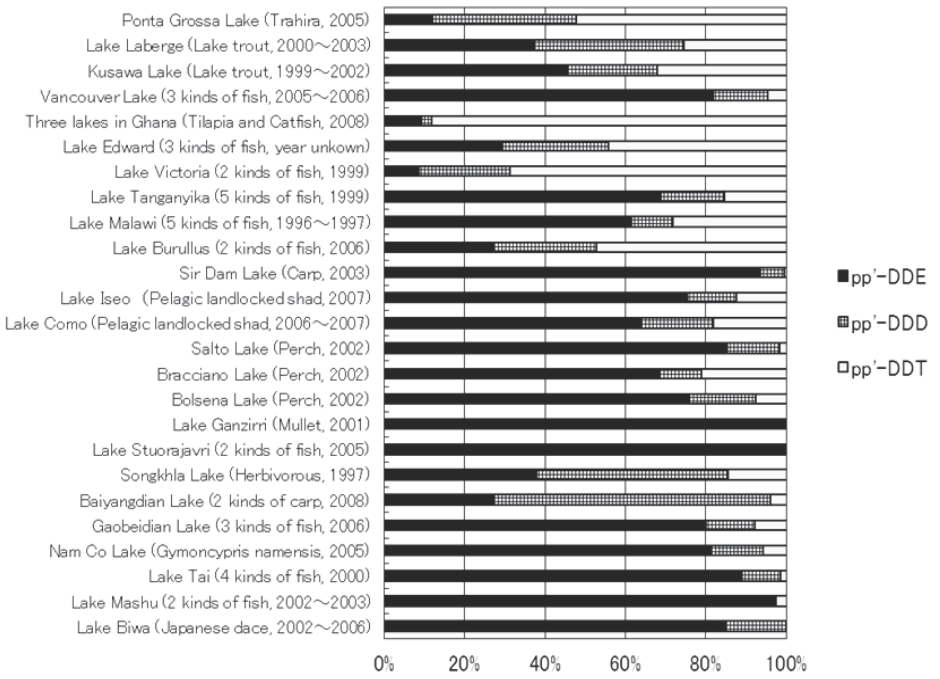

Figure 7. Composition of T-DDT in fish from lakes in the world

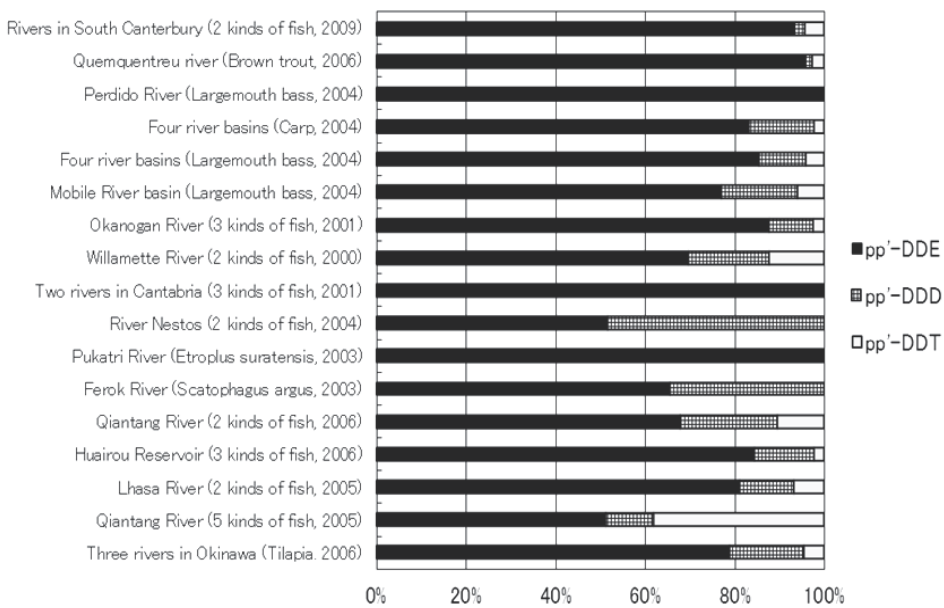

Figure 8. Composition of T-DDT in fish from rivers in the world

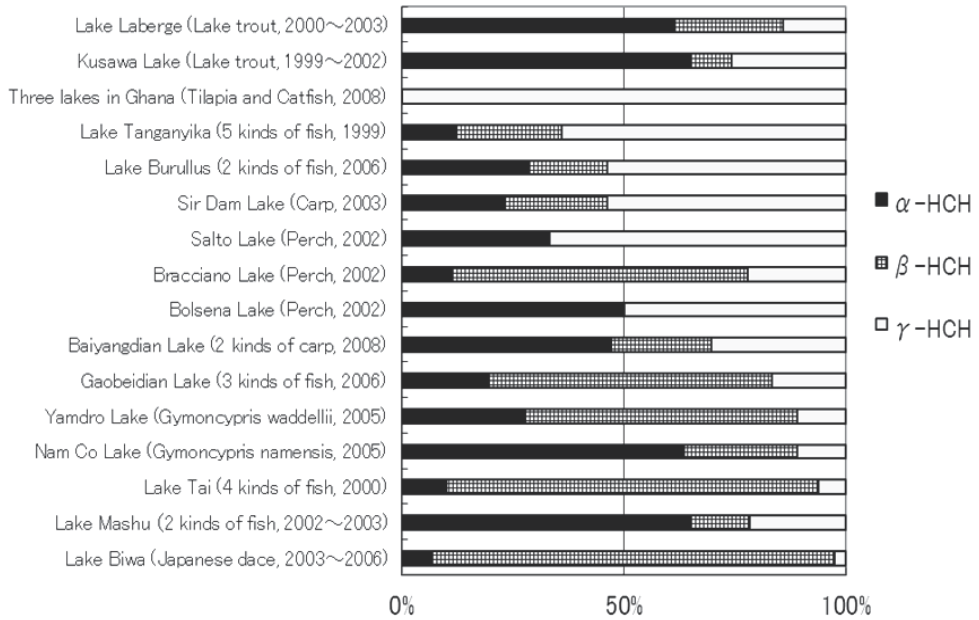

Figure 9. Composition of T-HCH in fish from lakes in the world

Biwa of Japan was higher than that in the lakes of China. There was a probability of high β-HCH percentage in Japan because β-HCH was highly persistent and the period after prohibition on the use of β-HCH was longer in Japan than in China. On the other hand, the percentage of β-HCH in Rainbow trout and Japanese dace from Lake Mashu in Japan was low. Composition of T-HC in Japanese dace from Lake Mashu (α-HCH 64~67 %, β-HCH 11 % and γ-HCH 22~25 %) was much different from that of Lake Biwa (α-HCH 6~8 %, β-HCH 89~92 % and γ-HCH 2~3 %). This is probably because HCH was loaded in Lake Mashu through the atmosphere [104] and the percentage of α-HCH in the water of Lake Mashu was much higher than that of Lake Biwa.

On the other hand, purified lindane (γ-HCH: more than 99 %) was used in Europe and America differently from Japan, China, etc. This is probably because of the high percentage of γ-HCH in Bolsena Lake and Salto Lake of Italy, Sir Dam Lake of Turkey, Lake Burullus of Egypt, Lake Tanganyika of Burundi and three lakes of Ghana.

Composition of T-HCH in fish from rivers in the world is shown in Figure 10 for the survey data in the 11 rivers in 5 countries of Europe and Asia from 2001 to 2006 [51, 68, 73, 77, 86, 92, 100, 101].

In China, high percentage of γ-HCH was detected in Qiantang River and Huairou Reservoir differently from the survey data in the lakes. The use of lindane was presumed in the two river basins. In Korappuzha River of India, the use of technical HCH was presumed and this corresponded well to the report that technical HCH had been used in China, India and former Soviet Union since 1979 [103]. Particularly high percentage of γ-HCH was detected in Nestos River of Greece and this corresponded well to the use of lindane in Europe and

America similarly in the case of the lake. For Okinawa Prefecture in Japan, the use of technical HCH in Shikaza River and Hija River and the use of lindane in Noha River were presumed from the composition of T-HCH shown in Figure 10. The high percentage of γ-HCH in Noha River did not correspond to the use of technical HCH in Japan.

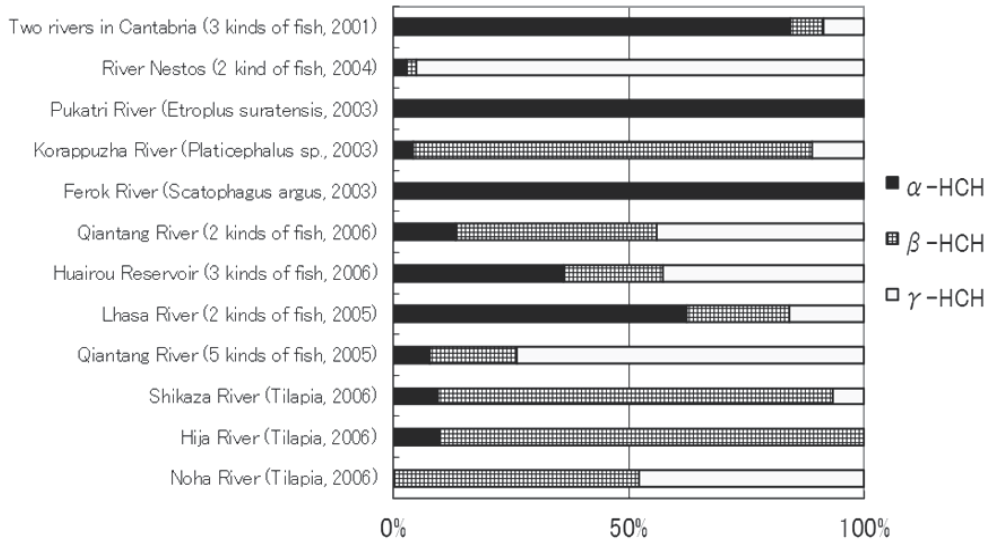

Figure 10. Composition of T-HCH in fish from rivers in the world

5. Conclusion

T-DDT concentrations in the fish from the lakes and rivers of America were relatively high, but those of Europe, Asia and Africa were relatively low. T-HCH concentrations in the fish were relatively low in both of the lakes and rivers in all over the world. T-DDT and T-HCH compositions were respectively compared among lakes and rivers from America, Europe, Asia and Africa. DDT was presumed to be used in Uganda, Egypt, Tanzania and Brazil in recent years or in the sampling date from the high percentage of *pp'*-DDT in the composition of T-DDT and its metabolites in the several kinds of fish from Lake Edward, Lake Burullus, Lake Victoria and Ponta Grossa Lake. No use of DDT was presumed in USA and European countries from the low percentage of *pp'*-DDT in the lake fish in the countries. Technical HCH was presumed to be used in Japan, China and India from the low percentage of γ-HCH in the composition of T-HCH in the lake and the river fish in the countries. On the contrary, Lindane was presumed to be used in the countries of Europe and Africa from the high percentage of γ-HCH in the lake and the river fish in the countries. Half-lives ($t_{1/2}$) of T-DDT in fish from lakes in Japan, Canada, USA and Sweden were calculated from the long-term monitoring data using an exponential decay model to evaluate the decline rate of DDT contamination in the lake environment. The $t_{1/2}$ values were 9 years for Lake Biwa in Japan, 11 years for Lake Ontario in Canada, 8 years for Lake Michigan in USA and 7 years for Lake Storvindeln in Sweden. There were no wide differences in the $t_{1/2}$ values of T-DDT in the fish among the four lakes.

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Chapter 3

Pesticide Residue Analysis in Animal Origin Food: Procedure Proposal and Evaluation for Lipophilic Pesticides

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Additional information is available at the end of the chapter

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1. Introduction

Pest control in intensive agriculture involves treatments with a variety of synthetic chemicals generically known as pesticides. These chemicals can be transferred from plants to animals via the food chain. Furthermore, animals and their accommodations can be sprayed with pesticide solutions to prevent infestations. Consequently, both contamination routes lead to bio-accumulation of pesticides in food products of animal origin as meat, fish, fat, offal, eggs, and milk [1].

Persistent organic pollutants (POPs) are mostly organochlorinated pesticides (OCPs) which have been banned for agricultural and domestic uses in Europe, North America and many countries of South America, due to their environmental persistence and potential adverse effect on wildlife and human health, according to the Stockholm Convention in 1980s. However, some OCPs are still used, as the *DDT* (for the control of the outbreak of the mosquito that spread malaria and as and as antifouling agent in some developing countries). Besides, the most commonly used acaricide, *Dicofol*, is made of *DDT*, so its formulated products always contain small amounts of *DDT* [2]. These harmful compounds persist in the environment and they are transported around the world, as Coscollà et al.[3] found in the air of France. OCPs, have a highly stable, low volatile, non-polar, lipophilic nature, and consequently exhibit considerable environmental persistence with a tendency to bio-accumulate, leading to the contamination of foodstuffs, especially those with a high fat content.

Also synthetic pyrethroid pesticides (PYRs) are effective broad spectrum insecticides although with low toxicity for mammalian and short-term environmental persistence, due to their rapid degradation, being ester cleavage the major process [4]. Pyrethroids range from

non-polar to low-polarity lipophilic compounds. Owing to their metabolism in animals, they tend to bio-acumulate in lipid compartments, becoming a potencial source of human exposure through foodstuffs [1].

Organophosphorus pesticides (OPPs), mainly used as insecticides, are esters of phosphoric acid with different substituents. They have a wide variety of physico-chemical properties, as polarity and water solubility. OPPs, less persistent than OCPs, use to be the first choice for treatment because they provide efficacious, safe and cost effective control of a wide range of pests. They can be absorbed by inhalation, ingestion and dermal absorption; and they can also concentrate along the food chain.

Triazines are among the most widely used herbicides in agriculture. They are degraded by chemical and biological processes in their respective hydroxytriazines. Triazines and their degradation products are low polarity compounds, weakly basic, stable in the environment and therefore, persistent. [l].

Other pesticides, such as carbamates, benzoylureas, quinoxalines, amines and fluorides, have been evaluated for analytical purposes in animal origin food.

Most usually studied pesticides in animal origin food are non polar compounds that tend to concentrate and remain in fatty food. Reported studies from literature [1] for products of animal origin, five groups of pesticides (OCPs, OPPs, PYRs, carbamates and triazines) were the most analysed. Few of them have been detected in various animal products (*DDT* and its metabolites *DDD* and *DDE, Hexachlorocyclohexane* α, β and γ isomers, *HCB, Aldrin, Dieldrin, Endrin, Heptachlor* and *HCE, Endosulfan* α *and* β *isomers*, *Chlordane, Simazine, Atrazine, Deltamethrin and Cypermethrin*). The 2009 European Union Report on Pesticide Residues in Food concludes that the majority of animal origin food was free of detectable residues, that 34 different pesticides were found and most of them were rather due to environmental contaminations with persistent pesticides, also *Deltamethrin, Cypermethrin* and *Clorpyrifos* were found in some samples [5]. In another study made in Spain, triazines (*Terbutilazine*, *Simazine* and *Atrazine*) were detected in 16% of raw cow´s milk samples [6].

The US Food and Drug Administration (FDA) categorize food as fatty food when the fat content is ≥ 2% of weight and non-fatty food when it is < 2 %. Besides that classification, Lehotay divided food into non-fatty (< 2% fat), **low**-fatty (2-20%) and high-fatty (>20%) [2]. In low-fatty samples, lipophilic and hydrophilic pesticides can occur, so analytical methods should cover a wide pesticide polarity range. However, in fatty food samples, occurring pesticides are fat soluble, so analytical methods should focus on non-polar pesticides [7].

For dietary intake purposes, it is desirable to include metabolites and degradation products of toxicological concern, for example the oxygen analogues of organophosphate pesticides that might be produced or persist in processed food. In most of cases, the MRL definition is established as the sum of the parent compound and its relevant metabolites [8].

As it has been said before, some non polar pesticides are fat soluble, facilitating their tendency to bio-accumulate in the food chain and to concentrate in human and animal tissues. The presence of these compounds in food could constitute a serious risk to human and animal health, and also to the environment. Different organizations have established

R (EC) 396/2005. Pesticide EU MRLs.	**Analyzed compounds**	**Fat, Meat and Liver (1)**	**Milk**	**Eggs**
Alachlor	Alachlor	0.01*	0.01*	0.01*
Aldrin and Dieldrin (Aldrin and dieldrin combined expressed as dieldrin**) (F)**	Aldrin	0.2	0.006	0.02
	Dieldrin			
Bifenthrin (F)	Bifenthrin	0.05* (0.1 fat bovine)	0.01*	0.01*
Chlordane (sum of cis- , trans and oxy-chlrodane) **(F)**	Chlordane-cis	0.05*	0.002*	0.005*
	Chlordane-trans			
Chlorpyrifos-methyl (F)	Chlorpyrifos-m	0.05*	0.01*	0.01*
Chlorpyrifos (F)	Chlorpyrifos	0.05* (chicken)	0.01*	0.01*
Cyfluthrin (cyfluthrin including other mixtures of constituent isomers (sum of isomers)) **(F)**	Cyfluthrin	0.05	0.02*	0.02*
Cyhalothrin-lambda (F)	Cyhalothrin-λ	0.5 (except 0.02* chicken)	0.05	0.02*
Cypermethrin (cypermethrin including other mixtures of constituent isomers (sum of isomers) **(F)**	Cypermethrin	Fat and meat 2 (except 0.1 chicken) Liver 0.2 (except 0.05* chicken)	0.05	0.05*
DDT (sum of p,p′-DDT, o,p′-DDT, p-p′-DDE and p,p′-TDE (DDD) expressed as DDT) **(F)**	DDT-pp	1	0.04	0.05
	DDT-op			
	DDE			
	DDD			
Deltamethrin (cis-deltamethrin) **(F)**	Deltamethrin	0.5 (except 0.1 chicken)	0.05	0.05*
Diazinon (F)	Diazinon	0.05*	0.01*	0.05*
Endosulfan (sum of alpha- and beta-isomers and endosulfan-sulphate expresses as endosulfan) **(F)**	Endosulfan-α	0.05*	0.05*	0.05*
	Endosulfan-β			
	Endosulfan sulphate			
Endrin (F)	Endrin	0.05	0.0008	0.005
Fenthion (fenthion and its oxigen analogue, their sulfoxides and sulfone expressed as parent) **(F)**	Fenthion	0.05*	0.01*	0.01*
Heptachlor (sum of heptachlor and heptachlor epoxide expressed as heptachlor) **(F)**	Heptachlor	0.2	0.004	0.02
	HCE-endo			
	HCE-exo			
Hexachlorobenzene (F)	HCB	0.2	0.01	0.02
Hexachlorociclohexane (HCH), α-isomer**(F)**	HCH-α	0.2	0.004	0.02

R (EC) 396/2005. Pesticide EU MRLs.	Analyzed compounds	Fat, Meat and Liver (1)	Milk	Eggs
Hexachlorociclohexane (HCH), β-isomer **(F)**	HCH-β	0.1	0.003	0.01
Lindane (Gamma-isomer of hexachlorociclohexane (HCH)) **(F)**	Lindane	0.02	0.001*	0.01*
Malathion (sum of malathion and malaoxon expressed as malathion)	Malathion	0.02*	0.02*	0.02*
Methidathion (F)	Methidathion	0.02*	0.02*	0.02*
Parathion (F)	Parathion	0.05*	0.05*	0.05*
Parathion-methyl (sum of Parathion-methyl and paraoxon-methyl expressed as Parathion-methyl)	Parathion-m	0.02*	0.02*	0.02*
Permethrin (sum of isomers)	Permethrin	0.05*	0.05*	0.05*
Pirimiphos-methyl (F)	Pirimiphos-m	0.05*	0.05*	0.05*
Pyrazophos (F)	Pyrazophos	0.02*	0.02*	0.1*
Trifluralin	Trifluralin	0.01*	0.01*	0.01*
Triazophos (F)	Triazophos	0.01*	0.01*	0.01*

Table 1. Analyzed pesticides, MRLs (mg/kg) (Reglament (EC) 396/2005. EU Pesticides database, accessed February 2012).
(F)-Fat soluble, (*)-Indicates lower limit of analytical determination, (1)-MRL or limit of analytical determination in animal meat, fat and liver (swine, bovine, sheep, goat, poultry-chicken)

strict regulation controls on the use of pesticides, handling to minimize the exposure of the population. Maximum Residue Levels (MRLs) are set by the European Commission to protect consumers from exposure to unacceptable levels of pesticides residues in food and feed. *Table 1* presents established MRLs for the studied pesticides in animal origin products, as *Regulation (EC) Nº 396/2005* establish [9]. Individual MRLs have not yet been required for fish. Depending on their water solubility, the maximum allowed levels for food of animal origin (meat, offals, milk, eggs) are regulated either on product or fat, as follow: When pesticides and/or metabolites are water soluble (log Pow < 3), the MRL is expressed as mg/kg of product. When they are fat soluble (log Pow ≥ 3), the MRL is expressed as mg/kg of fat or product [10]. Differences on fat content, type of foodstuffs and the ways to express those results are explained in *Table 2.*

Acording to the European Union legislation, some pesticides can be used for veterinary purposes. *Regulation (UE) Nº 37/2010* [11] establish MRLs for residues of pharmacologically active substances in foodstuffs of animal origin. This regulation includes pesticides used as veterinary drugs, as *Cypermethrin, Deltamethrin, Permethrin, Fenvalerate* and *Diazinon* are. In some cases, these MRLs are different from those from the *Reglament (EC) 396/2005*. *Cypermethrin* in bovine fat is an example, its MRL is 2 mg/kg in R 396/2005 and it is 0.2 mg/kg in R 37/2010.

Traditionally, pesticide residue analysis are carried out in a sequence of several steps, i.e. (i) extraction by organic solvent methods such as liquid-liquid partitioning [12-13], solid phase

micro-extraction [14], matrix solid phase dispersion [15] and supercritical fluid extraction [16]; (ii) clean-up by normal phase liquid chromatography (NPLC) [17] and/or gel permeation chromatography (GPC) [18-19], solid phase extraction (SPE) [20-21], and dispersive solid phase extraction (d-SPE) [22]; (iii) concentration according to analytical technique.

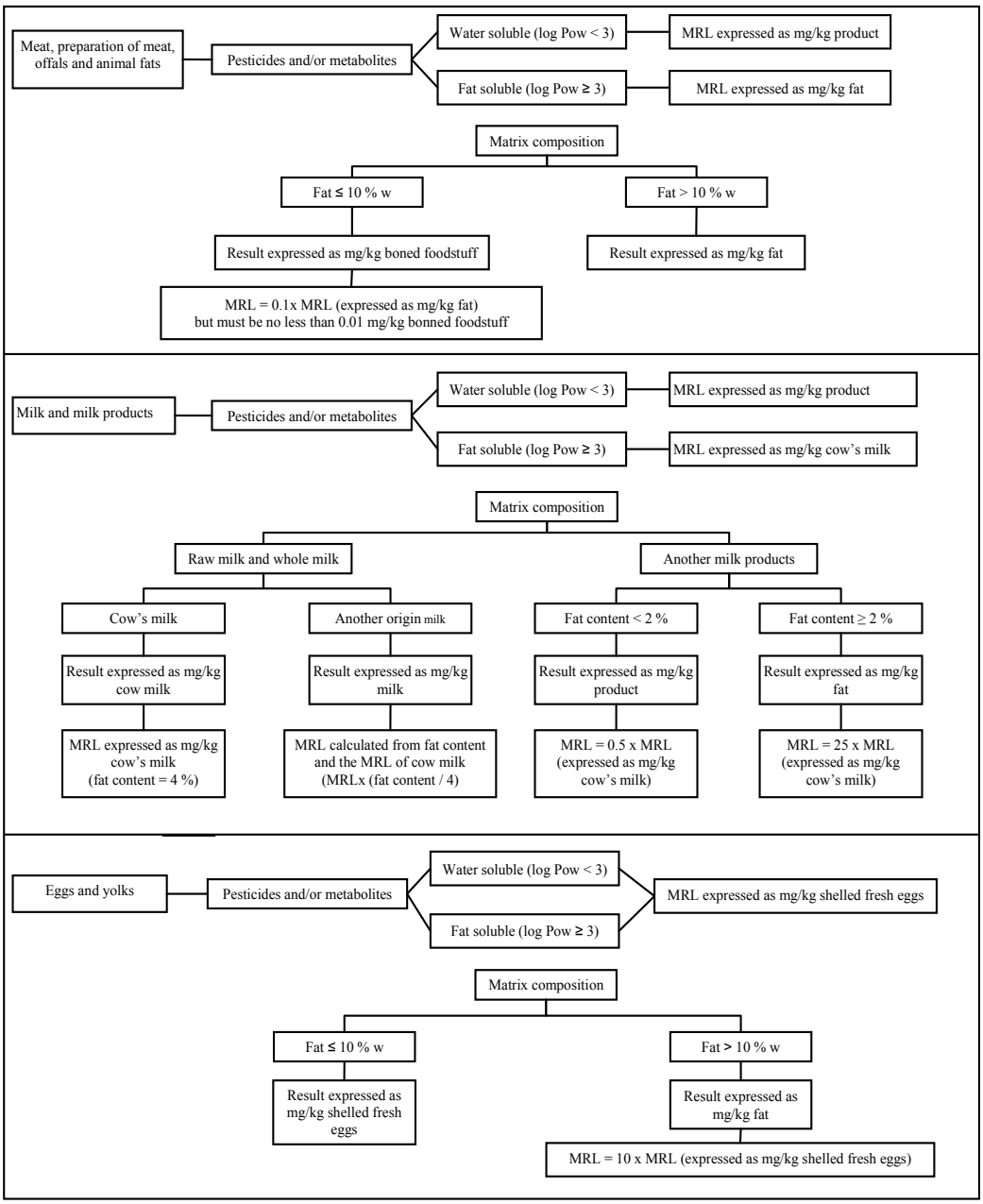


Table 2. Results expression and legal interpretation of MRLs depending on the products and substance properties

In recent years, QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) methodology (used for extraction and clean-up steps) has been applied with success on several non-fatty (< 2 %) food matrices such fruits and vegetables [23-24] and low-fatty (2-20 %) food matrices, such milk, egg and avocado [25].

In multi-residue analysis, the sample preparation process cannot be selective enough to remove chemical compounds of the matrix when they have similar properties to analytes. Modifications of QuEChERS method focused on adjusting solvents, salts volumes, water content and clean-up sorbents. Several modifications of the original QuEChERS method are present in literature, to adapt the method to specific applications. Recently modified QuEChERS methodology has been applied on meat based baby-food [26], olive oil, flaxseeds and peanuts [27-28].

The determination of pesticides can be carried out by sensitive analytical methods such as gas chromatography (GC) or liquid chromatography (LC) with a variety of selective detection methods. Chromatographic analysis traditionally used GC with electron capture detection (ECD) and nitrogen-phosphorus detection (NPD) for non-polar pesticides. Nowadays it is preferable to use mass spectrometry (MS) or tandem mass spectrometry (MS/MS) detection in order to verify peak identities. Automated large volume injection (LVI) systems, based on programmable temperature vaporizing (PTV) injection, are employed to improve the limits of detection for pesticides in food matrices [29-30].

In fatty samples, conventional methods for the analysis of pesticide residues usually involve laborious and time consuming clean-up steps. Moreover, analytical problems associated with lipids extraction when using Selected Ion Monitoring (SIM) mode detection are well known due to matrix interferences, they cause signal suppression or enhancement and make their identification difficult. Moreover, small amounts of remaining lipids in final extracts can harm columns, sources and detectors [28-29]. Therefore, to achieve obtained detection limits using GC-ECD, LVI systems are required when GC-MS is used, involving the introduction of a greater amount of co-extracted matrix material that can contaminate the inlet, column and MS source [30]. This fact makes the clean-up more exhaustive and therefore, conventional methods are tedious for routine analysis. When GC-MS/MS is used, smaller amount of sample can be injected on the system, due to its higher selectivity that minimizes interferences [31].

Governmental regulations demand an increasing number of pesticides to be included in the monitoring programmes. These regulations force laboratories to develop effective methods, capable of detecting an increasing number of pesticides with high certainly degree [32].

Last years, instrumentation and software tools have been developed in order to facilitate the analysts work. For example, Automated Mass spectral Deconvolution and Identification System (AMDIS) provided by the National Institute of Standards and Technology (NIST), has demonstrated the capability to detect target pesticides in matrices with high background of interfering compounds. The usual way to extract background spectra is by subtracting spectrum next to the target peak. The resulting component spectrum is compared with

spectra from database and reported if the quality match factor of the spectrum is over a certain pre-set value. Retention time of the target pesticides are locked to match the retention times in the AMDIS database. The software allows to pre-set a threshold level for the match factor. If it is too low or too high, the possibility of reporting false positive or false negative results increases [32].

One of the main problems when full scan working mode is used is the lack of sensibility compared to SIM or Multiple Reaction Monitoring (MRM) working modes, used in MS and MS/MS respectively. However, the main disadvantage of these methods is their restricted scope to target analysis, making necessary an optimization of each compound to obtain the MRM method transition lists. Available softwares facilitate this work by offering wide database libraries, which can be a starting point. One of those libraries is the Dynamic MRM Pesticide Database, which provides over 600 compounds with specific parameters for Agilent 6400 series LC/QqQ mass spectrometers. It allows re-optimization of compounds through the optimizer program and incorporation of the compounds into data acquisition methods for multi-residue analysis [33].

Over 1000 pesticides have been in use over the last century and new pesticides are being developed, so there is a great need to perform both targeted and non-targeted compounds screening in food. High mass resolution spectrometers (Time of Flight (TOF), tandem hybrid Quadrupole Time of Flight (Q-TOF), Magnetic Sector, and Orbitrap afford fast full-spectral sensitivity, at high-mass resolution with mass accuracy [33-35]. These full spectrum techniques offer the advantage of performing retrospective analysis. These tools allow a rapid and accurate screening of target and not-target compounds.

Non-target analysis can demonstrate the presence of not included compounds in the initial scope, leading us to identify those contaminants which can generate food safety problems and asses their inclusion in target lists [36].

Ideally, all compounds would have the same extraction efficiency, the same chromatography and equal response in MS. But actually, extraction efficiency, peak shapes and responses are different for each compound and each matrix. Detection capability must not be confused with the fact that not detected compounds are not present; this can only be done by validation studies showing that the specific method employed on specific matrices can detect the reported compounds as not present at the levels of concern [34,37].

Different platforms may be combined for the development of multi-residue analysis protocols combining the preliminary screening for the initial qualitative identification using full-scan with high resolution spectrometer platform followed by their quantitative MRM confirmation.

This is very interesting on research purposes and for defining future trends. However, from the point of view of the routine work of an official control laboratory, monitoring the presence of certain compounds proposed for the food safety authority is a priority, as well as monitoring the compliance with the MRL for the regulated analytes.

Our research is targeted on the evaluation of sensitive, selective, easy and quick methods for multi-residue analysis of pesticide residues in animal origin food. Also proposes on-going and effective systematic validation for evaluation methods.

The aim of this work focuses on: 1) to propose a multi-residue method for the determination of lipophilic pesticides in animal origin food, 2) the clean-up evaluation, 3) the instrumental analysis optimization, 4) the comparison GC-MS and GC-MS/MS data, 5) to propose of a screening methodology focusing on quantitative method validation for those detected analytes.

2. Determination of lipophilic pesticide residues in animal origin food: Procedure proposal

2.1. Method description

Steps to approach the analysis of lipophilic pesticides in animal origin food are: 1) Sample preparation: fat extraction from samples; extraction and purification of pesticide residues from lipidic fraction and (2) analytical determination.

The studied scope is shown in Table 1. All the included pesticides are classified as lipophilic (log Kow > 3), except *Malathion* (log Kow = 2.7), *Alachlor* (log Kow = 2.9) and *Parathion-m* (log Kow = 2.9).

2.1.1. Standards

Certified pesticide standards are purchased from Dr. Ehrenstorfer. Purity-corrected individual pesticide stock standards (200 μg/mL) are prepared: weighing 10 mg of pure standard, dissolving each compound in acetone, aliquoting, and storing in capped vials in freezer [23]. Adjusted aliquots (depending on the MRL for each pesticide) of the individual stock solutions are diluted with acetone to prepare three standard mixture solutions (organochlorines, organophosphorus, and pyrethroids). Working mixture solution in acetone (between 0.5 and 5 mg/L), spiking solution in n-hexane (between 0.2 and 2 mg/L), hexanic standards solutions (between 8 and 1500 μg/L), a internal standard solution containing *PCB-54* (100 μg/L) and surrogate solution containing *4,4'-DDT-D$_8$* (100 μg/L) are prepared and stored at -18 °C.

2.1.2. Sample preparation

a. Fat extraction

Animal fat is the most simple and pure matrix, it only needs to be melted and filtered through sodium sulphate. For other matrices, the extraction of the lipidic fraction is required.

Traditionally, this step has been carried out by Soxhlet and Sonication extractions, but Accelerated Solvent Extraction (ASE) allows faster extractions of larger sample series with lower consumption of organic solvents. In our method, the lipidic fraction from meat, offals,

fish, shellfish and dairy products matrices are extracted with ASE; except for eggs, which is extracted by homogenising with solvent, using "Ultraturrax" device. Each sample is spiked with surrogate standard, and some blank samples are spiked with spiking solution for validation and quality control purposes. Two approximations are considered: a) when the results are expressed as mg/kg of fat, the fortification is done in 1 g of melted fat, and b) when the results are expressed as mg/kg of product, the fortification is done in 10 g (5 g of fish) of homogenized sample. For dairy products, the sample volume is reduced by freeze-dried before analysis; for example 25 g of cow milk, corresponding to 1 g of fat, are freeze-dried and used at all. Spiked samples are let for 30 min before the beginning of extraction process.

Samples are mixed with an amount of diatomaceous earth enough to dry completely the sample and obtain a homogeneous powder consistency and dealing in ASE cells as needed. The ASE extraction is carried out using a Dionex ASE 200 and/or 350 with DCM/acetone (1:1) as the extraction solvent at a temperature of 100 ºC and pressure of 1500 psi, with at least two extractions per sample. In the case of eggs, mixed samples are centrifuged to separate the organic fraction from the rest of the matrix. The extracted solvent is reduced in volume under nitrogen stream.

A small part of moisture and powder of diatomaceous earth elute from the sample during the ASE lipid extraction step. In order to remove these impurities, the organic extract is purified by partitioning with hexane/MTBE (9:1) and 0.1M H_3PO_4 with 1% KCl, twice. Purified fraction is evaporated to obtain the lipidic fraction.

b. Extraction and purification of pesticide residues from lipidic fraction

One gram (when results are expressed as mg/kg of fat) or all (when results are expressed as mg/kg of product) the lipidic fraction is extracted twice with 8 mL of n-hexane saturated acetonitrile (18% (v/v)) each time and centrifuged, collecting the acetonitrile fraction. The acetonitrile extract is subjected to a pre clean-up step by remaining fat precipitation by cooling (4 °C overnight).

Clean-up step is d-SPE based on QuEChERS method for fatty samples [20 – 30, 38]. 8 mL of the defatted extract is introduced in a 15 mL polypropylene centrifuge tube containing 1,200 mg $MgSO_4$, 400 mg end-capped C_{18} and 400 mg PSA. It is mixed by vortex and centrifuged. Finally, 4 mL of cleaned supernatant is evaporated under nitrogen stream until near dryness, and then dissolved in a volume of internal standard solution (0.5 mL for GC-MS or 0.25 mL for GC-MS/MS). Then, the solution is transferred to autosampler vials for analysis [39].

Preparation of matrix-matched standards follows the same procedure. Standard solutions (0.5 mL in GC-MS analysis or 0.25 mL in GC-MS/MS analysis) are added before blank extract evaporation.

c. Analytical determination

GC-MS analysis is performed on an Agilent 6890 gas chromatograph coupled to a quadrupole mass spectrometer 5973 configured with a PTV inlet. A prototype deactivated borosilicate and multibaffle liner with fritted glass on interior walls purchased from Agilent

is employed. A retention gap of 5 m x 0.25 mm i.d. (deactivated fused silica) was placed between the injector and the analytical column to avoid a rapid analytical system contamination. Chromatographic separation is performed on a 30 m x 0.25 mm i.d. x 0.25 μm Agilent HP-5MS U.I. column. Helium (99.99%) is used as carrier gas at constant flow (1.2 mL/min). The PTV is configured in solvent vent mode. The automatic liquid sampler, in multiple injection mode, makes ten injections of 4 μL each into the PTV inlet with a 20 sec delay between injections to allow solvent evaporation. During the injection, the column head pressure is set to 0 psi, the steady flow of carrier gas through the liner causes the solvent to evaporate and to be swept with the carrier gas out through the split vent. After the ten injections, the column head pressure is restored and the vent flow is turned off. At this point, the inlet temperature is programmed up to 290 ºC to transfer all the analytes into the GC column. During the sample transfer, the oven temperature is held a 42 ºC. The GC temperature program is: initial 42 ºC, hold 5.8 min; rate 20 ºC/min to 80 ºC, hold 1 min; rate 5 ºC/min to 190 ºC, hold 5 min; rate 15 ºC/min to 270 ºC, hold 10 min and rate 25 ºC/min to 290 ºC, hold 10 min. Determination parameters are: Transfer line temperature 280 ºC, quadrupole temperature 150 ºC and ion source temperature 250 ºC. Ionization operates in electronic impact mode (EI) with 70 eV of electron energy. MS calibration is checked daily following the autotune test of sofware. Electron multiplier voltage is set at 400V above autotune. All the analyses are performed in SIM mode monitoring three ions for each analyte (*Table 3*). Data analysis is assured by MSDChemStation software D.02.00.275 (Agilent).

GC-MS/MS analysis is performed on a Trace GC Ultra and Triplus autosampler coupled to triple quadrupole mass spectrometer from Thermo-Finningan (TSQ Quantum). Separation is carried out on a HP-5MS U.I. capillary column (Agilent) 30 m x 0.25 mm i.d., 0.25 μm film. High-purity helium (99.99%) and argon C50 (99.99%) are used as carrier gas (constant flow of 1.2 mL/min) and as a collision gas, respectively.

The GC temperature program is: initial 90 ºC, hold 5 min; rate 25 ºC/min, to 180; rate 5 ºC/min to 200 ºC, hold 10 min; rate 5 ºC/min to 300 ºC, hold 15 min. Splitless injection conditions are as follow: split flow, 40 mL/min; splitless time, 1.00 min; surge pressure, 200 kPa; surge duration, 1.00 min; injection volume, 2 μL. The mass spectrometer operates in EI mode using 50 μA ionization voltage. The ion source temperature and the GC-MS/MS interface are set to 250 ºC and 300 ºC, respectively. MS calibration is verified daily with calibration gas (PFTBA). MRM working mode is used for all analytes. *Table 4* shows the two monitored transitions for each pesticide.

Data processing is performed using the QuanLab forms 3.1 software (Thermo-Finningan) witch allows the use of obtained MRM with different parents to confirm each compound.

3. Method development

3.1. Clean-up optimization

Acetonitrile is used as extraction solvent because: a) It is polar enough to minimize the amount of co-extractive fat, and b) it is non-polar enough to extract with efficiency lipophilic

pesticides from matrix. The addition of a small amount of a highly non-polar solvent as hexane is, improves the obtained recoveries of more lipophilic pesticides without increasing lipids co-extraction.

In our research, 2 classic clean-up procedures (GPC and fat oxidation), as well as some modifications based on QuEChERS procedures, were applied to fat samples and compared. Analytes and lipids quantitation were carried out to evaluate clean-up efficiency. Total lipids were determined by the sulfo-phospho-vanilline reaction followed by colorimetric determination, prior to each injection.

GPC sample treatment was discarded because a) it was observed that overlapping elution profiles from real samples and standard solutions, higher molecular weight pesticides (mainly pyrethoids) co-eluted with lipids, and b) high amount of chlorinated solvent (125 mL of dichlorometane for sample) was used. Those reasons leaded us to continue our studies testing alternative procedures in order to improve recoveries and minimize the use of organic solvents.

Sulfuric acid treatment was described as a good alternative for removing fat from matrix [40], so it was tested with representative chlorinated pesticides. After successive tests, it was concluded that acid removed fat with efficiency but it showed problems because of the partial (e.g., *Heptachlor* to *exo* and *endo HCEs*) or total (e.g., α and β *Endosulfan*) degradation of some analytes, as well as inconsistent recovery values for others; supporting what had been described by Esteve-Turrillas et al. [12] and Przybylski et al. [26] previously.

As a first approach, extract of 1 g of fat was evaporated and purified with 2 mL QuEChERS using constant amounts of PSA (400 mg) and $MgSO_4$ (1,200 mg) and different amounts of end-capped C_{18} (100, 200 and 400 mg). As a second approach, 2 g of fat was extracted and aliquots were taken achieving dilution factor 4 and purified with 15 mL QuEChERS with constant amounts of PSA (400 mg) and $MgSO_4$ (1,200 mg) and different amounts of end-capped C_{18} (100, 200, and 400 mg). Finally, 15 mL QuEChERS were tested with constant amounts of PSA (400 mg), $MgSO_4$ (1,200 mg), and end-capped C_{18} (400 mg), but two different amounts of sample were tested. *Figure 1* shows fat content after clean-up procedures.

When 2 mL QuEChERS were tested, obtained recoveries decreased as C_{18} amount was increased, while differences on the quantitation of remaining lipids were not relevant, but chromatograms without enough cleanliness were obtained. Experimentally, in some cases retention time drifts higher than 0.5% were found, preventing the determination of certain analytes. In addition, the extracts could not be injected into LVI without damaging the column. These facts led us to think that C_{18} retained analytes and the need of a dilution step or minimize matrix interferences [22]. Higher recoveries were obtained when dilution step was included and 15 mL QuEChERS were used, and remaining fat in vial before injection was one half less than what had been obtained previously.

Finally, the use of 1 g of fat sample instead of 2 g, made easier the quantitation, obtaining clearer chromatograms, reducing background noise, reaching the lowest fat content among

tested conditions and achieving required MRLs. This fact can be explained by the reduction of injected matrix that removed most of chromatographic interferences present when 2 g of sample were analyzed.

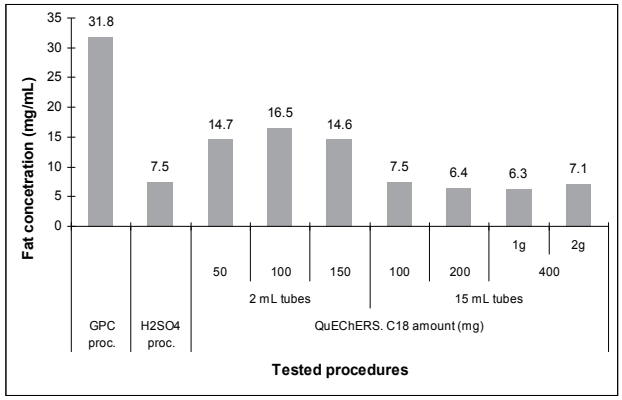


Figure 1. Fat content evaluation after clean-up procedures: GPC procedure, H_2SO_4 procedure, 2 mL QuEChERS and 15 mL QuEChERS.

These results leaded us to choose 15 mL QuEChERS with 400 mg of C_{18} using 1 g of sample and including a dilution step as the better procedure. This technique reached a sample concentration factor similar to the studied on GPC method (0.5 g/mL), obtaining clean extracts that could be injected into LVI (40 μL) without damaging chromatographic system. In addition, this technique consumed smaller solvent amount (13 mL acetonitrile + 3 mL n-hexane). Finally, acceptable recovery values were obtained for all studied pesticides [39].

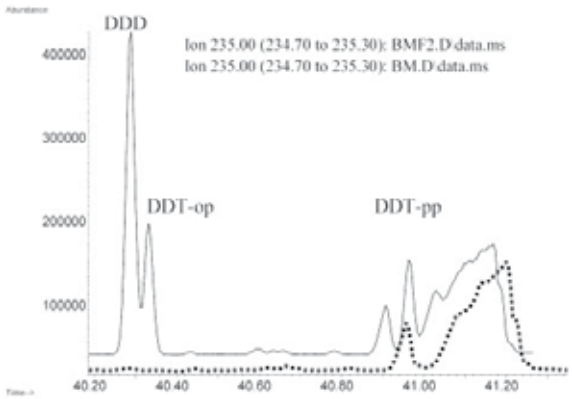
3.2. Instrument optimization

Chromatographic and spectrometric parameters were optimized with hexanic standard solution and matrix matched standard solution of all pesticides, due to the complexity of matrices. Final delay let to achieve the cleaning up of capillary column and to avoid losses by possible shifts of the retention times. Gradient slope optimization was performed with matrix matched solution. The application of a low gradient slope allows improving separation of co-extractives which could be co-eluted with analytes and allow the separation of isomers such as *HCHs*, as well as *DDD* and *DDTs* [41]. In *Figure 2*, shows two chromatograms where a couple of isomers are represented in both GC systems.

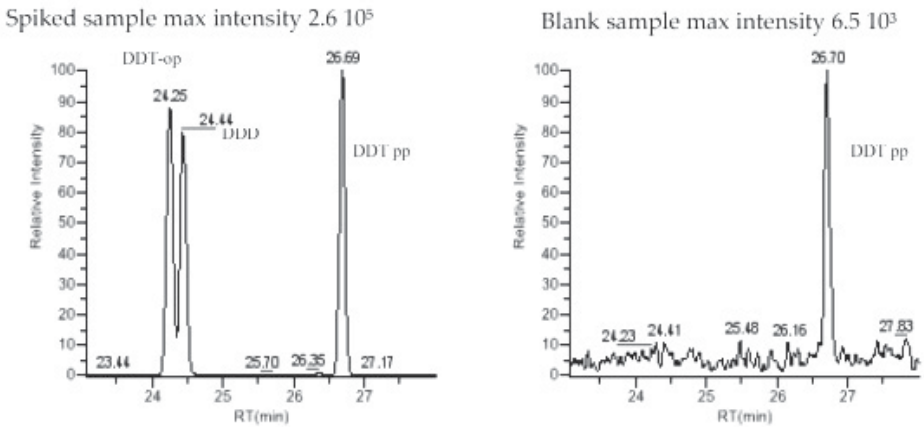
a. Ion selection in GC-MS system

As general criteria, mass spectrometry conditions were carefully selected to provide a compromise solution between sensitivity, selectivity and structural information, being the base ion chosen for quantitation purposes. However, most abundant ions could not be used in all cases, because of the interferences from matrix. Therefore, more selective and less sensitive ions must be used. The three most selective ions were chosen after the injection of matrix matched standard solution, in full scan mode; except for *Triazophos*, because of the

absence of a third selective ion with sensitivity enough to be detected at the lowest studied level.



a) GC-MS



b) GC-MS/MS

Figure 2. Chromatograms for *DDD, DDT-op* and *DDT-pp* in: a) blank sample (discontinuous trace), spiked sample at 0.05 mg/kg (continuous trace) with GC-MS(SIM) system and, b) blank sample, and spiked sample at 0.02 mg/kg with GC-MS/MS(MRM).

The method was divided into as many time segments as possible, in order to obtain the maximum signal for pesticide that gave the lowest response. Dwell time for each ion was set between 10 and 100 ms, depending on the number of ions monitored in each group and the sensitivity of each ion; so the minimum cycles/sec for each group was higher than 2.5. *Table 3* provides the SIM program used for the GC-MS analysis.

b. MRM selection in GC-MS/MS system

The use of MRM mode based on QqQ mass analyzer allows low analyte detectability and is one of the most selective approaches at present for trace analysis. However, for multiclass,

multi-residue methods, a precise optimization of MS/MS parameters is needed, in order to maximize the signal for each pesticide.

Group	Pesticide	RT (min)	Q (m/z)	q1 (m/z)	q1 ratio	q2 (m/z)	q2 ratio	Dwell time	Cicles/Seg
1	Trifluralin	28.7	306	264	65.0	290	12.4	25	3.36
	HCH-α	29.1	217	145	29.2	181	137.4	40	
	HCB	29.3	284	286	79.4	249	23.9	25	
2	HCH-β	30.4	221	181	188.6	147	35.8	60	3.09
	Lindane	30.5	181	217	87.6	145	19.6	60	
3	Diazinon	31.3	137	179	93.2	152	67.4	20	3.07
	PCB 54	32.3	292	220	56.0	255	6.5	80	
4	Chlorpyrifos-m	33.5	286	288	69.7	290	15.5	10	3.03
	Parathion-m	33.5	263	264	10.3	233	9.2	10	
	Heptachlor	33.7	272	274	79.7	270	53.7	60	
	Alachlor	33.9	160	188	90.6	146	32.8	20	
5	Pirimiphos-m	35.4	290	276	82.5	305	75.5	10	3.12
	Aldrin	35.8	265	293	57.2	261	95.7	45	
	Malathion	36.0	173	158	56.5	285	8.1	10	
	Fenthion	36.4	278	279	12.7	280	11.5	10	
	Chlorpyrifos	36.5	314	316	75.4	286	41.8	10	
	Parathion	36.6	291	186	19.1	235	16.4	10	
6	HCE-exo	37.7	353	355	79.9	351	52.1	30	3.09
	HCE-endo	37.9	353	355	81.1	263	42.1	70	
7	Chlordane trans	38.5	373	272	18.4	237	19.5	25	2.99
	Methidathion	38.6	145	302	3.7	146	6.7	25	
	Endosulfan-α	38.9	277	265	117.6	339	81.4	45	
	Chlordane-cis	39.0	373	374	13.4	272	17.9	25	
8	Dieldrin	39.6	263	277	81.0	279	77.1	70	3.08
	DDE	39.6	246	318	88.6	316	68.8	30	
9	Endrin	40.1	263	265	67.6	345	23.0	40	2.89
	Endosulfan-β	40.3	277	265	76.1	339	93.1	40	
	DDD	40.5	235	237	64.7	239	11.1	40	
	DDT-op	40.6	235	237	64.7	239	10.9	40	
10	Triazophos	40.9	172	313	31.8			30	3.02
	Endosulfan-sulfate	41.2	387	385	60.3	389	67.1	30	
	DDT-D_8	41.2	243	245	62.2	173	31.4	30	
	DDT-pp	41.2	237	235	69.0	212	11.7	10	
11	Bifenthrin	42.3	181	166	26.3	165	26.9	100	3.11
12	Cyhalothrin-λ	43.6	181	197	77.6	208	54.9	50	3.07

Group	Pesticide	RT (min)	Q (m/z)	q1 (m/z)	q1 ratio	q2 (m/z)	q2 ratio	Dwell time	Cicles/Seg
	Pyrazophos	44.0	232	373	70.0	237	58.9	50	
13	Permethrin	44.9	183	184	14.9	163	23.5	100	3.11
14	Cyfluthrin	46.4	165	226	81.9	199	66.2	50	3.08
	Cypermethrin	46.9	163	181	73.4	209	20.0	50	
15	Deltamethrin	51.5	181	253	92.9	255	45.4	100	3.10

Table 3. GC-MS SIM method parameters used for quantification and confirmation.

Group	Pesticide	RT (min)	Q transition (m/z)	CE (eV)	q transition (m/z)	CE (eV)	IR (Q/q)	ST (s)
1	Trifluralin	11.77	**306.00>264.27**	10	**263.96>159.60**	15	30	0.08
2	HCH-α	12.27	**216.85>180.93**	10	**218.86>183.17**	10	75	0.05
	HCB	12.44	**283.74>214.04**	30	**283.74>249.11**	20	90	
3	HCH-β	12.90	**180.84>144.88**	15	**218.87>183.15**	10	70	0.045
	Lindane	13.01	**180.84>144.88**	15	**218.87>183.15**	10	65	
	Diazinon	13.26	**304.05>179.23**	10	**179.02>137.00**	20	70	
4	PCB-54	14.14	**219.94>149.80**	30	**291.85>219.8**	20	100	0.05
	Chlorpyrifos-m	14.77	**285.85>271.12**	15	**285.85>92.89**	20	50	
	Pirimiphos-m	14.81	**262.94>108.85**	10	**262.94>79.00**	20	20	
	Alachlor	15.00	**188.03>160.14**	10	**188.03>130.06**	30	80	
	Heptachlor	15.05	**271.74>237.08**	15	**271.74>234.96**	15	20	
5	Parathion-m	15.77	**290.00>233.18**	10	**275.99>244.43**	10	15	0.033
	Malathion	16.16	**172.98>98.90**	15	**127.00>98.95**	10	90	
	Aldrin	16.39	**262.8>228.00**	20	**262.8>193.06**	25	170	
	Fenthion	16.58	**277.95>109.09**	20	**277.95>169.06**	15	90	
	Chlorpyrifos	16.62	**313.89>258.05**	15	**196.85>169.02**	15	85	
	Parathion	16.69	**291.05>109.15**	10	**291.05>80.85**	25	70	
6	HCE endo	18.31	**352.78>263.10**	15	**352.78>282.25**	15	25	0.02
	HCE exo	18.55	**182.84>119.02**	20	**182.84>155.09**	15	100	
7	Chlordane trans	19.67	**372.75>266.00**	20	**372.75>264.13**	20	65	0.11
	Methidathion	19.91	**144.88>58.26**	15	**144.88>84.95**	15	150	
	Endosulfan-α	20.46	**238.84>204.12**	15	**240.87>204.12**	15	30	
	Chlordane cis	20.67	**372.75>266.00**	20	**372.75>264.13**	20	65	
8	DDE	22.38	**245.94>176.14**	25	**245.94>211.13**	20	15	0.15
	Dieldrin	22.39	**276.84>240.57**	10	**262.81>193.08**	30	160	
9	Endrin	24.00	**280.96>245.15**	10	**262.79>192.89**	30	260	0.25
10	Endosulfan-β	24.74	**240.88>206.05**	15	**194.84>159.00**	10	85	0.13
	DDD	25.50	**235.07>165.09**	20	**236.95>165.07**	20	70	
	DDT-o,p	25.65	**235.07>165.09**	20	**236.95>165.07**	20	70	
11	Triazophos	26.94	**256.94>162.18**	10	**284.97>162.020**	10	40	0.07
	Endosulfan sulfate	27.47	**271.73>236.83**	15	**273.73>239.09**	15	55	

Group	Pesticide	RT (min)	Q transition (m/z)	CE (eV)	q transition (m/z)	CE (eV)	IR (Q/q)	ST (s)
	DDT-D_8	27.63	**243.03>206.14**	15	**243.03>172.6**	20	35	
	DDT-p,p	27.76	**235.07>165.09**	20	**236.95>165.07**	20	70	
12	Bifenthrin	30.93	**181.00>165.06**	25	**181.00>166.18**	10	65	0.22
13	Cyhalothrin-λ	33.56	**181.00>152.14**	20	**196.97>141.20**	10	55	0.1
	Pyrazophos	34.05	**232.07>204.18**	10	**221.05>193.07**	10	175	
14	Permethrin	35.45	**182.99>168.16**	10	**182.99>153.12**	15	100	0.22
15	Cyfluthrin	37.05	**226.07>206.21**	15	**162.98>90.98**	10	100	0.14
	Cypermethrin	37.23	**181.00>152.14**	20	**162.98>90.98**	10	70	
16	Deltamethrin	40.72	**252.90>92.76**	15	**181.00>127.03**	25	50	0.2

Table 4. GC-MS/MS(MRM) method parameters used for quantification and confirmation.

The first step was the selection of the parent ions (precursor ions), which were chosen after examining the full scan spectra of each pesticide. This step was performed using a hexanic standard solution. Not always the base peak was preferred (highest intensity), for some pesticides a more selective one was selected. For some pesticides, two parent ions were selected, one for each transition.

Secondly, three transitions were taken for each pesticide after checking the fragmentation of each parent ion, working in product scan mode with a hexanic standard solution. Finally, the MRM collision energy was optimized for each selected transition using matrix matched solutions, and the two most selective transitions were chosen. *Table 4* provides the selected conditions used in MRM program used for GC-MS/MS analysis.

An adjusted scan time (dwell time) was fixed for each transition, taking into account the recommendation that about 12-15 scans are needed across any peak to ensure representative peak shape and area.

GC-MS(SIM) method was optimized with animal fat samples. A LVI procedure based on a PTV injection was employed, in order to improve the detection limits of the target compounds in the analysis, The cleanliness of the obtained extracts with the developed method allows longer working sequences with acceptable drifts and a significantly reduce of instrumental maintenance. However, matrix interferences for some pyrethroids, as well as sensitivity limitations, were observed [39].

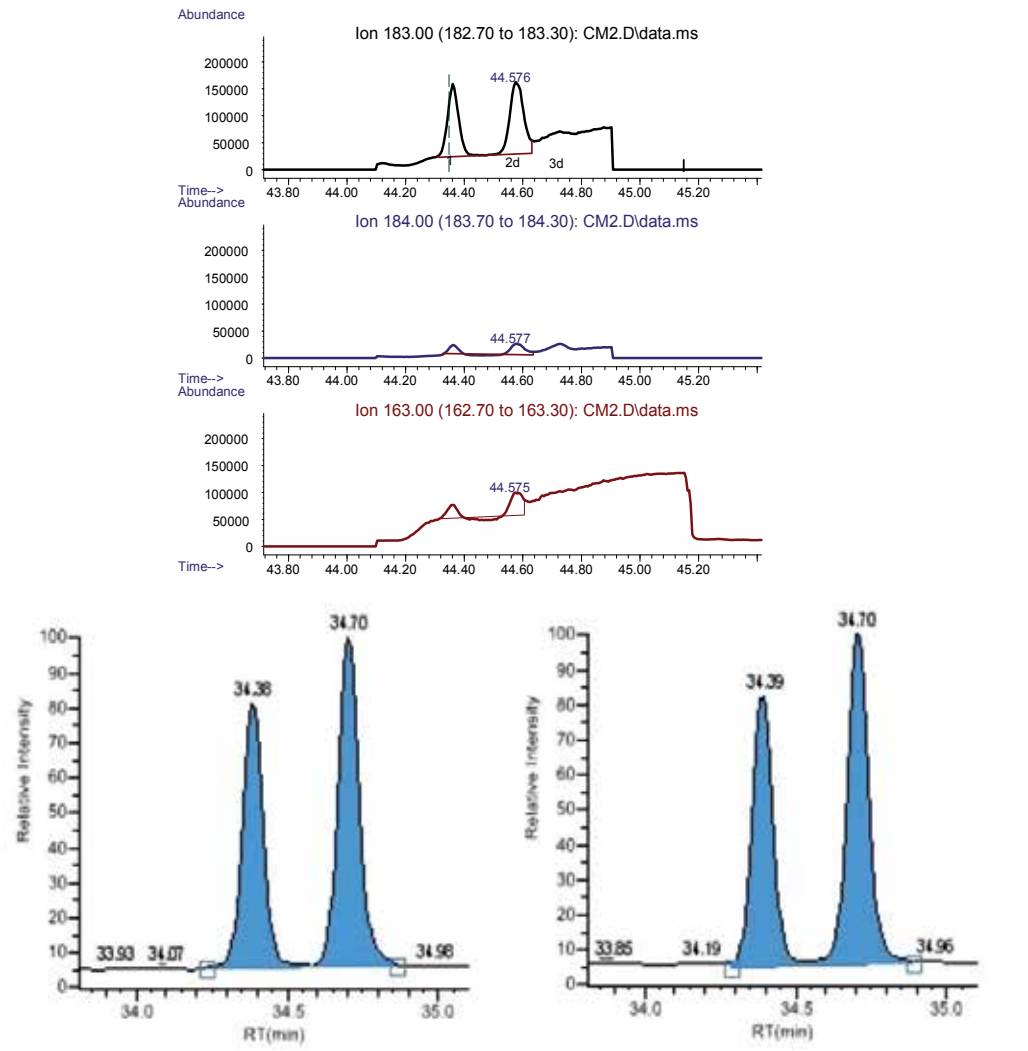
MS/MS offers additional specificity with secondary fragmentations, and thus circumvent co-elution problems. Besides, it provides a more definitive tool. Tandem MS also decreases matrix interferences, improves selectivity, achieves higher signal-to-noise ratio, and subsequently improves the detection limit [2, 31].

The optimized GC-MS/MS method achieved higher selectivity, lowers LODs and reduced the influence of the matrix, as for *Deltamethrin* and *Cyhalothrin-λ*, injecting the tenth part of sample that in GC-MS was.

Figure 3a shows the obtained chromatograms for *Permethrin* in a pork fat sample spiked at 0.05 mg/kg by GC-MS and GC-MS/MS analysis. *Figure 3b* shows the obtained

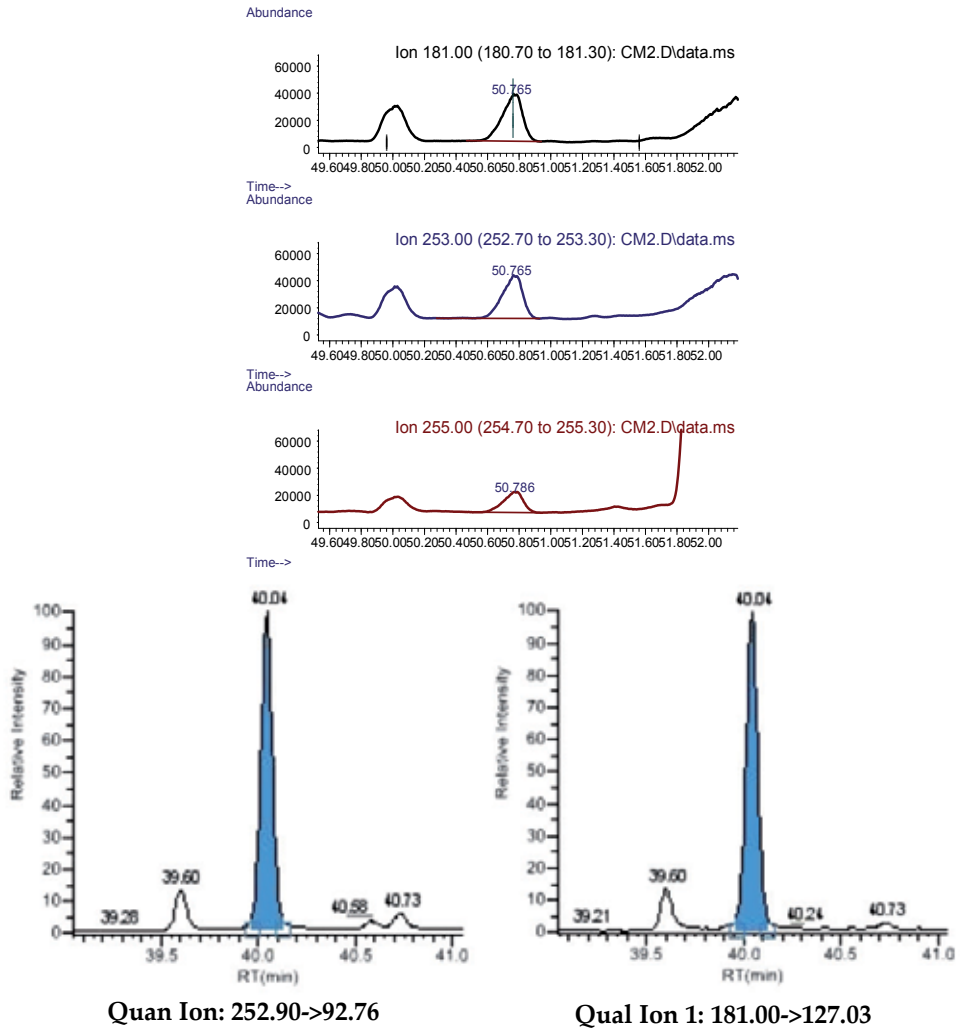
chromatograms for *Deltamethrin* in a pork fat sample spiked at 0.2 mg/kg by GC-MS and at 0.1 mg/kg by GC-MS/MS analysis. The additional MS stage of MS/MS instrument provides enhanced selectivity, reducing the noise from the matrix.

In addition, the reduction of sample volume injected, minimized problems caused by matrix components at the injector/column and detector sites.



Quan Ion: 182.99->168.16 **Qual Ion 1: 182.99->153.12**

(a)



(b)

Figure 3. a) *Chromatograms obtained for Permethrin in a pork fat sample at 0.05 mg/kg by GC-MS and GC-MS/MS analysis.* **b)** *Chromatograms obtained for Deltamethrin in a pork fat sample at 0.2 mg/kg by GC-MS and at 0.1 mg/kg by GC-MS/MS analysis.*

4. Method performance

A significant proportion of analyzed samples are free of pesticides in a detectable concentration and the most of the analytes are not detected, so a systematic quantitative study of them is a waste of effort and time.

The use of qualitative methods allow to discriminate samples and analytes where we should focus our efforts, applying quantitative methods only in those samples with previously detected analytes.

Primary sequences are screening sequences, composed of an appropriate number of unknown samples, a negative control and duplicated positive controls, formed by blank samples spiked at reporting level. Screening and confirmation can be performed in a single analysis in which the identification/confirmation criteria are applied to detected peaks. In any case, it is important to choose properly the detection criteria (cut-off): if it is too strict, we find too many false negatives and if it is too loose, we have many tentative detects, needing further following. We use as cut-off-value the obtained in validation studies from the mean response of spiked samples (V) and its standard deviation (S) (cut-off = V – 1.64 x S) [42]. Confirmation criteria, as Relative Intensity (IR) and Retention Time (RT) are adjusted with matrix-matched standards at LOQ level each sequence.

Quantitative sequences allow measuring the concentration of detected pesticides in selected samples as well as their confirmation, when necessary. In this case, multi level matrix matched calibration, adjusted by weighted linear regression, is used with this purpose.

5. Method evaluation

Document SANCO 1495/2011 [37] describes the method validation and analytical quality control requirements to support the validity of data used for checking compliance with maximum residue limits (MRLs), enforcement actions, or assessment of consumer exposure to pesticides.

5.1. Matrix effects

On the analysis of pesticide residues in fat and fatty food of animal origin, low concentration levels of residues in the presence of a great quantity of compounds from the matrix is expected. Practically, no clean-up method completely removes all the matrix components from a crude extract. The matrix components injected into chromatographic system may led to false positive or negative results, low analyte detectability, inaccurate quantitation and decreased method ruggedness [7].

That is the reason why there are several approaches in literature to reduction of matrix effect, as: a) the use of isotopically labeled internal standards, b) an exhaustive clean-up to remove the most of the matrix from the extracts, and c) the use of matrix-matched standard calibration. The first option is the most expensive one, especially in multi-component analysis. The second one has to be reached with selective extraction procedures or with more extensive sample clean-up, leading to time consuming procedures. The third one is the most widely and simple approach used in pesticide residue analysis.

Single MS detectors with low resolution can be not selective enough to distinguish interfering matrix compounds from selected ions. This fact becomes the main problem with compounds with non-specific ions with low m/z ratios. In that case, the use of chromatographic systems with increased sensibility and specificity, as MS/MS are, are a possible solution.

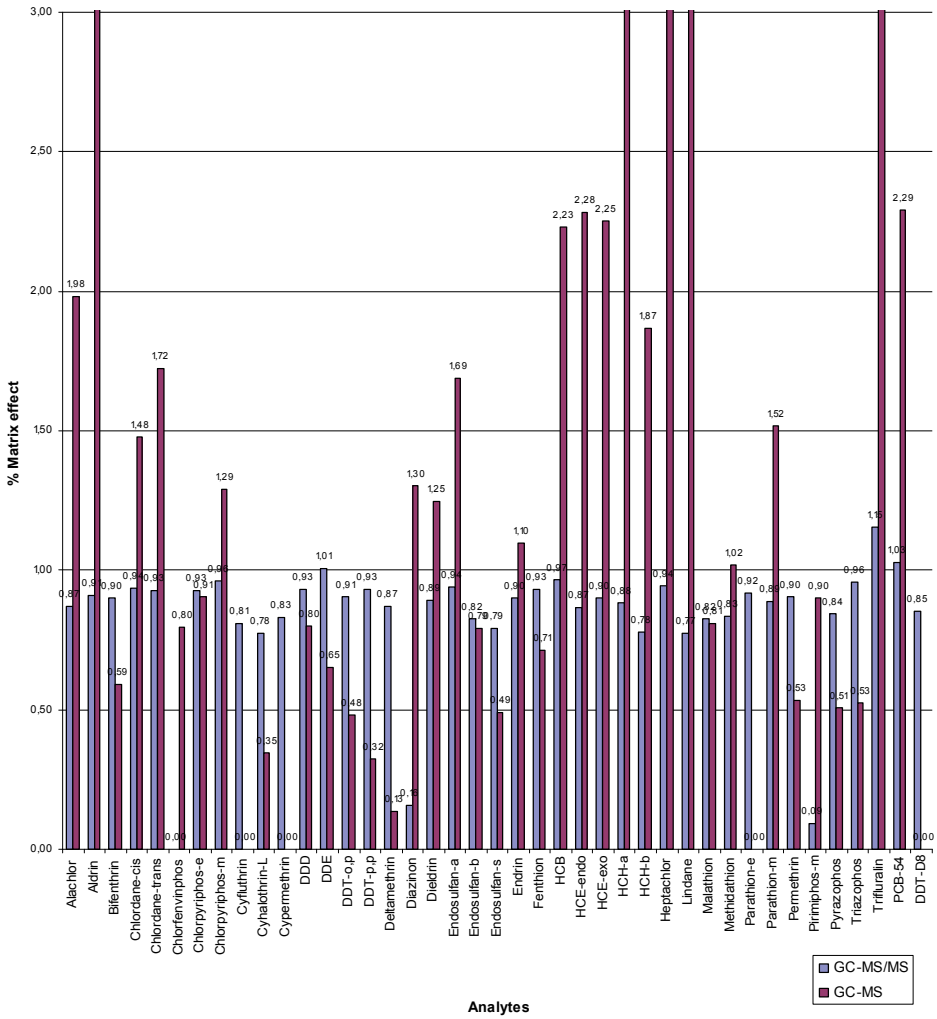


Figure 4. Experimental matrix effect (matrix matched slope/solvent slope) obtained with previous GC-MS and actual GC-MS/MS systems. Values below 1 mean signal suppression and values above 1 mean signal enhancement.

Matrix components can behave like interferences inducing a reduction of the signal corresponding to an analyte, presumably by decreasing ionization potential or also by inducing directly or indirectly its degradation [43-44]. Other pesticides can be greatly protected by the action of the co-extractives as powerful masking agents which block the active sites of analytical system, mainly in the injector or in the column. Mastovska et al. related that the addition of analyte protectants (ethylglycerol, gulonolactone, and sorbitol) on sample extracts and solvents standards corrected the matrix-induced enhancement and suppression [45]. The strength of this effect, can be evaluated by comparison of the calibration curve slopes obtained with solvent-based standards and with matrix-matched standards. An increased response represents a sensitivity enhancement, due to matrix, compared to the standard solution in solvent.

On this work, matrix-matched calibration standards were prepared adding standard solutions to blank extracts of a pork fat sample. Linearity was studied analyzing those matrix-matched standards. Matrix effects were evaluated by comparison of the solvent standard calibration curves and the matrix-matched standard calibration curves. Moreover, experimental matrix effect values with GC-MS/MS(MRM) were compared with those previously obtained with GC-MS(SIM), as *Figure 4* shows.

Obtained values show a great decrease on matrix effect when GC-MS/MS system is used. As a first approach, this fact can be explained by the use or a more selective method, avoiding most of the interferences and background noise present when GC-MS was used. On the other hand, the injection of a smaller amount of extract decreases the introduction of matrix and co-extractives which block the active sites of the analytical system and reduces matrix effects (enhancement or suppression).

5.2. Method validation

Sanco document [37] distinguishes between qualitative screening methods and quantitative ones. For the first ones, the Limit Of Detection (LOD) is defined as the lowest concentration for which has been demonstrated that a certain analyte can be detected in at least, 95% of the samples (i.e. a false negative rate of 5% is accepted). In the case of selectivity, the presence of false detects should be verified using unspiked samples (verifying the absence of interfering peaks whose response > 30% LOD). For each commodity group, a basic validation of a qualitative method should involve analysis of at least 20 samples spiked at the anticipated LOD. The selected samples should cover multiple matrices from the commodity group, with a minimum of two samples for matrix, and being representative for the matrix scope of the laboratory. Its application in routine analysis, on-going QC data should be acquired and the performance of the method should be periodically reassessed.

Quantitative methods must be tested to assess for sensitivity, mean recovery, precision, linearity, and Limit Of Quantitation (LOQ). This means that spiked recovery experiments to check the accuracy of the method, should be undertaken. A minimum of 5 replicates is required (to check the precision and accuracy) at the LOQ, and at least another higher level (HL). The LOQ is defined as the lowest validated spiked level meeting the method performance acceptability criteria (mean recoveries for each representative commodity in the range 70-120%, with an RSD ≤ 20%).

Experimental obtained values for the validation of the screening (qualitative) method are resumed on *Table 5*.

In fat and poultry meat samples, all the studied pesticides have been validated at initially proposed LODs. In the case of eggs, *Cyfluthin, Permethrin*, and *Methidation* could not be validated the proposed levels, 0.05, 0.05 and 0.02 mg/kg respectively. *Cyfluthrin* because a lack of sensibility on the confirmation transition; *Permethrin* because one of its isomers has an important interference; and *Methidathion* because of the inconsistent values on

confirmation transition. In shellfish, *Deltamethrin* and *Permethrin* had selectivity problems, and *Malathion* sensibility ones, so the proposed method is not valid for proposed LODs (0.01, 0.005 and 0.02 respectively). In liver matrices, *Malathion* and *Endosulfan-β* show interferences at LOD levels (0.002 and 0.005 mg/kg respectively). Finally, in milk products the screening method had not sensibility enough to detect *Alachlor* and *Parathion-m* at LOD levels (0.0004 and 0.0008 mg/kg respectively), as well as *Deltamethrin* and *Malathion* had some interferences at LODs (0.004 and 0.0008 mg/kg respectively).

Experimental obtained values for the validation of the quantitative method are resumed on *Table 6*.

Most pesticides gave 70 – 120 % acceptable recoveries with associated RSD < 20 %. Some organophosphorous pesticides show values out of these ranges: *Parathion*, *Parathion-m*, *Malathion*, and *Methidation* in both levels and *Triazophos* in high level. For those positive detections, alternative methods are necessary. *HCB* at high level gave low recovery (64%), but very reasonably consistent RSD (5%). The reason is a physicochemical partitioning factor between lipids and acetonitrile phases and the retention in C_{18} sorbent. Accurate quantification, of *HCB* will require either the use of internal standard that are matched more closely to the lipophilic analytes or the use of an extracted matrix matched calibration or to correct the results for the recovery factor. Improvements in the accuracy are feasible using deuterinated pesticides or ^{13}C labelled standard.

5.3. Quality control

Method verification is necessary during routine analysis (analytical quality control and on-going method validation).

For qualitative screening sequences, blank and a duplicate sample spiked at LOD level are introduced. Blank sample must agree with the blank criteria (no peaks with response higher than 30 % of the response at LOD level) and fortified samples classified as positive.

In quantitative sequences, introduced positives and blank controls and single recovery values must be in the range 60 – 140 %. Recoveries outside the mentioned range require re-analysis of the batch but can be acceptable in certain justified cases. Where the individual recovery is unacceptably high and no residues are detected, it is not necessary to reanalyse the samples to prove the absence of residues. However, consistently high recoveries should be investigated. If a significant trend occurs in recovery, or potentially unacceptable (RSD beyond ± 20 %) results are obtained, the cause(s) must be investigated. Linearity is checked for selected analytes by calculated the residuals, which must be within ± 20%.

The laboratory must participate regularly in relevant proficiency tests. When a low number of compounds (e.g.: < 90%) are analysed with respect to the pesticides present in the test sample, false positive(s) or negative(s) are reported or the accuracy achieved in any of the tests is questionable or unacceptable, the problem(s) should be investigated. Particularly for false positive(s), negative(s) and, or unacceptable performance, have to be rectified before proceeding with further determinations of the involved analyte/matrices combinations.

Matrix	Fat	Eggs	Milk	Shellfish	Chicken Meat	Liver
Compound name	LOD (mg/kg fat)	LOD (mg/kg fat)	LOD (mg/kg product)	LOD (mg/kg product)	LOD (mg/kg product)	LOD (mg/kg product)
Alachlor	0.01	0.01	NV	0.001	0.001	0.001
Aldrin	0.05	0.05	0.002	0.005	0.005	0.005
Bifenthrin	0.05	0.05	0.002	0.005	0.005	0.005
Chlordane-cis	0.05	0.05	0.002	0.005	0.005	0.005
Chlordane-trans	0.05	0.05	0.002	0.005	0.005	0.005
Chlorpyrifos-m	0.05	0.05	0.002	0.005	0.005	0.005
Chlorpyrifos	0.05	0.05	0.002	0.005	0.005	0.005
Cyfluthrin	NV	0.05	0.002	0.005	0.005	0.005
Cyhalothrin-λ	0.05	0.05	0.002	0.005	0.005	0.005
Cypermethrin	0.1	0.1	0.004	0.01	0.01	0.01
DDD	0.02	0.02	0.0008	0.002	0.002	0.002
DDE	0.02	0.02	0.0008	0.002	0.002	0.002
DDT-op	0.02	0.02	0.0008	0.002	0.002	0.002
DDT-pp	0.02	0.02	0.0008	0.002	0.002	0.002
Deltamethrin	0.1	0.1	NV	NV	0.01	0.01
Diazinon	0.05	0.05	0.002	0.005	0.005	0.005
Dieldrin	0.05	0.05	0.002	0.005	0.005	0.005
Endosulfan sulphate	0.05	0.05	0.002	0.005	0.005	0.005
Endosulfan-α	0.05	0.05	0.002	0.005	0.005	0.005
Endosulfan-β	0.05	0.05	0.002	0.005	0.005	NV
Endrin	0.04	0.04	0.0008	0.004	0.004	0.004
Fenthion	0.05	0.05	0.002	0.005	0.005	0.005
HCB	0.02	0.02	NV	0.002	0.002	0.002
HCE-endo	0.05	0.05	0.002	0.005	0.005	0.005
HCE-exo	0.05	0.05	0.002	0.005	0.005	0.005
HCH-α	0.05	0.05	0.002	0.005	0.005	0.005
HCH-β	0.05	0.05	0.002	0.005	0.005	0.005
Heptachlor	0.05	0.05	0.002	0.005	0.005	0.005
Lindane	0.015	0.015	0.0006	0.0015	0.0015	0.0015
Malathion	0.02	0.02	NV	NV	0.002	NV
Methidathion	NV	0.02	0.0008	0.002	0.002	0.002
Parathion	0.05	0.05	0.0008	0.005	0.005	0.005
Parathion-m	0.02	0.02	NV	0.002	0.002	0.002
Permethrin	NV	0.05	0.002	NV	0.005	0.005
Pirimiphos-m	0.05	0.05	0.0008	0.005	0.005	0.005
Trifluralin	0.01	0.01	0.0004	0.001	0.001	0.001

Table 5. Validation results for screening procedure applied to several animal origin matrices
NV, not validated compound

Compound	LOQ Level				Higher level			
Name	mg/kg	n	R%	RSD%	mg/kg	n	R%	RSD%
Alachlor	0.01	8	106	10	0.10	6	105	8
Aldrin	0.05	8	81	16	0.50	6	76	6
Endosulfan-α	0.05	8	97	8	0.50	6	93	5
HCH-α	0.05	8	99	11	0.50	6	96	7
Endosulfan-β	0.05	8	108	11	0.50	6	97	5
HCH-β	0.05	8	117	15	0.50	6	107	19
Bifenthrin	0.05	8	100	14	0.50	6	96	8
Cyfluthrin	0.05	8	106	20	0.50	6	99	20
Cypermethrin	0.1	8	103	13	1.00	6	94	16
Chlordane-cis	0.05	8	96	13	0.50	6	91	7
DDD	0.02	8	95	18	0.20	6	90	6
DDE	0.02	8	78	17	0.20	6	84	6
Deltamethrin	0.1	8	109	15	1.00	6	99	18
Diazinon	0.05	8	103	13	0.50	6	105	6
Dieldrin	0.05	8	98	11	0.50	6	95	3
Chlorpyrifos	0.05	8	104	11	0.50	6	103	5
Parathion	0.05	8	123	14	0.50	6	110	21
Endosulfan sulphate	0.05	8	119	15	0.50	6	113	20
Endrin	0.04	8	98	14	0.40	6	93	4
Fenthion	0.05	8	115	14	0.50	6	107	11
HCB	0.02	8	71	18	0.20	6	64	5
HCE-endo	0.05	8	101	10	0.50	6	98	5
HCE-exo	0.05	8	100	10	0.50	6	99	5
Heptachlor	0.05	8	89	11	0.50	6	88	8
Cyhalothrin-λ	0.05	8	106	16	0.50	6	96	16
Lindane	0.015	8	103	8	0.15	6	98	10
Chlorpyrifos-m	0.05	8	104	13	0.50	6	102	4
Parathion-m	0.02	8	128	23	0.20	6	120	28
Pirimiphos-m	0.05	8	106	14	0.50	6	105	4
Malathion	0.02	8	126	20	0.20	6	124	26
Methidathion	0.02	8	123	15	0.20	6	116	25
DDT-op	0.02	8	101	10	0.20	6	96	8
Permethrin	0.05	8	92	16	0.50	6	90	9
Pyrazophos	0.02	8	109	13	0.20	6	102	15
DDT-pp	0.02	8	96	16	0.20	6	91	5
Chlordane-trans	0.05	8	94	14	0.50	6	92	4
Triazophos	0.01	8	111	36	0.10	6	130	42
Trifuralin	0.01	8	104	13	0.10	6	100	7

Table 6. Validation results obtained in fat sample at two levels studied (LOQ and HL).
N = nº of spiked samples; R = recovery (%) and RSD = relative Standard deviation (%)

6. Application to the analysis of real samples

The method was applied for routine analysis of lipophilic pesticides. Samples of different animal origin were collected during 2010 and 2011 from several farms from Comunidad Valenciana (Spain) and analyzed. 167 samples of animal fat (19% from swine, 19% from bovine, 29% from sheep, 25% from chicken, 5% from rabbit and 3% from horse) were analyzed. Sixteen samples were contaminated by *DDE* with values above the LOD (0.02 mg/kg): fourteen of them were sheep fat, with *DDE* values between 0.021 and 0.175 mg/kg; and two of them were bovine fat, with 0.210 and 0.221 mg/kg of pesticide. Only one sample showed the presence of *HCB* in a higher concentration than the LOD (0.02 mg/kg); it was a porcine fat with 0.036 mg/kg of pesticide. The rest of the samples presented concentrations below the LOD values.

Pesticides from other matrices were analyzed too: eggs (20), milk (4), fish (2), shellfish (4), liver (10), and chicken meat (10). All of them showed absence of pesticides above LOD level.

7. Future research

Our working team intends to continue its research on the determination of pesticides in animal origin food following two ways:

a. The extension of the laboratory scope in both, analytes and matrices; by the increase of analyzed pesticides and to extend and complete the list of the included substances on the definition of residue. In the case of matrices, the main objective is to increase the number of covered matrices.
b. Instrumental development on GC-MS/MS by the comparison of negative chemical ionization and EI ionization modes. The improvement of selectivity and sensibility by the use of back-flush technology. The complementation of GC chromatography with the use of UHPLC-MS/MS system, to extend the list of analyzed pesticides with other families as triazines, carbamates, etc., also included on the definition previously mentioned.

8. Conclusions

Official control laboratories are required to handle a large number of analytes in different matrices with validated methods. That is the reason that quick, and easy methods are required.

In this chapter, extraction and clean-up efficiency have been evaluated (due to the success of the analysis of lipophilic pesticides in fatty matrices critically relies on the efficiency of the clean-up) in order to: a) reduce instrumental maintenance, b) reduce solvents consumption, and c) minimize time and effort consumption in sample treatment. This evaluation, lead us to choose modified QuEChERS procedure as the best one.

Previously observed matrix interferences in GC-MS for some pyrethroids, as well as sensibility limitations, have been reduced with the use of a more selective instrument, as GC-MS/MS is. Matrix-matched standard calibration is still necessary for an adequate quantification although an important decrease on matrix effects have been observed when using GC-MS/MS equipment.

Furthermore, an efficient data processing system has been developed; as a first approach by the use of screening sequences, discriminating all the samples without detected analytes; and secondly, applying a quantitative method to the previously ones detected as positive samples. This working methodology allows faster data processing, concentrating our efforts mainly in those samples which need to be carefully evaluated because of their potential damage on human health.

All these incorporations have become a powerful tool to improve laboratory efficiency, developing a method that allow the multi-residue determination of 38 representative pesticides including organochlorine, organophosphate, and pyrethroid pesticides in animal origin food samples. Thereby, this method can be used in routine analysis laboratories for national and community monitoring programs of different families of pesticides in animal origin food with equipments that allows screening as a first approach, quantitation and confirmation when necessary.

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Chapter 4

Removal of Organic Pollutant from Water by Modified Bentonite

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Additional information is available at the end of the chapter

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1. Introduction

Bromoxynil is a nitrile herbicide, widely used in the region for annual weed control. Its application creates environmental contaminations. For instance, it was detected in air samples [1], water samples [2], and soil samples [3]. It application may create health problems [4]. Furthermore, bromoxynil has been reported as a toxic agent to important green algae in the ecosystem [5], more over, it is recently indicated that bromoxynil was among eight-herbicide mixture, that produced negative effects on microbial communities even at low concentrations [6].

Application of commercial formulations of bromoxynil (emulsion concentrate, and/or suspension concentrate) in the ecosystem may induce species shifts within the communities and could affect the structure and the function of the aquatic communities [7].

Several methods have developed to remove organic pollutants from water. This includes adsorptive techniques [8], photochemical methods [9-11] activated carbon [12], carbon nanotubes [13], gamma-MnO2 [14] and combined nano MgO-nanofiltration technique [15]. Worldwide, very little information about removal of bromoxynil from water are available, beside the fact that no attempt has been made in Gaza to remove or attenuate bromoxynil concentration in the ecosystem.

The objectives of this study are: 1) to design suitable organo-clay complexes and 2) to optimize the aquatic pH to enhance removal of bromoxynil from water.

2. Materials and methods

Materials. Ca^{2+}-bentonite clay (M48) with cation exchange capacity (CEC) equivalent to 960 mmol(+) kg^{-1} (= 0.96 meq g^{-1}) [16] was used. The organic salts used are: N-cetylpyridinium bromide (NCP^+Br^-) and N-hexadecyl tributylphosphonium bromide ($HDTBP^+Br^-$). The

organic pollutant used is Bromoxynil (HPLC grade, 3,5-dibromo-4-hydroxybenzonitrile). Those materials were purchased from Sigma Chemical Co., Germany.

Synthesis of organo-Bentonite complexes. The organo-Bentonite complexes were synthesized by simple ion-exchange reaction. In this procedure, 5 mmol of the solid organic salt was added to 1L of 1% (w/v) aqueous suspension of Bentonite clay (M48) under stirring conditions for 3 days [16].

Bromoxynil stock solution was prepared by dissolving 31 mg in 2-3 mL methanol and diluting to 1 L with deionized water.

2.1. Removal of bromoxynil from water

In this experiment 0.5 mg of bromoxynil was added to various glass centrifuge tubes containing 10 mg of different organo-Bentonite. The tubes were shaken for 48h and then the supernatants were collected by centrifugation at 20000g. The remaining concentration of bromoxynil in the supernatants were determined by HPLC.

2.2. Influence of bromoxynil concentrations on the removal process

Thirty ml of bromoxynil concentrations ranged between 0.66-31 mg/L were transferred to 30-mL glass centrifuge tubes containing 0.005 g organo-Bentonite. The tubes were kept under continuous rotary agitation for 48 hours to maintain aqueous suspension during the experimental time. This experiment was maintained at pH7. Control samples were made by performing the same procedures under the same conditions without adding the organo-Bentonite to each tube. The aqueous solutions were collected by centrifugation at 20,000 g for 0.5 h.

2.3. Influence of pH on removal of bromoxynil

Deionized water was used to perform the removal experiments at pH 7, whereas few drops of acetic acid were added to the stock solution of bromoxynil to adopt the pH to 3.

The removal experiments were performed as described above using HDTBP-Bentonite complex.

2.4. Influence of Temperature on removal of bromoxynil

To measure the effect of temperatures on the removing process of bromoxynil, the experiments described above were performed at 5, 17 and 40 $\pm$ 2 ^{o}C using NCP-Bentonite complex as a removing materials

2.5. Release of bromoxynil from organo-bentonite complexes

To insure removal of bromoxynil from water, the used organo-bentonites in the above mentioned experiments were collected, air dried and used for bromoxynil extraction. In this experiments the air dried organo-bentonites were transferred to test tube containing 5 ml of

water methanol mixture as described in mobile phase (section 2.8). The test tubes were transferred to an ultrasonic machine functioning at high speed for 3 min. the solution was collected and additional 5 ml of water methanol mixture was added again and the same procedure was repeated to insure maximum extraction. Concentration of bromoxynil in the solution was determined as mentioned above.

2.6. Measurements of bromoxynil

The concentrations of bromoxynil in the supernatants were determined by Waters 717 HPLC with UV detector (detection wavelength 283 nm). Column: Nova-Pak C18 (inner diameter 3.9 mm, length 150 mm), flow rate: 1 mL min-1. The mobile phase was methanol/water 50/50 (v/v). The amount of bromoxynil adsorbed was calculated from the depletion of the bromoxynil concentration in the aqueous solutions.

2.7. Data analysis

The removal data were collected as an average of 3 replicate and the standard deviation was calculated and used as error bars to discriminate differences among treatments. Presenting the standard deviation as error bar is the best method to determine significant differences among adsorption isotherms. It is well known that overlapping of error bars indicating no difference whereas small or extremely small error bars indicate significant difference.

3. Results and discussion

The cationic quaternary ammonium/phosphonium salts used in this study are solid materials at room temperature, and surface-active agents (surfactant), The molecular structures include an aliphatic part and/or an aromatic ring. In a diluted solution (< 0.1 mmol/g) the adsorbed amounts of surfactant on bentonite surfaces were nearly similar (Data not shown), whereas as high added concentration (>0.5 mmol/g) the extent of adsorption became a function with the size and shape of the surfactant. Large organic cations can effectively displace inorganic cation such as Ca^{2+} and/or Na^{+} from mineral surfaces of clay by ion exchange [17]. More detailed results are presented in El-Nahhal and Lagaly [16].

Removal of bromoxynil from water by different organo-bentonite complexes are shown in Figure 1. It can be seen that the removed amounts of bromoxynil on raw Bentonite (clay) or modified Bentonite with NCP and/or HDTBP, are: (14.6±3.65); (84.65± 8.12) and (46.48± 27.25) mg/g respectively.

The largest removed amount of bromoxynil was observed on bentonite modified with NCP followed by HDTBP. The explanation of these results is that low removed amount using raw Bentonite is due to the hydrophilic surfaces of Bentonite and hydrophobic nature of bromoxynil. Modification of Bentonite surfaces with NCP or HDTBP may have created a microscopic organic phase on Bentonite surfaces as in hexadecyltrimethyl ammonium-smectite [18]. This situation may acts as a solubilizing (partitioning) medium for removing nonionic organic compounds from water

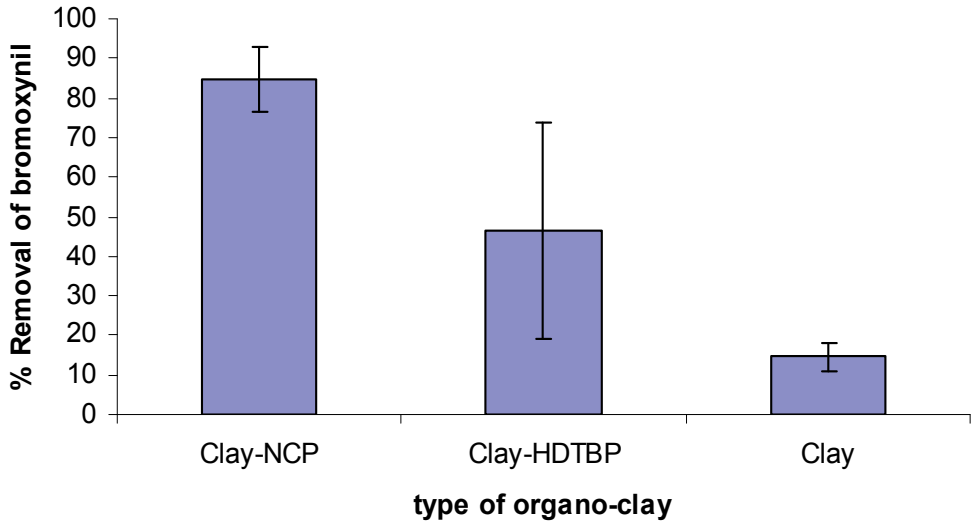

Figure 1. Removal of bromoxynil from water by different organo-bentonites. Error Bars represent standard deviation.

Effects of various concentrations of bromoxynil on the removal process are shown in Figure 2. It is obvious that at low concentration of bromoxynil, the removed amount by using organo-bentonite complexes exceed 90% of bromoxynil from water, whereas at high bromoxynil concentrations (25 mg/l), the removed amount of bromoxynil reduced to 48.02% and 76.57% using Clay-HDTBP or Clay-NCP respectively. Using hydrophilic clay the removed amount of bromoxynil did not exceed 8% and was not affected by the concentration of bromoxynil.

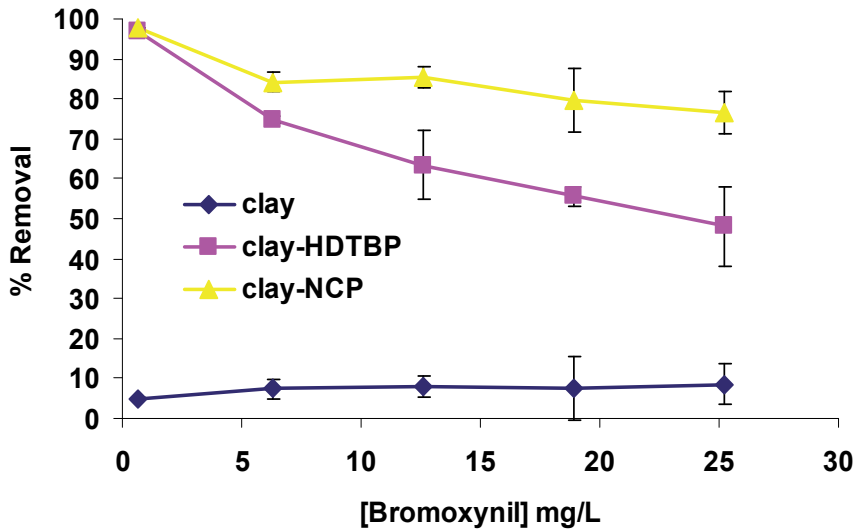

Figure 2. Influence of Bromoxynil concentration in the removal process. Error bars represent standard deviation

The explanation of these results is that at low concentration of bromoxynil the molar concentration of the adsorption sites equal to 0.0096 meq/l. Under these condition, the molar ratio between bromoxynil molecules and the adsorption sites is nearly similar. At a high concentration of bromoxynil the molar ratio between bromoxynil molecules and the adsorption sites tend to increase. Accordingly, the number of free adsorption sites tends to decease, under this condition the removed amount of bromoxynil tends to decease. Furthermore, one can realize from the data in Figure 2 that Clay-NCP is more potent than Clay-HDTBP for removal of bromoxynil from water. The explanation of these results is that Clay-NCP contains an aromatic ring in its chemical structure which may enhance the interaction between bromoxynil molecules and Clay-NCP molecules. El-Nahhal et al., [19] demonstrated the efficacy of the chemical structure in enhancing the adsorption of organic molecules from water. Under this condition, hydrophobic interaction between the adsorbed molecules of bromoxynil and those in water may be enhanced. Lagaly (1994) showed similar phenomena for HDTMA.

In addition, using different fractions of organo-bentonite for removing bromoxynil showed optimal removal at low fraction of organo-bentonite complexes (Data not shown). The explanation of these results is that at low fraction of organo-bentonite complex the adsorption sites are diluted in the solution and available for interaction with bromoxynil from water whereas at high concentration of organo-bentonite complexes the percent removal tends to decrease due to possible aggregation of organo-bentonite complexes in the water clay suspension. Accordingly, the availability of free adsorption sites is limited for removal of bromoxynil. Consequently, the concentration of the adsorption site in water should be optimized. It was shown that the best results were obtained at a very diluted organo-Bentonite complexes (Data not shown).

Influence of pH values in the removed amount of bromoxynil from water is shown in Figure 3.

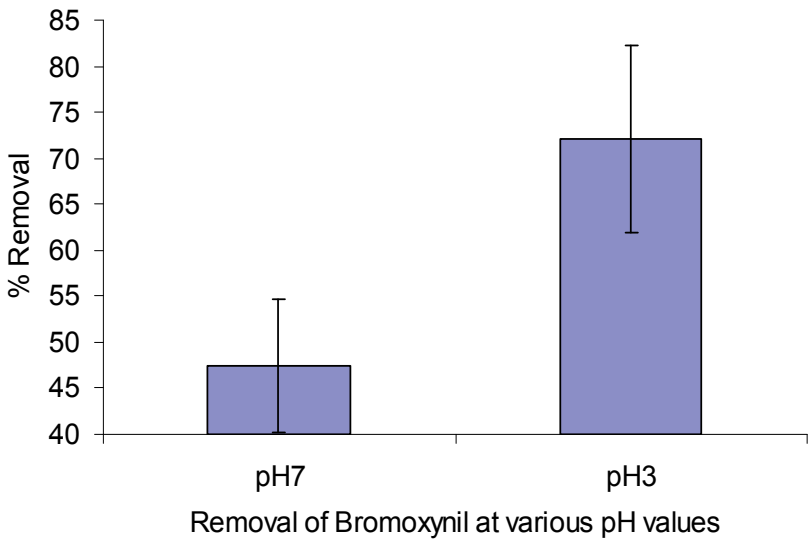

Figure 3. Influence of pH values in the removal of bromoxynil from water by Bentonite-HDTBP. Error bars represent standard deviation

It is obvious that the removed amount of bromoxynil at pH 3 is several times higher than the removed amount at pH 7. The explanation of these results is that bromoxynil has an acid dissociation constant pKa value of 4.06 [20], accordingly the degree of ionization in the solution is depends on both the pKa value and the pH of the solution in which it is dissolved, a relation described by the Henderson-Hasselbalch equation for weak acids:

$$pKa-pH=\log [nonionized]/[ionized] \quad (1)$$

Employing Eq 1 at pH 3, one can realize that the nonionized fraction equals to 11.48 times higher than the ionized fraction whereas at pH 7 the nonionized fraction equals to 0.001 of the ionized faction. As obvious at pH 3 bromoxynil molecules remain in the nonionized from (Hydrophobic form) and adsorbed directly to the organo-bentonite (Figure 3) whereas at pH 7 bromoxynil molecules tend to be ionized (Hydrophilic form) in this case it remains in the solution due to formation on anionic form (Figure 4). This explanation is in accord with the higher removed amount of bromoxynil at pH 3 (Figure3).

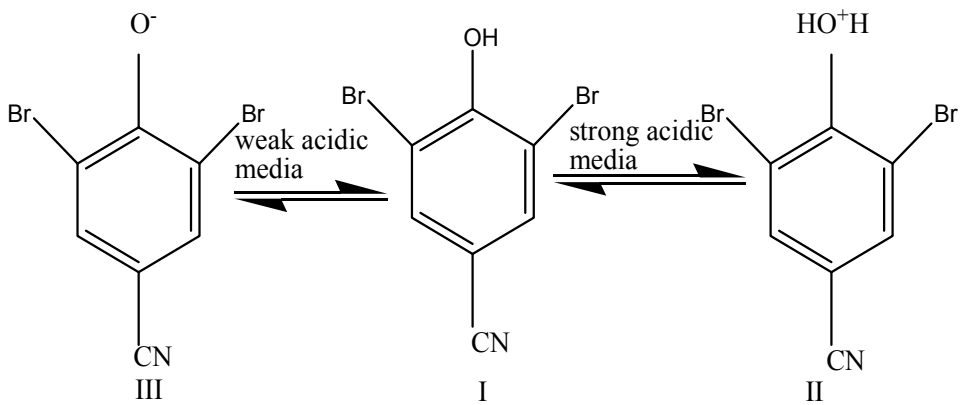

Figure 4. Proposed ionic forms of bromoxynil according to pH values.

The presented results agree with previous reports [21-22] who found high adsorbed amount of bromoxynil in soil and wheat char at low pH.

Removal of bromoxynil at various temperatures are shown in Figure 5. It can be seen that removed amount of bromoxynil is more pronounced at 17 °C than at 40 or 5 °C (Figure 5).

The explanation of these results is that at a low temperature (e.g. 5 °C) the chemical potential of bromoxynil molecules is reduced and the molecules tend to from crystals due to low solubility in water (0.13 g/l, 20 °C). This agrees with the general concept of solubility in chemistry. In contrast, at high temperature the system absorbs heat energy and an increase in the chemical potential may have occurred. This step makes more bromoxynil molecules

available for removal from water due to dynamic mobility in the aqueous solution. The optimal removed amount appears to be at 17 °C.

Extraction experiments using ultrasonic machine showed that nearly the same amount of bromoxynil that removed from water (Figure 1) was extracted from the matrixes used in this study (Data not shown). These results indicate the efficacy of the used matrixes in removing of bromoxynil. These results are in agreement with recent report [23] who showed that bromoxynil was strongly encapsulated in organo-clay complex and used for slow-release formulations under field conditions.

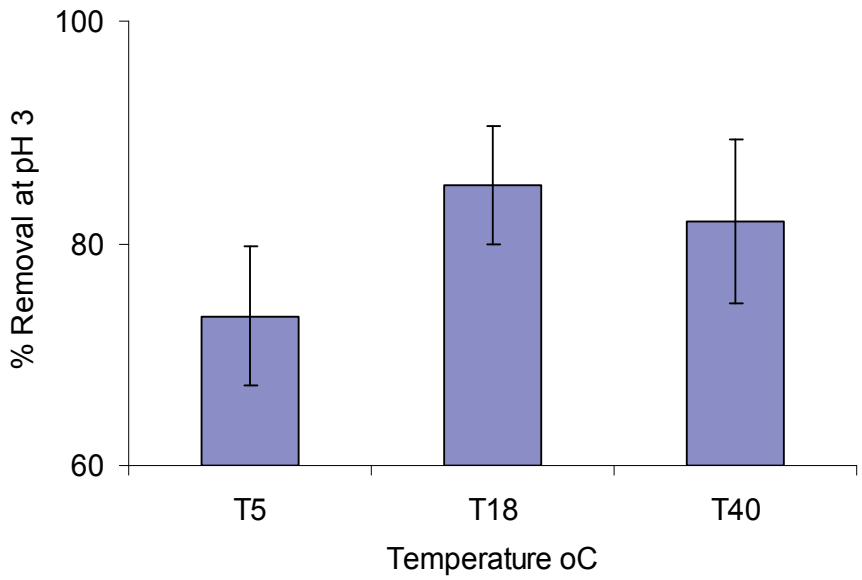

Figure 5. Removal of bromoxynil at different temperatures. Error bars represent standard deviation

4. Concluding remarks

The study reveals that exchanging Bentonite surfaces with organic cations increases their capacity to remove bromoxynil (organic pollutant) from water.. The rationale of this work is that removal of bromoxynil from water can be enhanced by modifying the Bentonite surfaces with NCP and HDTBP. The results showed that highest removed amount of bromoxynil was obtained at pH 3 and at 17 °C (Figures 3, 5). Extraction of bromoxynil from the used matrixes was nearly similar to the amount removed in Figure 1. The environmental relevance of this work emerges from the fact that organo-bentonite complexes can be used to remove organic pollutants from water and develop environmentally acceptable herbicide encapsulation for safe application [24].

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Chapter 5

Removal of Residual Pesticides in Vegetables Using Ozone Microbubbles

Masahiko Tamaki and Hiromi Ikeura

Additional information is available at the end of the chapter

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1. Introduction

The present agriculture has enabled mass and stable production by using of agricultural pesticides. However, agricultural pesticides can have an adverse effect on the environment in addition to being harmful to humans, animals and fishes. The health hazard to the farmer as well as the residue in crops is also a global problem. Recently, the safety of crops including contamination with agricultural pesticides is a major concern to both the producer and consumer, and the development of a method to remove the pesticides before marketing has been eagerly awaited. In Japan, about 600 agricultural pesticides are included in the Positive List established in 2006. Since agricultural crops cannot be marketed when they contain pesticides exceeding the residual limit, the development of a measure for eliminating residual pesticides in crops is now an important issue (Yamaguchi, 2006).

Ozone (O_3) is the natural substance in the atmosphere and one of the most potent sanitizers against a wide spectrum of microorganisms (Khadre et al., 2001). O_3 is generated by the passage of air or oxygen gas through a high voltage electrical discharge or by ultraviolet light irradiation (Mahapatra et al., 2005), then has a strong oxidative power, and is used for sterilization, virus inactivation, deodorization, bleaching (decoloration), decomposition of organic matter, mycotoxin degradation and others (Cataldo, 2008; Karaca and Velioglu, 2009; Karaca et al., 2010; Takahashi et al., 2007a). In addition, O_3 is changed to oxygen by autolysis and does not harm the flavor of vegetables and fruits (Li and Tsuge, 2006). Therefore, O_3 is considered to be most suitable for removing residual pesticides from vegetables and fruits and controlling microbes of food safety concern (Selma et al., 2008; Gabler et al., 2010).The threshold concentration of O_3 for continuous human exposure is 0.075 μL/L (US Environmental Protection Agency, 2008). Although there are many studies on the removal of pesticides using O_3 for water purifications in waste water, there are

several reports on the use of O_3 to remove residual pesticide in vegetables and fruits (Daidai et al., 2007; Hwang et al., 2001a; Hwang et al., 2001b; Hwang et al., 2002; Karaca and Velioglu, 2007; Ong et al., 1995; Wu et al., 2007a; Gabler et al., 2010).

Microbubbles (MB) are less than 50 μm in diameter and have special properties such as generation of free radicals, self-pressurization and negative charge, and their use in the field of food science and agriculture is attracting attention (Sumikura et al., 2007; Takahashi et al., 2007b). Millibubbles generated by using an air pump are 2-3 mm in diameter, rapidly rise in water and burst at the water surface. Therefore, the solubility of the gas in water is very low. On the other hand, MB rise in water slowly and the interior gas is completely dissolved in water (Takahashi et al., 2007a).

Up to now, growth promotion of lettuce in hydroponic cultures with air MB (Park and Kurata, 2009) and inactivation of *Escherichia coli* using CO_2 MB (Kobayashi et al., 2009) have been reported. In addition, reports on disinfecting wastewater using ozone (O_3) MB have discussed their strong disinfectant activities and relative long-term durability in water (Sumikura et al., 2007; Chu et al., 2007; Chu et al., 2008a; Chu et al., 2008b).

There are two types of O_3 MB (OMB) generators, a decompression type and a gas-water circulation type. In the former, a sufficient amount of gas is dissolved in water under a 3-4 atmospheric pressure to cause a supersaturated condition (Figure 1-A). Under such a condition, supersaturated gas is unstable and escapes from the water generating a large amount of air bubbles, which are MB. In the latter, gas is introduced into the water vortex, and the formed gas bubbles are broken into MB by breaking the vortex (Figure 1-B) (Takahashi, 2009).

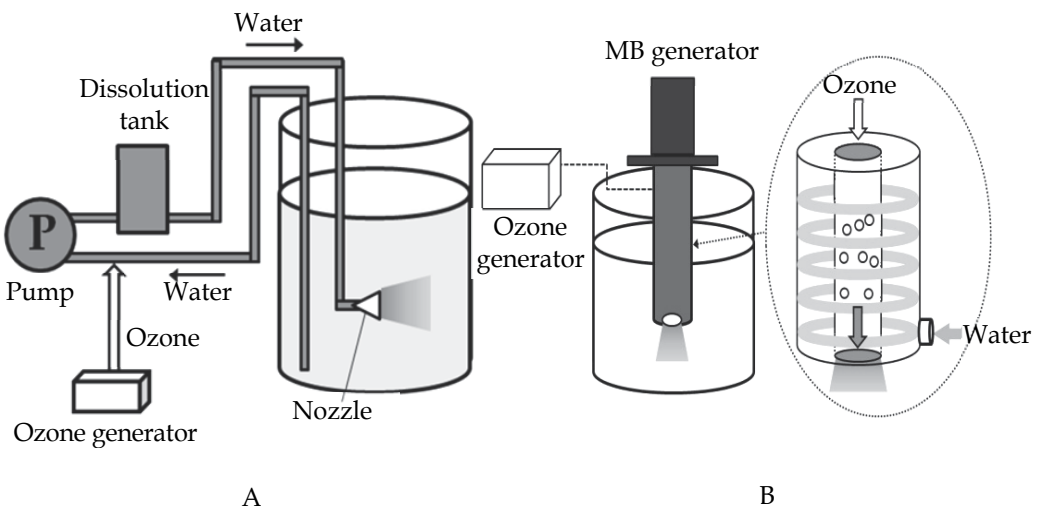

Figure 1. Schematic diagrams used for MB generation. (A) The decompression-type MB generator, (B) the gas-water circulating-type MB generator.

By the way, although there have been several studies on the use of O_3 millibubble for removing residual pesticides from vegetables and fruits, few studies have reported on the use of OMB to remove them. Therefore, since microbubbled gas is highly soluble in water and O_3 is a powerful decomposer of organic matter, OMB were expected to remove residual pesticides efficiently from vegetables and fruits.

No comparative studies exist on the effects of OMB generated by different methods on the removal of residual pesticides in vegetables. In this study, we examined 1) the effects of OMB generated by different methods and 2) the effects of OMB dissolved by different concentrations on the removal of pesticide (fenitrothion, FT) infiltrated into vegetables with different shapes. Since FT has been utilized widely as pesticide and acaricide, or mixture with organophosphorus agent, carbamate, pyrethroid and antibiotic in Japan, we used it in this study.

2. Materials and methods

2.1. Materials

Lettuce, cherry tomatoes and strawberries used in this study were purchased from a supermarket in Kawasaki city. A common organic phosphorous pesticide, fenitrothion, (Sumithion emulsion, 50% of MEP) and d_6-fenitrothion were obtained from Sumitomo Chemical Co. Ltd. (Osaka, Japan) and Kanto Chemical Co. Ltd. (Tokyo, Japan), respectively. The structure of FT ($C_9H_{12}NO_5PS$, MW 277.25) is shown in Figure 2. FT agent contains 50 % of *O,O*-Dimethyl-O-(3-methyl-4-nitrophenyl) thiophosphate, 50% of organic solvent and surfactant agent, and generally it is used by 1000-fold dilution with tap water.

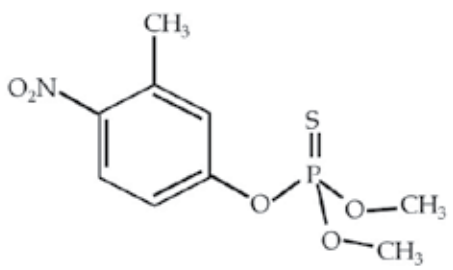

Figure 2. Chemical structure of fenitrothion

2.2. Treatment with agricultural pesticide

FT was 1000-fold diluted in tap water, and three drops of a spreading agent (Haiten power, Hokko Sangyo, Co., Ltd. Tokyo, Japan) were added. The concentration of FT solution was 500 ppm. Lettuce leaves and fruits of cherry tomatoes and strawberries were immersed in this solution (60 L) for 1 min and left in a cool dark room for 24 hr to infiltrate FT into vegetables. Thereafter, they were washed in tap water for 1 min and treated with O_3 as follows.

2.3. O_3 treatment

2.3.1. Experiment 1

Forty liters of tap water was pooled in a cylindrical vessel (55 cm×32 cm i.d.) and kept at 20 °C in a room to remove chlorine in tap water for 24 hr. We confirmed that all the chlorine had been removed 24 hr later by a chlorine comparator (Photometer CL, OYWT-31, OYALOX Co., Ltd., Tokyo, Japan). OMB was generated in dechlorinated water by using a MB generator of a gas-water circulation type (FS101-L1, Fuki Co. Ltd., Saitama, Japan) or a decompression type (20NEDO4S, Shigen-Kaihatsu Co. Ltd., Kanagawa, Japan) combined with an O_3 generator (ED-OG-A10, Ecodesign Co. Ltd., Saitama, Japan) at a flow rate of 2.5 L/min. Under this condition, no more than 2.0 ppm of O_3 could be dissolved. Therefore, O_3 generation was stopped when the concentration of dissolved O_3 reached 2.0 ppm and three kinds of vegetables were immersed in the solutions for 0, 5, or 10 min. Solution temperature was kept at 20 °C in all treatments. In addition, the concentration of dissolved O_3 was measured with a dissolved O_3 meter (OZ-21P, DKK-TOA Co. Ltd., Tokyo, Japan), by shaking the electrode with 10 cm/s in solutions. Analyses were run in triplicate.

2.3.2. Experiment 2

O_3 millibubbles (OMLB) were generated in the dechlorinated water with an ED-OG-A10 O_3 generator at a flow rate of 2.5 L/min. The maximum amount of O_3 that could be dissolved under these conditions was 0.2 ppm, so O_3 generation was stopped when the concentration of dissolved O_3 reached 0.2 ppm. Then the vegetables were immersed in this solution for 0, 5, or 10 min.

OMB were generated in the dechlorinated water using a gas-water circulation type MB generator together with the ED-OG-A10 O_3 generator described above. Ozonated water solutions were produced containing 0.5, 1.0, or 2.0 ppm dissolved O_3. Then, the vegetables were immersed in these solutions for 0, 5, or 10 min. Treatment with OMB solution containing 0.2 ppm dissolved O_3 was not fully tested because a preliminary experiment showed that its pesticide-removing activity was not significantly different from that of the OMLB solution.

A further treatment was set up where MB were continuously generated during vegetable immersion in the ozonated solutions (bubbling OMB). In these treatments O_3 microbubbling was continued to maintain the concentration of dissolved O_3 at 2.0 ppm for 0, 5, or 10 min vegetable immersion.

A control treatment was also conducted where the vegetables were immersed in dechlorinated water. The solution temperature was maintained at 20 °C during all treatments and the concentrations of dissolved O_3 were measured using an OZ-21P O_3 analyzer with a DO_3 electrode. All analyses were performed in triplicate.

2.4. Residual pesticides analysis

Lettuce leaves or fruits of cherry tomato or strawberry (20 g) and 100 μL of 20 ppm d_6-FT as internal standard were added with liquid nitrogen, homogenized for 3 min by a blender

(18000 rpm, Nissei Co. Ltd., Aichi, Japan), and then extracted by shaking in 100 mL of acetone for 30 min. After the extraction was filtered with a glass filter under reduced pressure and was evaporated until about 5 ml by a rotary evaporator, the extraction was added 5 mL of distilled water, and then was poured into a diatomite column (CHEM ELUT-20mL, UNBUFFERED, 100/PK, VARIAN Technologies Japan, Co. Ltd., Tokyo, Japan). The column was washed with 10 ml of hexane at twice, the pesticide followed by 120 ml of hexane to elute. The elution was evaporated to dryness by a rotary evaporator and refused in 2 ml of acetone, and this solution (10 μL) was injected into Gas Chromatograph-Mass Spectrometry.

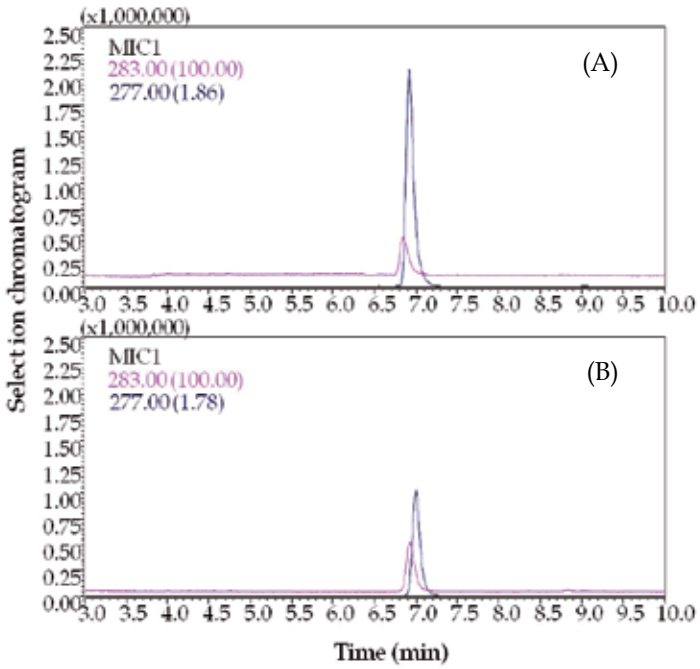

Figure 3. GC-chromatogram of fenitrothion and d_6- fenitrothion before and after OMB treatments. (A) before O_3 treatment. (B) after O_3 treatment.

A Shimadzu GC-MS QP2010 (Shimadzu Co., Ltd., Kyoto, Japan) was used for the analysis, with ionization achieved by electron impact at 70eV. The capillary column used was an Inertcap 1MS capillary column (30 m × 0.25 mm i.d.; J&W Scientific, Folsom, CA). The operating conditions were: injection port temperature, 250°C; interface temperature, 280°C; column oven temperature, 200 °C for 5 min, ramped at 1 °C /min to 215°C, followed by 20°C/min to 280°C; helium carrier gas (flow rate of 30 cm/s); 10 μL injection volume. The split/splitless injector was operated in the splitless mode for 0.5 min after injection of the sample. The selected ion of the labeled standard (d_6-FT) was analyzed in the single ion monitoring (SIM) mode and the intensity calculated by a Shimadzu GC-MS solution. SIM of d_6-FT used the ion: for d_6- FT, m/z=283; for FT, m/z=277 were quantification. Analyses were run in triplicate. Chromatogram and mass spectra of FT and d_6-FT were shown in Figure 3 and 4, respectively.

2.5. Statistical analysis

Mean separation of O_3 concentration, residual percentage of FT and concentration of FT in vegetables between treatments were determined by Turkey-Kramer test at $P < 0.05$ and standard division of the mean (SD).

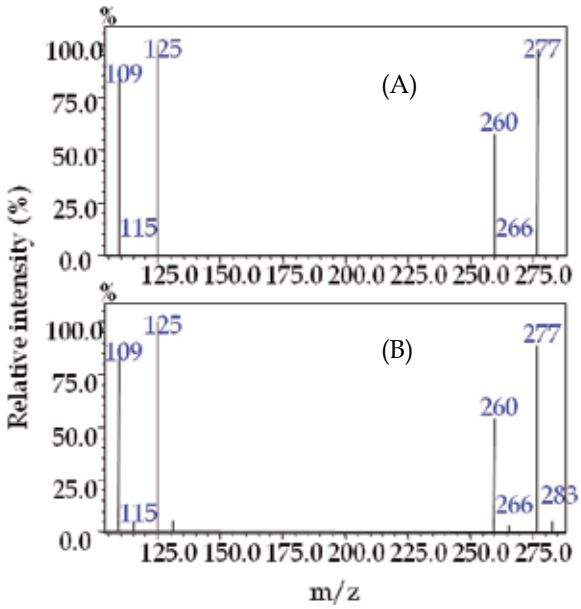

Figure 4. Mass spectrum of fenitrothion and d_6- fenitrothion (A) fenitrothion, (B)d_6-fenitrothion

3. Results and discussion

3.1. Removal of residual pesticide, fenitrothion, in vegetables by using OMB generated by different methods (Experiment 1)

Figure 5 shows the change in the concentration of dissolved O_3 in solutions after the start of OMB treatments with the gas-water circulation type and the decompression type. In both solutions, measured in the absence of vegetables, the concentration of dissolved O_3 decreased gradually with time, and the concentration at 5 and 10 min after the start of OMB treatments was 1.3 and 1.0 ppm in the gas-water circulation type solution, and 1.6 and 1.4 ppm in the decompression type solution, respectively. Thus, the concentration of dissolved O_3 was kept higher in the decompression type solution than in the gas-water circulation type solution. The half-life of dissolved O_3 by using an air pump is reported to be 2.27 min in tap water at 25°C (Dhillon et al., 2009). That by using the gas-water circulation type was about 10 min and that by using the decompression type was much longer, though the solution temperature was 20°C in this study.

Figure 6 shows the residual percentage of FT in lettuce (A), cherry tomatoes (B) and strawberries (C) at 5 and 10 min after the immersion into the solutions of OMB treatments

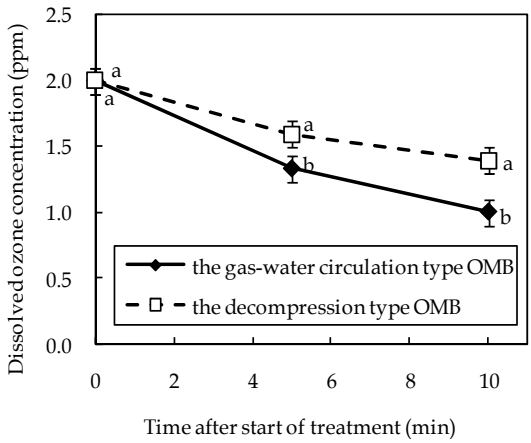

Figure 5. Change in the DO_3 concentrations after OMB treatments by using the gas-water circulating-type and the decompression-type.
Vertical bars represent the standard division of the mean (n=3).
Different letters indicate a difference significant at the 5% level by Turkey-Kramer test between treatments.

by using the gas-water circulation type and the decompression type. In lettuce, the concentration of residual FT after washing in water was 212.21 ppm (Table 1), and the concentration rapidly decreased after the start of both the treatments of the decompression type and the gas-water circulation type, reaching 44 and 55% at 5 min, and 33 and 45% at 10 min, respectively. Thus, in lettuce, both the treatments of the decompression type and the gas-water circulation type removed residual FT effectively, and the decompression type was more effective than the gas-water circulation type. The dissolved O_3 in the solutions of OMB treatments generates hydroxyl radicals that are highly effective at decomposing organic molecules like the residual FT (Sumikura et al, 2007; Takahashi et al., 2007b), and hydroxyl radicals are generated by the collapse of OMB in solutions (Chu et al., 2008a). The decompression type would have generated a high enough concentration of dissolved O_3 to produce a large amount of hydroxyl radicals. However, the gas-water circulation type was lower effective than the decomposing type, because the concentration of dissolved O_3 was lower and fewer hydroxyl radicals would have been generated.

In cherry tomatoes, the concentration of FT after washing with water was 3.02 ppm (Table 1), and the residual FT percentage at 5 min after the start of OMB treatments of the decompression type and the gas-water circulation type was 89 and 97%, respectively, showing a low pesticide-removing effect. At 10 min after the start of OMB treatments, it was 84 and 95%, respectively. Thus, the decompression type was slightly more effective than the gas-water circulation type. The most likely explanation for the lower reduction of residual FT in cherry tomatoes is that the dissolved O_3 and hydroxyl radicals could not penetrate through the thick pericarp of the cherry tomatoes and not reach the sarcocarp, and were inactivated by contact with the pericarp.

In strawberries, the concentration of FT after washing with water was 37.80 ppm (Table 1), and the residual percentage of FT at 5min after the start of OMB treatments of the

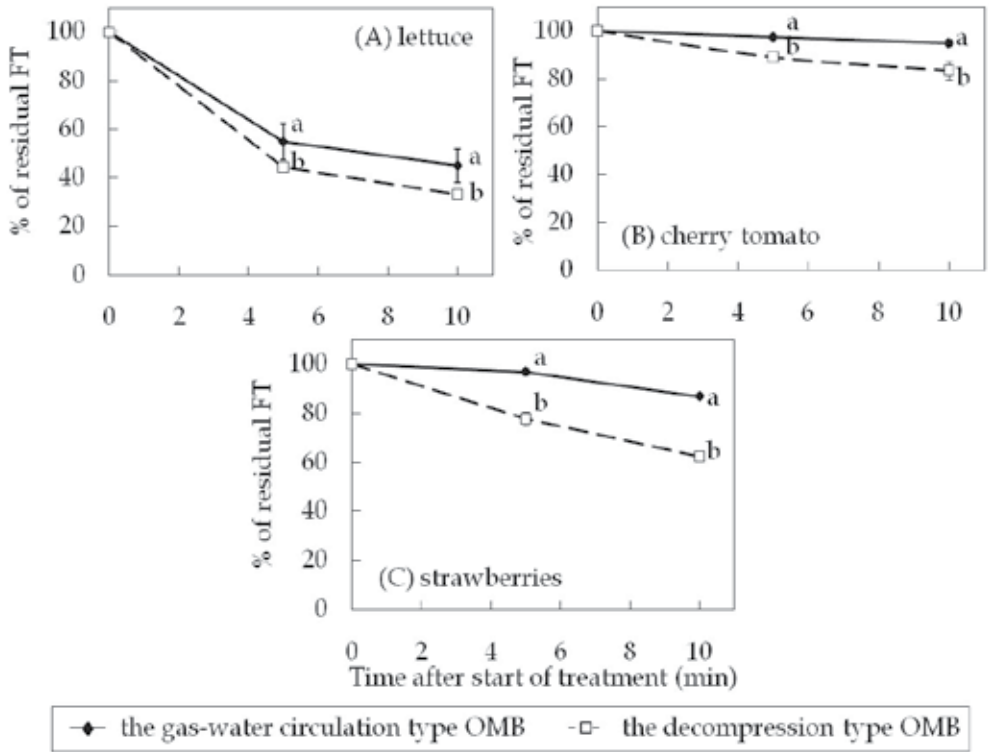

Figure 6. Residual fenitrothion percentages for lettuce (A), cherry tomatoes (B), and strawberries (C) after immersion in solutions containing OMB generated by using the gas-water circulating-type and the decompression-type.
Vertical bars represent the standard division of the mean (n=3).
Different letters indicate a difference significant at the 5% level by Turkey-Kramer test between treatments.

decompression type and the gas-water circulation type was 78 and 97%, respectively. That at 10 min after the start of OMB treatments of the decompression type and the gas-water circulation type was 62 and 87%, respectively, showing that the pesticide could be removed effectively by using the decompression type. The amount of FT removed in strawberries was higher than that in cherry tomatoes at both types of OMB generators. We think that strawberries have a rougher surface and larger surface area than cherry tomatoes and then can contact with O_3 efficiently, removing FT easily in the sarcocarp.

The decompression type had a high FT-removing effect on all vegetables examined even though the initial concentration of dissolved O_3 was 2.0 ppm. The difference in the pesticide-removing effect between the decompression type and the gas-water circulation type may be caused by the difference in the size and the number of the bubbles (Takahashi, 2009). The diameter of the MB generated using the decompression type shows about 10 μm, and the number of bubbles smaller than 50 μm in diameter amounted to several thousand per ml (Takahashi et al., 2007a). On the other hand, the diameter of the MB generated using the gas-water circulation type shows about 40 μm, and the number of MB smaller than 50 μm in

		Concentration (ppm)						Coefficient of variation (%)	
	Time	The gas-water circulation type OMB	±	SD	The decompression type OMB	±	SD	The gas-water circulation type OMB	The decompress-sion type OMB
lettuce	before[z]	346.64	±	5.75	346.64	±	5.75	1.66	1.66
	0	212.21	±	31.24	212.21	±	31.24	14.72	14.72
	5	117.77	±	4.30	95.11	±	1.96	3.65	2.06
	10	96.50	±	0.79	92.75	±	3.93	0.82	4.24
tomatoes	before	3.41	±	0.14	3.41	±	0.14	4.05	4.05
	0	3.02	±	0.17	3.02	±	0.17	5.70	5.70
	5	2.75	±	0.04	2.70	±	0.11	1.50	4.08
	10	2.75	±	0.12	2.53	±	0.23	4.25	9.00
Straw-berries	before	43.50	±	2.12	43.50	±	2.12	4.88	4.88
	0	37.80	±	4.58	37.80	±	4.58	12.11	12.11
	5	34.80	±	4.12	29.35	±	2.18	11.84	7.44
	10	32.25	±	3.07	23.59	±	0.15	9.53	0.62

[z] before washing in tap water.

Table 1. Concentration of residual FT for lettuce, cherry tomatoes, and strawberries after immersion in solutions containing OMB generated by using the gas-water circulating-type and the decompression-type.

diameter amounted to several hundred per ml, which is less than that of the decompression type (Takahashi et al., 2003). These findings show that the decompression type had a strong pesticide-removing effect, which could be attributed to the larger number of small OMB that could more easily infiltrate into the vegetables than the gas-water circulation type. There have been no reports on the effects of OMB generated by different methods on the removal of residual pesticides in vegetables. This is the first report showing the pesticide-removing effect of OMB with the different methods of generation. In this study, we tested that whether vegetable containing in high concentration of pesticide was removed or not, and so we confirmed that vegetables were removed efficiently by treatment with the OMB. In near future, we should be attempted to confirm safety of vegetable treated by OMB.

3.2. Removal of residual pesticides in vegetables using OMB dissolved by different concentration (Experiment 2)

Experiment 1 was conducted, in the absence of any vegetables, to determine how dissolved O_3 concentrations changed in the ozonated water solutions over time at 20°C. Figure 7

shows how the concentration of dissolved O_3 changed over a 10 minute period (the maximum length of subsequent vegetable treatments), once the ozonated water solutions had been prepared. Over the 10 min period the concentration of dissolved O_3 in the MB solution with a starting concentration of 2.0 ppm (2.0 ppm OMB solution) decreased steadily to 1.0 ppm Similarly, the concentrations of dissolved O_3 in the 0.5 and 1.0 ppm OMB solutions, and the 0.2 ppm OMLB solution also decreased steadily, with all dissolved O_3 lost from the 0.5 ppm OMB and 0.2 ppm OMLB solutions within 10 minutes.

Figure 8 shows the reduction in residual FT in lettuce treated with the OMB and OMLB solutions. Before treatment with OMLB or OMB solutions, but after washing with tap water, the concentration of residual FT in lettuce was 212.2 ppm (data not shown). The amount of residual FT decreased with increasing treatment time and dissolved O_3 concentration. The residual FT in lettuce was reduced to 67%, 55% and 45% after 5 minutes treatment with the 1.0 ppm OMB, 2.0 ppm OMB, and 2.0 ppm bubbling OMB solutions, respectively. After 10 minutes treatment the respective amounts of residual FT had been further reduced to 49%, 45% and 42%. The similarly high reductions in residual FT achieved with the 1.0 ppm OMB, 2.0 ppm OMB, and 2.0 ppm bubbling OMB, indicates that immersion of lettuce in an OMB solution containing 1.0 ppm or more dissolved O_3 may be sufficient to effectively remove residual FT from the lettuce, possibly because lettuce has thin leaves. In contrast, after 10 minutes treatment with the OMLB and 0.5 ppm OMB solutions the residual FT had only been reduced to 87% and 78%, respectively.

The dissolved O_3 in the OMB treatment solutions generates hydroxyl radicals that are highly effective at decomposing organic molecules like the residual FT (Sumikura et al., 2007; Takahashi et al., 2007b). Hydroxyl radicals are generated by the collapse of OMB in solution, and so the 1.0 ppm OMB, 2.0 ppm OMB and bubbling OMB solutions would have had a high enough concentration of dissolved O_3 to produce a large amount of hydroxyl radicals. However, the OMLB treatment was not nearly so effective because the concentration of dissolved O_3 was much lower and so far fewer hydroxyl radicals would have been generated.

Figure 9 shows the reduction of residual FT in cherry tomatoes for each treatment. The starting concentration of residual FT in the cherry tomatoes was 3.0 ppm (data not shown), prior to O_3 solution treatments. Removal of residual FT by the various treatment solutions was much less in the cherry tomatoes than in the lettuce. After 10 minutes treatment residual FT had been reduced to 65% in 2.0 ppm bubbling OMB solution, but remained at >90% for all other treatments. The most likely explanation for the lower reduction of residual FT in the cherry tomatoes is that the dissolved O_3 and hydroxyl radicals could not penetrate through the thick pericarp of the tomatoes and to reach the sarcocarp, and were inactivated by contact with the pericarp. The greater effectiveness of the bubbling OMB solution was probably because the concentration of dissolved O_3 remained high and so hydroxyl radicals continued to be generated throughout the treatment.

Figure 10 shows the reductions in residual FT in strawberries for each treatment. The starting concentration of residual FT in strawberries was 37.8 ppm (data not shown). After 10 min of

treatment, the greatest reduction in residual FT was in the 2.0 ppm bubbling OMB treatment where 75% residual FT remained. The other treatments ranged from 85% residual FT remaining in the 2.0 OMB treatment to 91% in the OMLB treatment. The amount of FT that

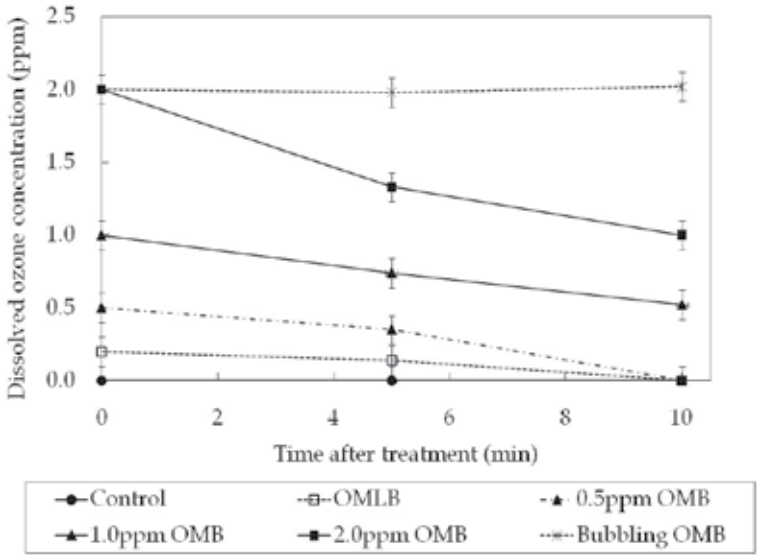

Figure 7. Change in the concentration of dissolved O_3 after the start of the O_3 treatments, in the absence of vegetables.
Vertical bars represent one standard deviation of the mean.

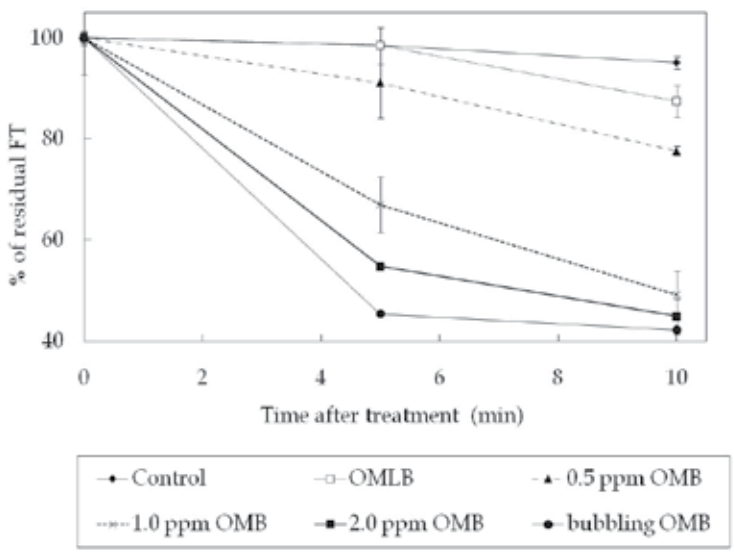

Figure 8. Change in the residual FT in lettuce treated with the OMB and OMLB solutions.
Vertical bars represent the standard deviation of the mean.

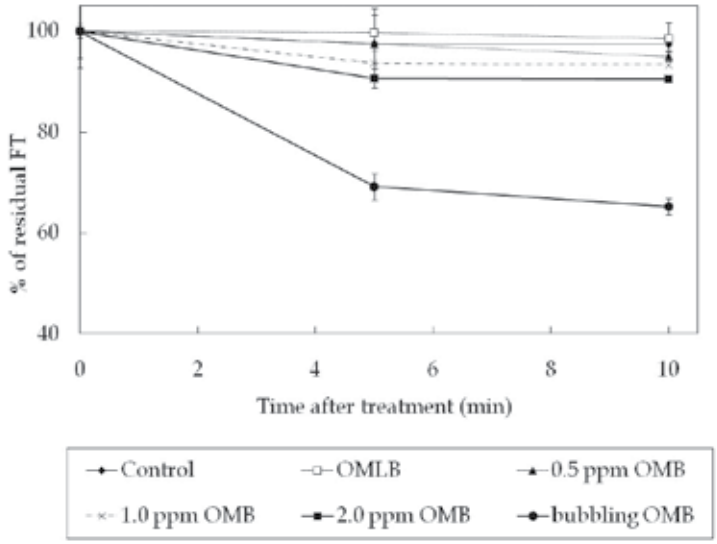

Figure 9. Change in the residual FT in cherry tomatoes treated with the OMB and OMLB solutions. Vertical bars represent the standard deviation of the mean.

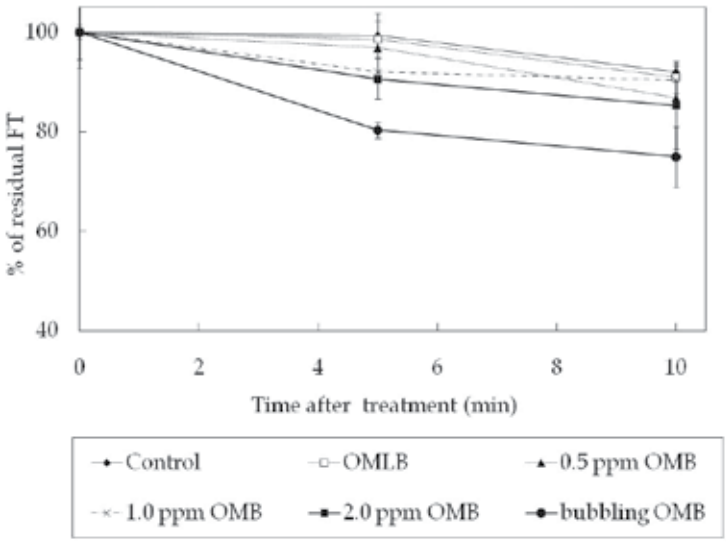

Figure 10. Change in the residual FT in strawberries treated with the OMB and OMLB solutions. Vertical bars represent the standard deviation of the mean.

was removed with the 2.0 ppm bubbling OMB solution was lower than that in the cherry tomatoes. Strawberries have a rougher surface and larger surface area than cherry tomatoes and this may cause O_3 and hydroxyl radicals to lose their specific activity upon contact with the surface of strawberries, preventing them from removing FT in the sarcocarp.

There have been several studies on the decomposition of pesticides by O_3 treatment (Daidai et al., 2007; Hwang et al., 2001a; Hwang et al., 2001b; Hwang et al., 2002; Karaca and Velioglu, 2007; Ong et al, 1995; Wu et al., 2007a). For example, it was reported that 140 ppm FT was completely decomposed within 40 min in 13% ozonated solution produced by millibubbling (Tanaka et al., 1992). Another study reported that 53% of diazinon, 55% of parathion, 47% of methyl parathion, and 61% of cypermethrin were removed from the brassicaceous vegetable "Pakchoi" (*Brassica campestris* L. ssp. *chinensis* Makino) treated with 0.1 ppm of these pesticides and then immersed in ozonated solution containing 2.0 ppm dissolved O_3 for 30 min (Wu et al., 2007b). A further study demonstrated that 2.0 ppm residual captan, azinphos-methyl, and formetanate HCl on the surface of apples after harvest were reduced effectively by immersing them in 0.25 ppm O_3-millibubbled solution for 30 min (Ong et al., 2007a). Although these studies show that residual pesticides can be removed from vegetables and fruits by immersion in ozonated solution, prior to our study there had not been any reports on using these techniques to remove residual pesticides from fruity vegetables such as tomatoes and strawberries. Interestingly, one report showed that 11 kinds of pesticides (alachlor, atrazine, bentazon, butylate, carbofuran, cyanazine, 2,4-dichlorophenoxyacetic acid, malathion, metolachlor, metribuzin, and trifluralin) could be removed by O_3 generation (454 g O_3/day) and UV irradiation, but several hours of treatment were necessary (Philip et al., 1987).

Clearly, residual pesticides in leafy vegetables can be removed by immersion in ozonated solution, but the concentrations of residual pesticide in the earlier studies were low. In the present study, high concentration of residual FT founded in lettuce (>200 ppm) could be reduced to less than 100 ppm in 5–10 min by treatment with 1.0–2.0 ppm OMB solution. Such a large reduction may be possible because the chemical structure of FT is similar to diazinon, which can be easily decomposed by hydroxyl radicals (Kouloumbos et al., 2003), and so the oxidative powers of O_3 and hydroxyl radicals may act in concert to effectively degrade FT. This effective joint action was only possible in the MB generated solutions because the millibubble generated solutions could not achieve high enough dissolved O_3 concentrations and not generate hydroxyl radicals.

This study showed that OMB can remove high concentrations of residual pesticides within a short time from not only leafy vegetables but also fruity vegetables. Thus, OMB could be useful for removing residual pesticides from a wide range of vegetables. In near future, we should be attempted to confirm the quality and safety of vegetable and fruits treated by OMB.

4. Conclusion

The effectiveness of OMB for removal of residual pesticides varies with the methods of the OMB generation. The decompression type was more effective than the gas-water circulation type on removing the residual pesticide in vegetables, which could be attributed to the larger number of small OMB that could more easily infiltrate into vegetables than the gas-water circulation type.

OMB quickly and effectively removed high concentrations of residual FT from lettuce. In addition, continuously bubbled OMB effectively removed residual FT from fruity vegetables with a thick pericarp and sarcocarp, such as cherry tomatoes and strawberries. Unlike millibubbles, MB allow O_3, which is highly insoluble in water, to be easily dissolved in water at high concentrations. As a result, OMB solutions are more effective than OMLB solutions at removing residual pesticides from vegetables because the OMB solutions combine the oxidative power of O_3 with the generation of hydroxyl radicals from the collapsing OMB.

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Chapter 6

New Trends in Pesticide Residues Control and Their Impact on Soil Quality and Food Safety

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Additional information is available at the end of the chapter

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1. Introduction

The concept of sustainable development represents the totality of forms and methods for the socio-economic development, whose foundation is primarily to ensure a balance between the socio-economic systems and the natural capital.

This concept has become, bringing into focus the complex relationship between development and environment, the quality of human activity and consciousness, of present and future.

The increasing needs of food as a result of world population growth have led to intensive use of chemicals, particularly of fertilizers and pesticides. Despite of the beneficial role on increasing production, some pesticides have been shown to have a negative effect on human health by means of bioaccumulation as a result of residues persistence in food.

Nutrition trends, especially in recent decades have turned to integral grains and unrefined flours with a high nutrient content and also to increase the fiber weight in the diet, with beneficial effects on the human health, thus leading to increased consumption of such products with diversified sorts of integral bread grains.

The importance of these varieties is primarily derived from their chemical composition, high contents of proteins and essential amino acids, mineral substances and lipid compounds, and also from the fact that, in the production process, they do not require large inputs, are not demanding from the conditions of cultivation, fertilization, tolerance to diseases and pests, and can survive at harsh climate.

In this chapter the correlation parameters of grain quality and safety of biological factors, climate (in terms of climatic influence) and technological parameters will be studied in order to identify potential safety concerns of persistent pesticide residues remained in cereals after subjecting them to different treatments on vegetation under cultivation technology.

To follow-up on the evolution status of soil fertility, the application of measures to improve its physiological and biochemical properties is need, as well as the rational use of the chemicals applied in order to maintain soil productivity on the required parameters.

As part of the scientific and technical data, aspects on the agrochemical and biological characteristics of soil are of high importance to the rational application of fertilizers and pesticides. It is also necessary to know the basic principles of micro-organism life in soil, as well as knowledge of the biochemical processes that ensure the fertility of cultivated and uncultivated land.

Soil pollution can be of diverse nature: physical, chemical, biological and radioactive. It can be quantified by assessing the qualitative and / or quantitative reduction of agricultural production as compare to normal situation. Among the above mentioned pollution types, the most frequent and harmful are those caused by the use of fertilizers and pesticides.

High toxicity of some plant protection chemicals applied in various forms (powder, dust, granules, capsules, solutions, suspensions, aerosols, foams, gas, steam, paste) may cause direct poisoning of plants and animals, generating changes and disturbances of equilibrium. The pesticides penetrating the plants or into the soil undergo chemical reactions and biochemical transformations on the plant surface or inside their tissues.

On the other hand, pesticides in the soil or subsoil are absorbed through complex processes such as: transfer of load, ionic exchange, and hydrophobic bonds. Soil particles, by means of clay-humic complex, represent an adequate support for both pesticides adsorption and for some of their degradation byproducts, the adsorptive capacity depending on soil texture.

The effect of pesticides on soil microbiological activity was studied by examining microorganism response after the application of different doses of insecticides, fungicides and herbicides. To certify the statistical dependence of the numerical variation of populations of micro-organisms, enzyme activity and soil chemical properties, correlations have been performed among the values of these indices.

Research has continued and the response of soil micro-organisms was examined, following the application of doses of pesticides, according to the requirements of application recommended. Thus, it was found that, in compliance with pesticide application dose, soil microorganisms are not affected.

Biological properties of soils, such as enzymatic activity and the total number of microorganisms in the soil, serve as a tool for assessment of soil quality change under the influence of various agrochemical processes, regarding the state of fertility for the diagnosis and certification of influence of the application of chemical fertilizers and pesticides on the global biological activity of soil. Moreover, it is important to know the activity of microorganisms and their seasonal variation and to quantify their involvement in reducing the residual effect of pesticides.

Research of chemical fertilizers influence on physical, chemical and biological soil indices can offer the possibility to find practical solutions to prevent on the one hand, the

emergence of negative changes in soil chemistry and, on the other hand, to intervene efficiently for the optimal recovery of agrochemical indicators.

2. Influence of chemical fertilizers and pesticides on soil properties and ecological implications

Environmental protection in agricultural production is of particular importance for the national economy and especially to ensure an optimal framework of social life in rural and urban communities. In recent decades there has been a continuous chemicalization of agriculture to maintain a high level of crops, while obvious depletion of soil nutrients in natural substances. An important place in agricultural process is occupied by the use of a wide range of pesticides. They are particularly useful to solve problems for which they were created and applied.

However, their accumulation in soil, flora and fauna, and uncontrolled migration in the environment of residues resulted from the recovery processes can produce some hazardous effects on both plants and the ecosystem as a whole. In this case, accumulation and uncontrolled migration in soil and groundwater of pesticides can cause significant disturbances to the environmental factors.

Fertilization with chemical fertilizers and pesticide treatments must take into account soil and climatic conditions to prevent some harmful phenomena on plant production.

Plant nitrate pollution is caused by excess mineral nitrogen applied as nitric and has a harmful action on living organisms.

Forage plants (ryegrass, cocksfoot) and especially feed grains (green mass) have a higher concentration of nitrates. Moreover, many plants with thick stems (sunflower, crucifers) accumulate nitrates. Root crops contain more nitrates in leaves than in roots. Legumes do not normally accumulate nitrates. Pollution plant products are made through absorption by plants of pesticides in soil, where they are found in excessive amounts. Absorption intensity depends on the nature of pesticides, plant species and soil type.

Plant pollution by nitrates and pesticides is made through their absorption from the soil, at their rate, the forage plants are consumed by animals and thus the animal products are contaminated (meat, milk, etc.).

Maintaining the maximum allowable levels of nitrates, pesticides and heavy metals in soil can be done by preventive measures such as reducing industrial pollutants, control of wastewater used for irrigation, rational use of manure from industrial growth of pigs, cattle and poultry, etc.

The main problems involved in the dynamics of pesticides in soil (adsorption, volatilization, absorption, degradation, migration, accumulation, etc.) are the subject of extensive laboratory and field studies of both theoretical and experimental profile. In recent years, theoretical and experimental investigations have contributed to highlighting the results of certain scientific and practical value. These results were reflected in the following areas:

- analysis of the main factors determining migration of pesticides in soil;
- setting specific coefficients based on the influence of different pedoclimatic factors on the processes involving pesticides in physical - chemical and biological complex of soil;
- development of criteria for building a forecasting model for soil pollution; define model forecast for pollution of various characteristic types of soil;
- development of criteria for building a forecasting model of groundwater pollution, define model forecast for groundwater pollution in static and dynamic regims;
- qualitative and quantitative determination of coefficients specific to pollution process (diffusion, retention, degradation, etc.).

2.1. Risk assessment of pesticide pollution on soil microbiological activity

Research was conducted to examine the response of soil microorganisms, as a result of application of specific pesticide doses, according to the recommended requirements of application. Each soil sample was treated with one of the following pesticides at recommended dosages: insecticide at the rate of 0,0025g, fungicide at the rate of 0,01g and herbicide at the rate of 0,01ml. Thus, it was found that, in compliance with pesticide application doses, microorganism life in soil is not significantly affected.

Days of incubation sample	Total number of germs (T.N.G.)
Control 0 insecticide	6.8×10^6 6.8×10^6
Control 5 insecticide	1.9×10^7 4.1×10^7
Control 10 insecticide	3.2×10^7 5.4×10^7
Control 15 insecticide	1.1×10^6 1.4×10^7
Control 20 insecticide	0.6×10^6 4.9×10^6

Table 1. Effect of insecticide on the number of total microorganisms per gram of dry soil

As shown, normal dosage of insecticide applied to soil has little effect on microflora at the 10th day of incubation, with no increase in the total number of germs as compared to control. In the following days of incubation a small increase in the number of microorganisms in soil treated is observed as compare to control. This shows that microorganisms are able to multiply in the presence of insecticide.

Days incubation samples	Total number of germs (T.N.G.)
witness 0 fungicide	6.8×10^6 6.8×10^6
witness 5 fungicide	3.1×10^7 4.7×10^7
witness 10 fungicide	3.8×10^7 4.9×10^8
witness 15 fungicide	2.3×10^6 1.9×10^8
witness 20 fungicide	1.2×10^6 4.4×10^7

Table 2. Effect of fungicide on the number of total microorganisms per gram of soil

The number of germs indicates that soil on which the fungicide was applied contains microorganisms capable to metabolise this fungicide. In reference soil a decrease in the number of microorganisms during the incubation was observed.

Days of incubation	Total number of germs (T.N.G.)
Control 0 herbicide	7.5×10^6 7.5×10^6
Control 5 herbicide	1.9×10^5 4.4×10^7
Control 10 herbicide	3.5×10^6 4.9×10^7
Control 15 herbicide	7.9×10^6 2.7×10^7
Control 20 herbicide	6.4×10^6 2.0×10^7

Table 3. Effect of SDMA herbicide on the number of total microorganisms per gram of dry soil

In soil treated with herbicide the microorganisms can grow and multiply.

On the whole, one can remark that normal doses of insecticides, fungicides and herbicides have little effect on total number of germs in the first incubation period, but afterword there

is an increase. Therefore, microorganisms are able to multiply in the presence of insecticides and fungicides, while in the presence of herbicides they are multiplied rapidly.

It is important to know the activity of microorganisms and their seasonal variation due to their involvement in reducing the residual effect of pesticides.

Note: Decis Mega 50 EW (emulsion in water), Substance active: deltametrin 50 g/l

Pyrethroid insecticide for pest control in wheat, apple, cherry, plum, apricot, peach, cabbage, eggplant, tomato, lettuce, potato, rapeseed, vines, corn, sunflower, sugar beet.
Wheat: using the dose of 0.15 l / ha in 300-400 liters of water to combat bedbugs cereals (*Eurygaster integriceps*) Balos beetle (*Lema melanopa*) and red worm straw (*Haplodiplosis marginata*)
Apple: 0.0125% (0.187 l / ha in 1500 liters of water) to combat worm moth (*Cydia pomonella*). 0.015% (0.225 l / ha in 1500 liters of water) for aphids (*Aphis* trees), skin fruit moth (*Adoxophyes reticulana*), San Jose Scale (*Quadraspidiotus perniciosus*)
Cabbage: 0.02% (0.12 l / ha in 600 liters water) to combat headed eagle (*Mamestra brassicae*) L1-L3 larvae, aphids (*Brevicoryne brassicae*)
Beet sugar: 0.15 l / ha to fight ladybug leaves of maize (*Tanymecus dilaticollis*), beet flea (*Chaetocnema tibialis*) and ladybug beet (*Bothynoderes punctiventris*).

Aliette 80 WG(water dispersible granules), Substance active: aluminum fosetyl 800 g/kg

Systemic fungicide for disease control in vegetables, fruit trees, hops.
Apple, pear, quince: 0.3% for fire fighting of rozaceelor bacterial (*Erwinia amylovora*).
Cucumber: 0.2% (2 kg / ha) to control blight (*Pseudoperonospora cubensis*)
Hops: 0.2% (4 kg / ha in 2000 liters of water) to combat blight (*Pseudoperonospora* humuli).

SDMA-is a systemic herbicide for weed control in wheat, corn and grass.

Application rate of 1L/hectar in 250-350 liters of water.

3. Methods for identifying pesticide residues

3.1. Determination of compounds of interest

Achieving quality parameters depends on several factors such as genetic potential quality of the cultivated variety which materializes only in terms of ensuring all the elements of culture technology, taking into account the natural and climatic background of the area.

Nutritional value of proteins in certain grains (synthesis species of rye and wheat, a variety of higher production potential and adaptability as compared to other cereals) is higher than that of wheat. Thus, to improve the quality parameters of bakery products the utilization of these types of cereals has been studied, choosing a species with high resistance to low temperatures, and using modern methods of investigation in order to determine the content of pesticides in such cereal species. For cereals investigated, certain quality and safety parameters have been correlated with biological, climatic (in terms of climate impact) and technologic factors.

In specific phenological/evolutionary moments important for vegetation period various combinations of treatments with foliar fertilizer (phosphorus or nitrogen), fungicides, insecticides and herbicides have been applied.

In order to determine the compounds of interest regarding the identification of pesticide residues in commercial products the active substances present in treated grain samples have been analyzed. Treatments applied to these grain varieties under study aimed at combating pests, fungi that cause plant diseases and weeds that destroy crops.

Quality assessment of bread-making grains was based on measurements performed by standard physico-chemical methods to characterize the integrity and quality of cereal species under study, quality of meal mixtures and quality of the finished product made from whole wheat.

3.2. Choosing QuEChERS extraction method

Extraction method chosen is QuEChERS method suitable for pesticide determination in dry samples as whole wheat, wheat flour and bran according to the literature and based on solid phase extraction, with the advantage of retaining co-extracted substances in the extract and pesticide releasing. The method adapted to wheat matrix used to determine pesticide residues is highly competitive for GC-MS analysis and is based on the following reasons: time savings, lower volumes of solvents, validated in analytical conditions of repeatability, reproducibility, specificity and accuracy.

3.3. Determination of performance parameters

Determination of pesticide residues in food matrix in compliance with regulations in force laid down determination of performance parameters of the method (linearity, repeatability, reproducibility, accuracy and recovery coefficient), both for standard methods and those with small improvements.

For qualitative determination a series of callibration solutions has been injected into gas chromatograph in order to identify retention times for each compound of interest based on chromatograms obtained by using the SCAN module. This SCAN identification of compound has been performed based on NIST library spectra. This involves determination of the mass spectrum from GC / MS analysis of pesticide standard together with the mass spectra of possible compounds found in the spectra library.

For quantitative determination in SIM mode a calibration curve of analyzed compounds was drawn and thus the concentration range was established for which linearity was established. As the concentration range of samples is fairly wide two different calibration curves have been chosen, as follows:

- low concentration range: 0.02 ppm - 0.2 ppm;
- high concentration range: 0.2 ppm - 1.5 ppm

To highlight the method precision - repeatability and reproducibility, determinations were performed simultaneously on pesticide residues, establishing average standard deviation (RSD%).

To verify the method accuracy, we determined the recovery coefficient (± CR), for two compounds analyzed (eg amidosulfuron, tebuconazole), achieving thus a sample enriched with standard concentrations.

3.3.1. Relationship between pesticide residues content detected and some quality parameters of grain studied

Achieving increased production per unit area and obtaining appropriate quality agro-food products is a constant concern of specialists. Food value of agricultural products can be determined both by physical and chemical methods. Research conducted for chemical testing of wheat quality is refered to the protein content and quality, but also to the ripeness and freshness of the finished product.

Analysis of the results obtained by combining the two methods best reflects the technological indices of wheat flour. Thus, one can track the contribution of various factors (climate - climatic conditions of experimental years 2008 to 2011, biological - and technological species - fertilization and vegetation treatments) to achieve qualitative characteristics of cereals in the study, each factor having a specific influence on grain quality. Percentage estimation of these factors is shown in the table bellow.

Factor/ Parameter	Climatic conditions	Species type	Treatment	Interactions	Fertilization
Mass/storage Volume (kg/hl)	57	33	5	3	2
Protein Content, %	76	6	1	5	12
Sedimentation Index, %	49	2	-	6	43
Wet gluten, %	84	8	1	3	4
Distortion Index, mm	57	15	-	3	23
Gluten index, %	82	7	1	3	5
Fall index, sec	24	39	-	37	-

Table 4. Contribution of various factors on qualitative characteristics of cereals

A critical role is played by knowledge of the relationship among the main quality parameters of grain species studied. In this respect, the strongest influence on the quality indicators is given by the species x year interaction (climatic conditions), then species x fertilization, while there is an insignificant influence from species x treatment interaction.

Since the factor "year", from climatic point of view, had the most important influence in achieving quality parameters (according to variant analysis), for better highlighting the quality of grain species, the influence of environmental conditions have been analysed, as it was identified by the species x year interaction (in terms of climate) for the main quality parameters.

The genetic potential is valorized only by a proper fertilization and at given levels of precipitations and normal temperatures.

Analyzing the evolution of quality parameters as function on fertilization dose, the control sample and sample of species analyzed, one can conclude that additional fertilization affects the following quality parameters: ash, protein, sugars, total fibers, cellulose enzyme activity and energetic value. In terms of appearance, fertilization significantly affects the volume of finished product.

Bread volume is related to its form which is obtained by determining the ratio of product height (H) and diameter (D). The ratio H / D allows characterizing aspects referring to product volume and shape.

As referring to agro-fund, the results highlight the crucial role of the additional dose of fertilizer in terms of parameters characterizing the finished product quality.

Providing additional dose of fertilizer correlated with favourable weather conditions, the ratio grain: wheat may reach up to 70%.

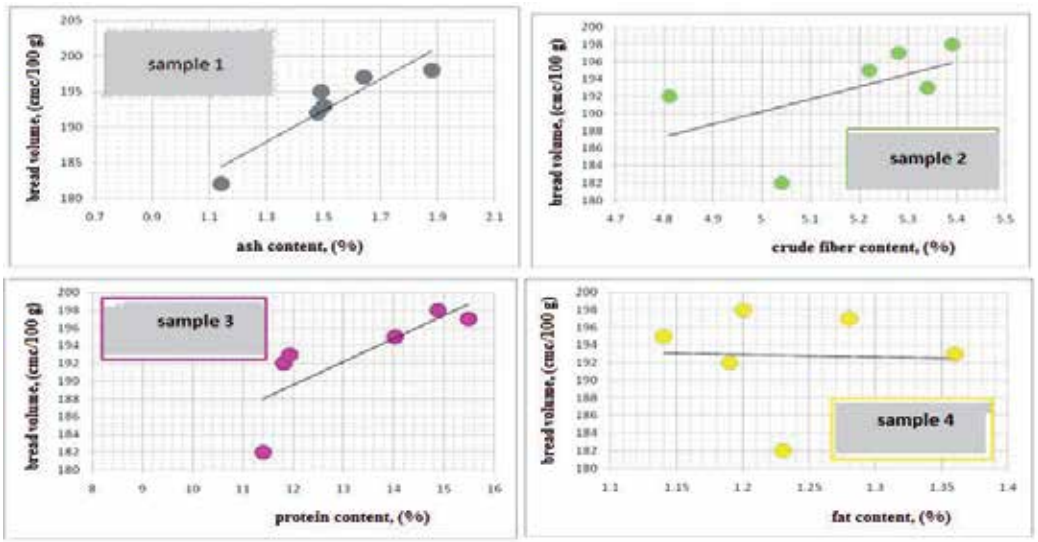


Figure 1. Correlations among some quality parameters of flour and bread volume of finished product

Volume change of finished bakery products (bread) is determined by the variation of content as follows: ash (80 - 85%), starch (70 - 75%), protein (60 - 65%), sugar (35 - 40%), fiber and energy value (25 - 30%) and it is not influenced by the fat content (0%). There is a strong link among the volume of finished bakery products and ash, starch or protein of interest, and a connection of medium interest between the sugar content and the volume of finished product bakery (bread), among the fiber content, energy value and volume. There is no association between fat content and bread volume, these variables being uncorrelated.

3.4. Determination of pesticides by modern methods of investigation

Protocol requires the extraction of pesticides using acetonitrile and extract purification using solid phase extraction (D-SPE) on a C18 column as sorbent material (octadecyl), followed by gas chromatographic determination coupled with mass spectrometry (GC-MS).

The compounds identified in samples containing pesticide residue are: amidosulfuron, (0.01 - 0.04 mg/kg), propioconazol (0.03 mg/kg), tebuconazole (0.11 - 0.19 mg/kg).

Variations in pesticide content and comparison with pesticide regulations on MRLs (maximum residue limit), and relative proportions of the analyzed samples are presented in the following figures:

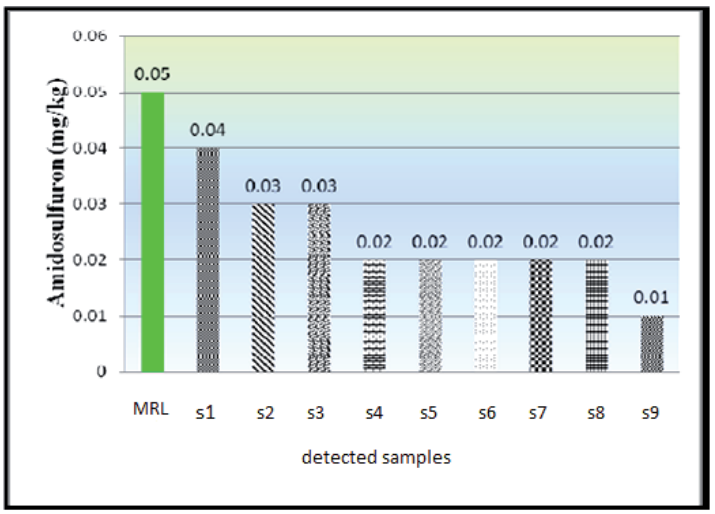


Figure 2. Amidosulfuron content as compared with MRL

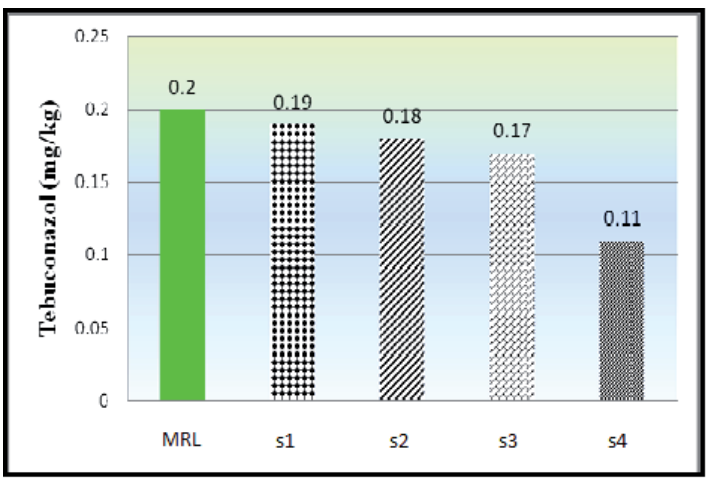


Figure 3. Tebuconazol content copared with MRL

The relationship between some quality parameters and content of pesticide residues detected allows explain to what extent this pesticide content, even it is bellow the maximum residual limit (MRL), can be influenced by some quality parameters of grain analysed. To measure the intensity of these relationships between pesticide residues and various quality parameters, the linear correlation coefficient was calculated.

Analyzing the data using the regression equations represented in Fig. 4, one can say that there are insignificant correlations between amidosulfuron content and moisture (r = 0.24), protein content (r = 0.35) or gluten content (r = 0.14) and a moderate correlation between amidosulfuron content and mass per storage volume (r = 0.54).

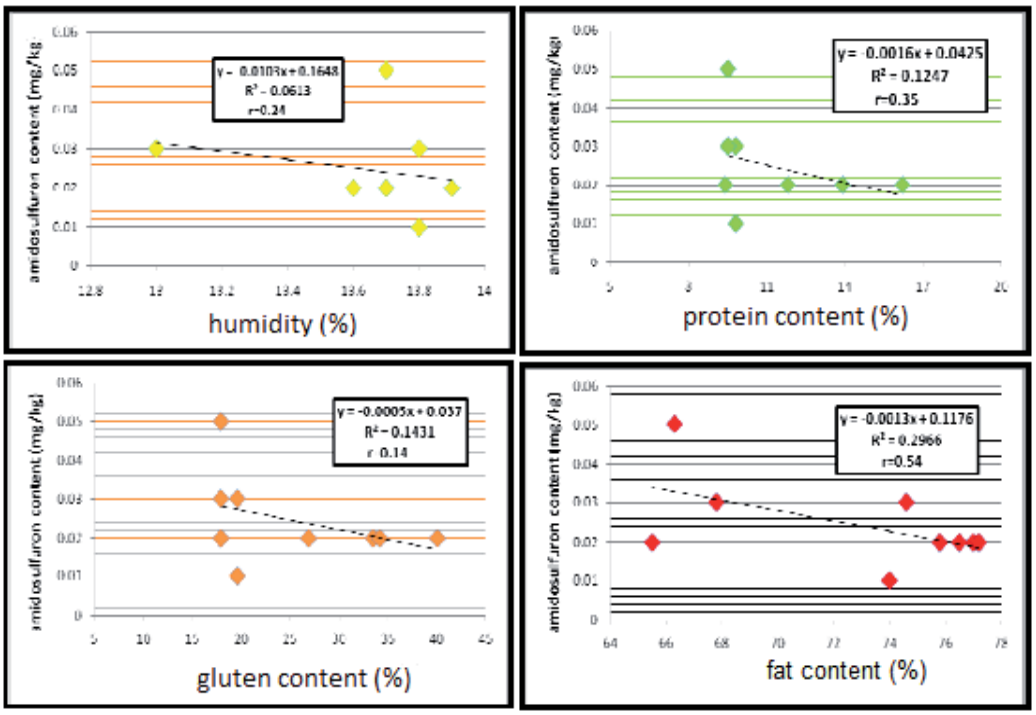


Figure 4. The relationship between some parameters of quality and content amidosulfuron

4. Methods for organochlorine insecticides analyses in soil, water, plants and their monitoring

4.1. Quantitative determination of organochlorine insecticide residues by GC-ECD and GC-MS techniques

For qualitative and quantitative organochlorine insecticides determination in soil two methods for multi-residues analyses have been applied: gas-chromatography with electron

capture detector (GC-ECD) and gas-chromatography with mass spectrometer detector (GC-MS). Standard stock solution of 1000 mg/L (19 organochlorine insecticides) has been prepared from which dilutions up to 1 mg/L were made.

Standards solutions for calibration curve were prepared in the range of 0.025 mg/L – 0.5 mg/L with intermediate points of 0.05; 0.1 and 0.25 mg/L, and were injected into GC-ECD.

No. crt.	Organochloride insecticide	Retention time (minute)
1.	HCB	10.93
2.	Lindane (ɣ-HCH)	11.57
3.	Heptachlor	13.99
4.	α- HCH	10.68
5.	β- HCH	11.43
6.	δ- HCH	12.32
7.	Heptachlor-endo	17.47
8.	Heptachlor-exo	17.24
9.	α - endosulfane	19.08
10.	β - endosulfane	22.36
11.	Endosulfan sulfate	25.09
12.	pp′- DDD	23.19
13.	pp′- DDT	25.61
14.	pp′- DDE	20.62
15.	op – DDT	23.32
16.	Alochlor	13.93
17.	Aldrin	15.38
18.	Dieldrin	20.52
19.	Endrin	21.80

Table 5. Retention time of organochlorine insecticides from mixture

Since at these retention times some interferences from also soil can occur, a second GC-MSD method has been tested, being reccomended and frequently used in pesticides residue analyses. In this context, standard solutions were prepared for 11 organochlorine insecticides in the range of 0.1 - 0.5 mg/L and were injected into GC-MS.

For the quantitative analysis of pesticide residues from stock solution of 1000 mg/L containing 19 organochlorine pesticides a set of intermediate solutions has been prepared having the following concentrations: 0.05; 0.1 and 0.25 mg/L. After injection into GC-MS autosampler the calibration curves for organochlorine insecticides were obtained.

Qualitative and quantitative determination of insecticides in water and plant samples is performed by GC-MS technique in the similar conditions as for soil sample analyses.

Nr. crt.	Organochloride insecticide	Retention time (minute)	Specific ions
1.	HCB	9.61	
2.	Lindan (ɣ-HCH)	10.41	181, 219
3.	Heptaclor	12.25	337,237, 194
4.	α- HCH	9.53	181, 219
5.	β- HCH	9.63	
6.	δ- HCH	11.40	181, 219
7.	Heptaclor-endo	14.73	337, 237, 194
8.	Heptachlor-exo	14.77	
9.	α - endosulfan	15.86	195, 269, 241, 339
10.	β - endosulfan	17.65	
11.	Endosulfansulfat	19.36	272, 289
12.	pp'- DDD	18.06	235, 165, 237
13.	pp'- DDT	19.45	235, 165, 237
14.	pp'- DDE	16.57	
15.	op – DDT	18.09	
16.	Aloclor	13.23	
17.	Aldrin	13.35	263, 257, 293, 220
18.	Dieldrin	16.78	263, 277, 345
19.	Endrin	17.53	245, 279, 263, 345

Table 6. Retention times and specific mass for ions detected

Methods of organochlorine insecticides analyses					
in SOIL		from WATER		from PLANTS	
Extraction	Purification	Extraction	Cleaning	Extraction	Cleaning
Static extraction - in a mixture of acetone-hexane 1:1. Dynamic extraction - a mixture of acetone, petroleum ether, methylene chloride 1:1:1.	For both extraction methods the same purification method was used with silicagel 60, having particle size of 63 – 200 μm. The recovery rate of first extraction procedure is between 40-60%, and in the dynamic extraction is between 70-90%.	with petroleum ether or hexane 5:1 in water as solvent.	water being a simple matrix, there is no need the cleaning of organic extract.	with acetone/ water 1:2.	for plant samples the liquid-liquid cleaning is enough.

Table 7. Multiresidual analysis methods for organochlorine analyses in soil water and plants

4.2. Organochlorine pesticide residues in soil and water

4.2.1. Transfer and translocation

A study was performed in Arges River area, Romania, regarding the presence, persistency and accumulation of these organochlorine insecticides in surface water samples, underground water samples (drills), soil samples (0 – 10 cm; 10 – 20 cm; 40 – 60 cm) and vegetable plants (cabbage-fruit and tomatoes – for endosulfane).

Water samples extraction is done by direct contact with dichloromethane, after drying the sample with sodium sulfate hydricum. Cleaning was done on celite column with hexane.

In underground water samples very small concentration levels have been detected, below those which might affect the human health, as one can see in the following tables:

Sample	Lindan	Aldrin	alfa-endosulfan	DDT
P1	0.050	0.041	0.053	0.039
P2	0.054	0.033	0.153	0.053
P3	0.040	0.045	0.054	0.040
P4	0.042	0.014	0.080	0.065

Table 8. Organochlorine concentration levels in surface water (μg/L)

Sample	Lindan	Aldrin	alfa-endosulfan	DDT
P1	0.005	0.006	0.002	0.010
P2	0.007	0.008	0.009	0.012
P3	0.025	0.009	0.007	0.014
P4	0.023	0.004	0.005	0.011

Table 9. Organochlorine concentration levels in undergroundwater (μg/L)

Sample	Layer depth (cm)	Lindan	Aldrin	alfa-endosulfan	DDT
P1	0 – 10	0.547	0.451	0.518	0.708
	10 – 20	0.117	0.106	0.127	0.120
	20 – 40	0.034	0.024	0.017	0.035
	40 - 60	nd	nd	nd	nd
P2	0 – 10	0.551	0.442	0.500	0.665
	10 – 20	0.112	0.113	0.114	0.127
	20 – 40	0.032	0.027	0.028	0.032
	40 - 60	nd	nd	nd	nd

Sample	Layer depth (cm)	Lindan	Aldrin	alfa-endosulfan	DDT
P3	0 – 10	0.557	0.513	0.492	0.617
	10 – 20	0.122	0.092	0.095	0.120
	20 – 40	0.031	0.026	0.026	0.035
	40 - 60	nd	nd	nd	nd
P4	0 – 10	0.572	0.468	0.506	0.649
	10 – 20	0.118	0.092	0.088	0.111
	20 – 40	0.029	0.031	nd	0.043
	40 - 60	nd	nd	nd	nd

nd - nedetectabil

Table 10. Organochlorine concentration levels in soil (mg/kg)

4.3. The accumulation of studied insecticides in vegetables as effect of treatments with endosulfane on soil and plants

In order to follow the persistency and accumulation of endosulfane residues in cabbage and tomatoes controlled treatments with Thionex 3I CE have been applied in concentration of 0.2 mg/kg for cabbage and tomatoes, 20 days before harvesting.

At each sampling period a number of 5 units of cabbage-fruit and 1 kg of tomatoes were taken. For each sampling period 3 samples were analysed, and average values are presented in the tables below.

Days after treatment (no.)	Residue în mg/kg (average values)	Residue în mg/kg (average values)
3	5.42	5.42
7	3.00	3.00
14	1.70	1.70
21	0.71	0.71
28	0.44	0.44
42	< 0.025	< 0.025

Table 11. Degradation dynamics of endosulfane insecticide in cabbage and tomatoes

Treatments were applied to soil with Chinese cabbage and radish crops, in small plants phase, protected crops, in dose of 1.8 mg/kg (ppm) (lower dose) and 18 mg/kg (ppm) (upper dose). A chromatogram of a mixture of organo-chlorinated insecticides including endosulfan and its isomers has been registered.

Determination of endosulfane and its congeners content was done at harvest in leaves and root for radish, in fruit for cabbage and in soil.

Year	Doze (ppb)	Residue mg/kg		
		α- endosulfan	*β- endosulfan*	*Endosulfan-sulfat*
2003	1800 *	< 0.50	< 0.50	1.50
	18000 **	0.10	0.40	nd
2004	1800	< 0.50	< 0.50	0.80
	18000	0.10	0.40	3.40
Year	**Doze (ppb)**	**accumulation**	**Residue mg/kg**		
			α- endosulfan	*β- endosulfan*	*Endosulfan-sulfat*
2003	1800	leaves	< 1	< 1	< 1
	18000		0.30	0.80	nd
2004	1800		< 1	< 1	< 1
	18000		0.10	0.20	3.60
Year	Doze (ppb)	accumulation	Residue mg/kg		
			α- endosulfan	β- endosulfan	Endosulfan-sulfat
2003	1800	root	< 0.5	< 0.5	2.00
	18000		0.20	1.40	20
2004	1800		< 0.5	< 0.5	1.30
	18000		0.10	0.50	14.60

* =1800 ppb- lower doze; ** = 18000 ppb- upper doze

Table 12. Concentration of endosulfane in cabbage and radish

Reffering to endosulfane accumulation at smaller dose there are no differences between the 3 congeners, both in leaves and in root. In case of upper dose it might be remarked the difference between the accumulation level in root comparing to the level from leaves.

The biggest amounts were detected in case of endosulfane-sulfate congener.

Year	Dose (ppb)	Residue mg/kg		
		α- endosulfan	*β- endosulfan*	*Endosulfan-sulfat*
2003	1800 (low)	5.3	58.3	323.5
2004	1800	1.8	18.6	115.30
Year	**Dose (ppb)**	**Residue mg/kg**		
		α- endosulfan	*β- endosulfan*	*Endosulfan-sulfat*
2003	18000 (high)	21.60	209.60	820.30
2004	18000	20.10	230.60	1049.80

Table 13. Persistency of endosulfane residue in soil cultivated with cabbage and radish

Accumulation level of endosulfane insecticide in the cabbage soil crop was surveyed only at lower dose, while for radish crop only at higher dose. The results show for cabbage crop the presence in quantifiable levels of all 3 congeners.

These results indicate the necessity of a very good control on long persistency pesticide use. The most frequent cases of environmental factors pollution are are caused by ignorance, negligence and improper use of pesticides.

The results show that the accumulation of these pollutants in soil, plants and underground waters is influenced by chemical structure of compound, and physico-chemical properties of soil as well.

The monitoring of organochlorine insecticide residues in soil and water was carried out in samples from the same area. The distribution of organochlorine insecticide residues in the four soil layers is different, in the upper layer being more than 80% from total toxic content, while for the last layer the toxic values are below detection limit.

Determination made for persistency and accumulation level in different vegetables of organochlorine insecticide residues of endosulfane type indicate preferential accumulation in root and less in leaves.

5. Use of chemical and biochemical sensors for analytical control of pollutants

Enzymatic systems were used to determine pesticide content based on cholinesterase. A set of chemical and biochemical sensors has been achieved and tested in the presence of organic solvents for analytical control of pollutants in environmental samples. Operational parameters of biosensors have been optimized for the determination of organophosphorus insecticides and a method for preconcentration and oxidation of insecticides has been developed.

5.1. Determination of organophosphorus insecticides using a potentiometric biosensor based on acetylcholinesterase (AChE)

Two organophosphorus compounds have been determined using a potentiometric reusable biosensor. Acetylcholinesterase (AChE) was immobilized on a cellulose ester membrane, using for this purpose glutaraldehyde and bovine serum albumin (BSA). The membrane was then fixed on a flat surface glass electrode. The degree of inhibition of the enzyme is dependent on the concentration of pesticide in the sample. Inhibited enzyme was reactivated using pyridine-2-aldoxime metyliodide (2-PAM).

AChE catalyzes the hydrolysis reaction of the substrate, acetylcholine, to obtain as reaction products choline and acetic acid.

The latter produces a pH variation of membrane solution, which is measured using a glass electrode. When insecticides inhibit the enzyme immobilized on the membrane one can be seen a reduction in variation of pH in the presence of substrate.

This biosensor based on AChE immobilized on a cellulose ester membrane has a good operational and storage stability. Organophosphorus insecticides used in these experiments

were: ethyl paraoxon and dichlorvos. After each measurement of enzyme inhibition, reactivation of the enzyme biosensor was made by incubating for 10 min in a solution of 2-PAM 0.02 mol/L prepared in 0.002 M phosphate buffer, pH = 8. Comparing the percentages of inhibition obtained at the same concentrations of paraoxon, dichlorvos it was found a greater capacity of enzyme inhibition of paraoxon than of dichlorvos.

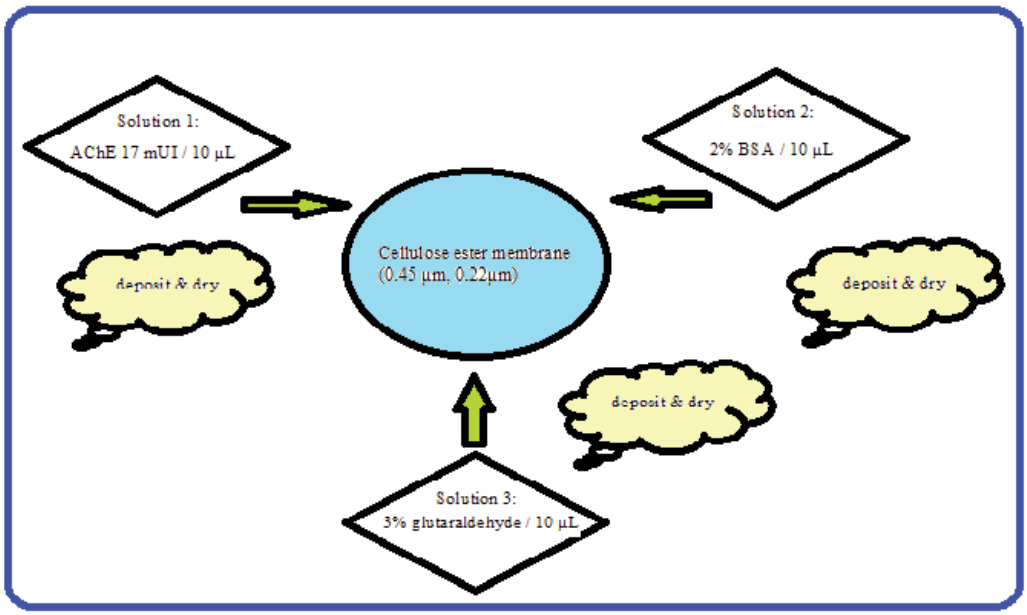
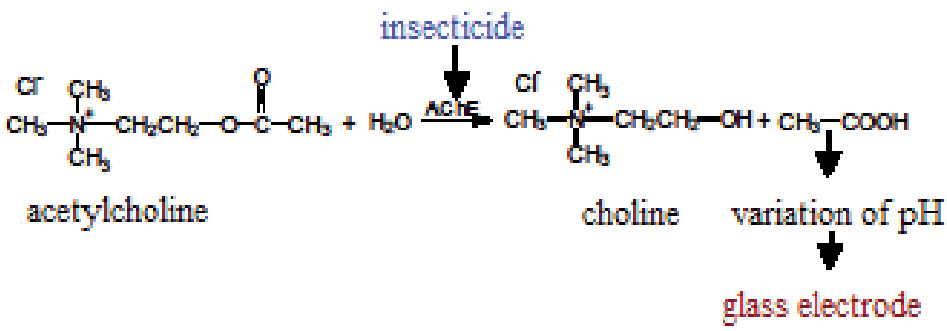
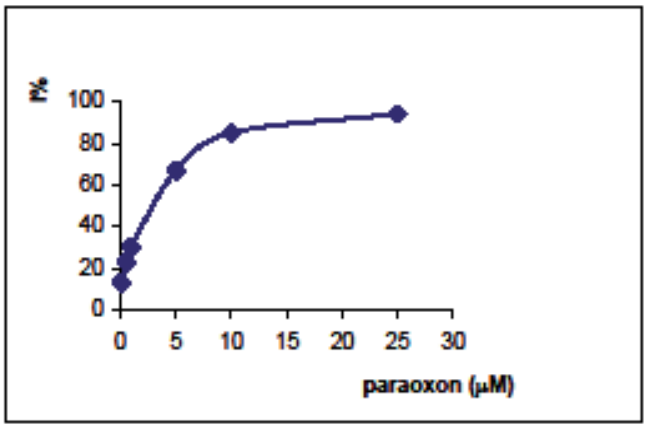


Figure 5. AChE immobilization on cellulose ester membrane



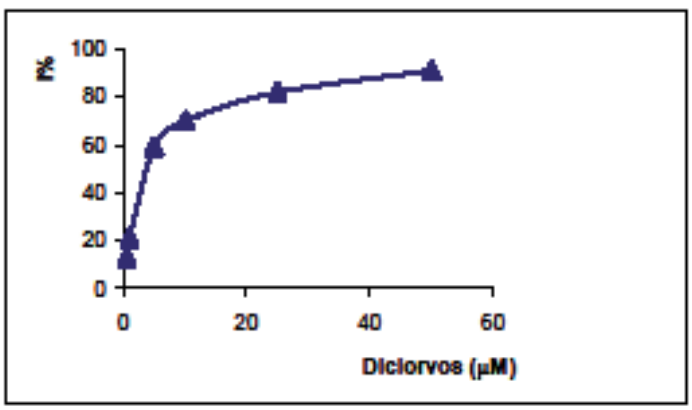
Scheme 1. The principle of potentiometric detection of organophosphorus insecticides using a biosensor with AChE

The detection limit obtained with biosensors built was 0.5 µM for dichlorvos and 0.1 µM for ethyl paraoxon. This method requires no sample pre-treatments and biosensor can be easily used to identify and evaluate the toxicity of samples analyzed.



Working conditions: 0.002 M phosphate buffer plus 0.1 M KCl, pH = 8; 17 mUI AChE/membrane, incubation for 20 minutes.

Figure 6. Inhibition curve of the biosensor based on AChE for paraoxon



Working conditions: 0.002 M phosphate buffer plus 0.1 M KCl, pH = 8; 17 mUI AChE/membrane; incubation for 20 minutes.

Figure 7. Inhibition curve based on AChE biosensor for dichlorvos

5.2. Immobilisation of acetylcholinesterase (AChE) using sol-gel process

AChE immobilization was followed in sol-gel matrix to achieve an amperometric biosensor for the detection of organophosphorus insecticides. We optimized conditions for immobilization of AChE in sol-gel matrix for amperometric biosensor development of the mediator tetracianochinodimetan (TCNQ). Two types of precursors have been used: tetra-metoxi silan – TMOS, and metyl tetrametoxisilan - MTMOS.

Sol-gel solution was obtained by mixing TMOS and MTMOS precursors with 1 mM HCl solution, PEG600 and water. The resulting mixture was 20 minutes sonicated for homogenization and then kept at 40°C for 12 hours before use, in order to assure silane

hydrolysis. From these experiments the optimal composition of the sol-gel matrix for AChE immobilization has been established.

Sol-gel				
TMOS	MTMOS	H_2O	HCl	PEG_{600}
20 µl	10 µl	44 µl	40 µl	5 µl

Table 14. Composition optimal sol-gel

Sensitive layer		
Enzyme solution	Sol-gel	Graphite power with TCNQ + 3% HIEC
25 µl	25 µl	25 µl

Table 15. Composition optimal for submitting sensitive layer

Determination of immobilized enzyme activity, and inhibition tests were performed by amperometric measurements in a cell with a volume of 10 mL. Screen-print electrodes were modified with an electrochemical mediator sensitive for the oxidation of thiol compounds, TCNQ, which allows measurements at low potential.

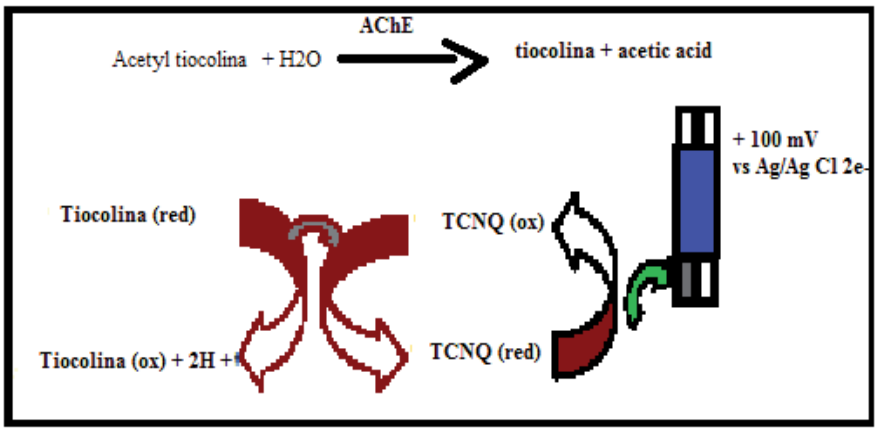


Figure 8. Amperometric detection of tiocoline produced in the enzymatic hydrolysis of acetiltiocoline

For detection of insecticides, inhibition measurements were performed in three stages:

i. Measuring the initial biosensor response to acetiltiocoline injection and averaging the results obtained;
ii. Sensor incubation for 10 or 20 minutes into insecticide solution;
iii. Measuring the residual response and averaging the results obtained.

Percent inhibition is calculated as:

$$I\% = (I_0 - I)/I_0 \times 100 \quad (1)$$

where: I% = percentage of inhibition; I_0 = current value before inhibition; I = current value after inhibition.

For immobilization of AChE different mixtures of TMOS, MTMOS, PEG600, 1 mM HCl and H_2O have been tested for obtaining hydrolysated precursors. In this way the operational stability of biosensors has been studied.

To prepare the sensitive layer of biosensors the following mixtures of reagents were used: (i) graphite powder with TCNQ in HEC, (ii) AChE in buffer and (iii) precursor hydrolysated.

Another feature studied was storage stability of the biosensors with enzyme immobilized in sol-gel. Good results were obtained by biosensors conservation in freezer at a temperature of - 20°C in vacuum.

The influence of substrate concentration on the biosensor response has been also studied. Substrate concentration chosen for measurements of enzyme activity was 1 mM. For inhibition tests three insecticides have been used: paraoxon as reference insecticide, oxon clorpirifosmetyl as one of the most toxic insecticides and dichlorvos.

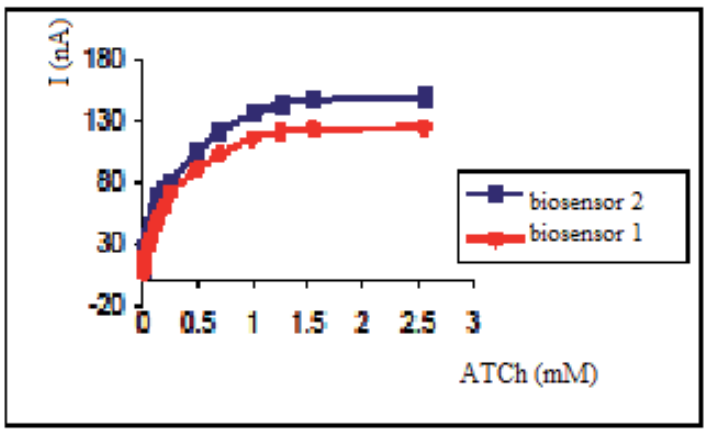


Figure 9. Influence of substrate concentration on the biosensor response based on AChE

Working conditions: 0.1 M phosphate buffer plus 0.1 M KCl, PH = 8.2 mUI / AChE / electrode, 100 mV vs Ag / AgCl.

For each of biosensors a decrease of signal after incubation phase could be observed which implies a decrease in AChE activity immobilized on the electrode.

Inhibition curves are presented for the three insecticides using the two biosensors 1 and 2.

The best results were obtained with the biosensor 2 for the incubation time of 20 minutes. For paraoxon was obtained a detection limit of 0.008 μM.

In case of dichlorvos a detection limit of 0.02 μM was obtained.

The best limit of detection was obtained for chlorpyrifos-methyl Oxon, i.e. 0.001 μM.

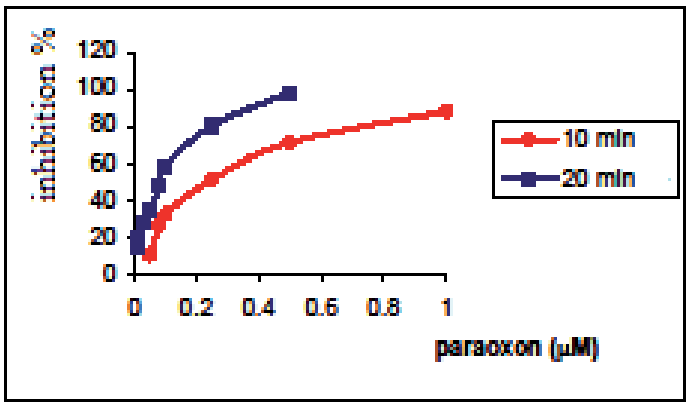


Figure 10. Influence of paraoxon concentration on first biosensor response with AChE immobilized in sol-gel; each experimental point is the average of 3 measurements

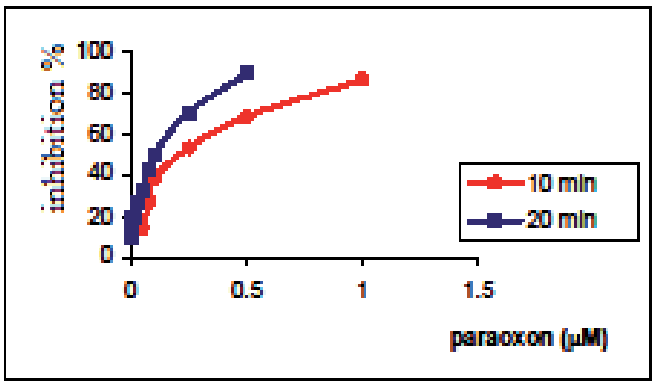


Figure 11. Influence of paraoxon concentration on the second biosensor response with AChE immobilized in sol-gel

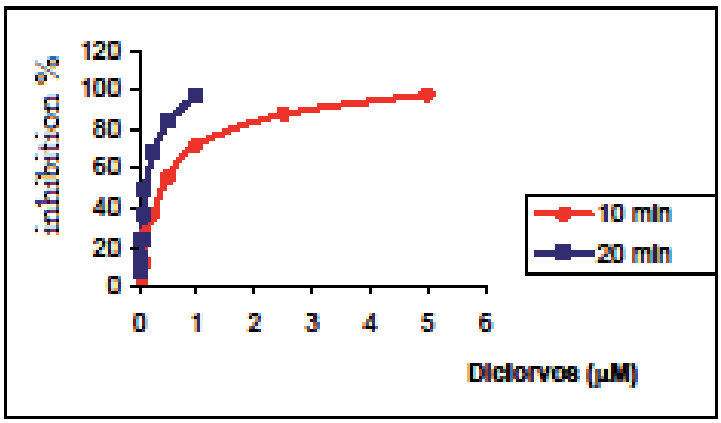


Figure 12. Influence of the concentration of dichlorvos on type 1 response with AChE biosensor immobilized in sol-gel, each experimental point is the average of 3 measurements.

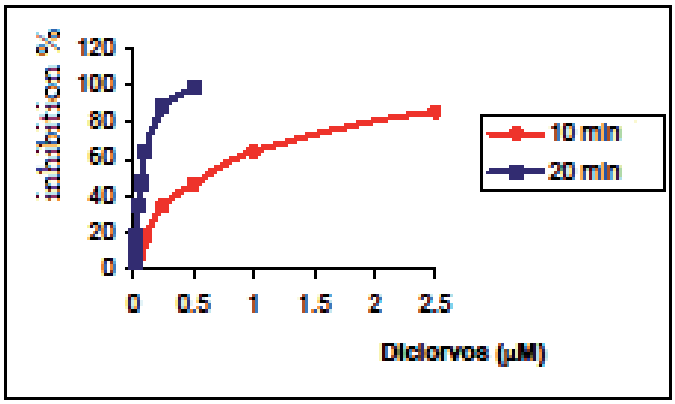


Figure 13. Influence of dichlorvos concentration on second type biosensor response with AChE immobilized in sol-gel

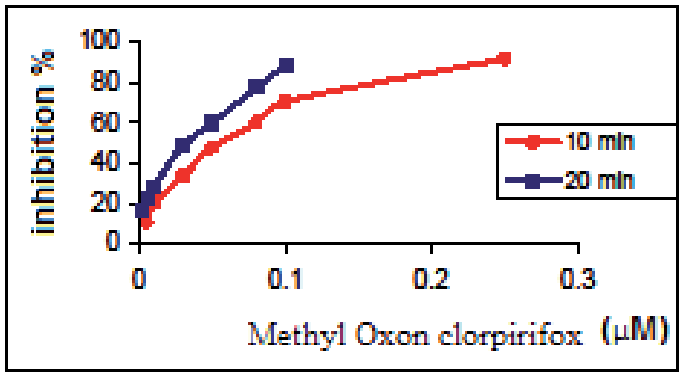


Figure 14. Influence of the concentration of chlorpyrifos methyl Oxon on the biosensor response with AChE immobilized in sol-gel

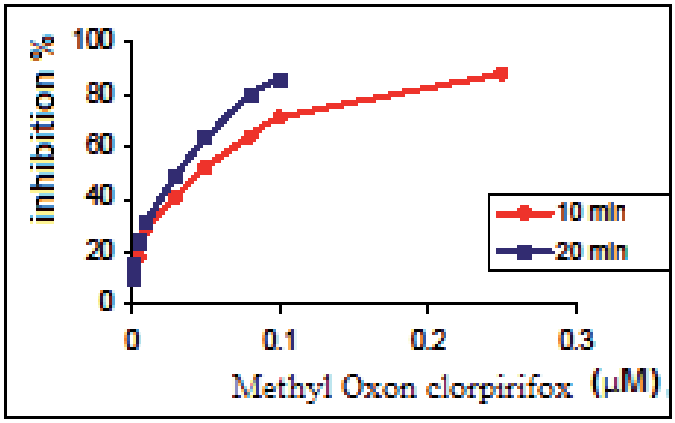


Figure 15. Influence of the concentration of chlorpyrifos methyl 2 Oxon on biosensor 2 response with AChE immobilized in sol-gel

Comparing the percentages of inhibition obtained at the same concentrations of paraoxon, dichlorvos and chlorpyrifos-methyl Oxon a greater capacity of enzyme inhibition was found for chlorpyrifos methyl Oxon, followed by paraoxon and dichlorvos.

5.3. Preconcentration and oxidation of organophosphorus insecticides

To improve detection limits and selectivity of analyzes performed with biosensors a column containing XAD2 has been used. In this way organophosphorus insecticides can be preconcentrate and oxidized. Solid phase extraction is widely used to determine insecticides, so this method can be also adopted for biosensors, provided that the organic solvent used in elution insecticide to be compatible with the enzyme and the transducer. Depending on the hidrofobicity of organic solvent and possible restraining effect of immobilization method on the enzyme, enzyme activity can be strongly affected. This is the reason for which the enzyme was immobilized in an optimized sol-gel matrix.

The biosensor thus o optimized shows good operational stability. The average response of a single biosensor was 245 nA with good reproducibility of the biosensor response.

Biosensor response increases with increasing substrate concentration until a plateau (saturation of the enzyme). The influence of biosensor incubation in phosphate buffer solution (PBS) containing organic solvents has been also investigated as function on substrate concentration. For each graph different biosensors have been used.

The diagrams obtained at biosensors incubation for 10 min in PBS containing 10% ACN, methanol and ethanol do not show significant changes compared with the pattern obtained with the biosensor that was not in contact with organic solvents (Fig. 16).

The effect of the organic solvent on AChE activity has been studied by incubating the biosensor for 10, 15, and 20 minutes in 0.1 M phosphate buffer solution containing 5, 10, 15% (v / v) ACN, ethanol and methanol (Fig. 17). It was found that the effect of distortion of the enzyme denaturation increases with time of biosensor incubation.

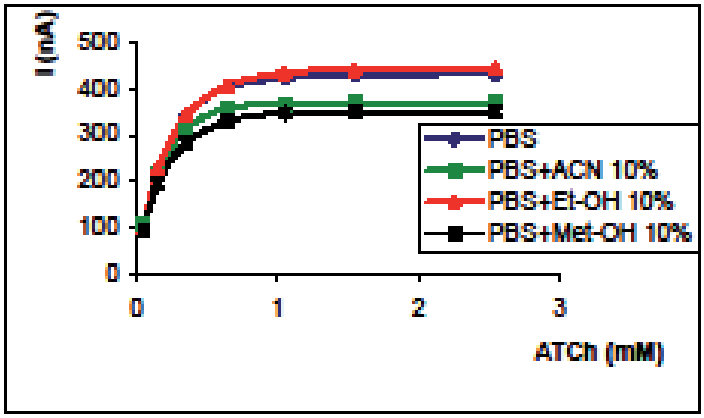


Figure 16. Effect of biosensor incubation in 0.1 M PBS containing 10% ACN, 10% Et-OH and 10% Met-OH on calibration curve for ATCh

Working conditions: phosphate buffer (PBS) 0.1 M KCl plus 0.1 M; 2mUI AChE / electrode, applied potential 100 mV vs. Ag / AgCl, incubation time 10 minutes.

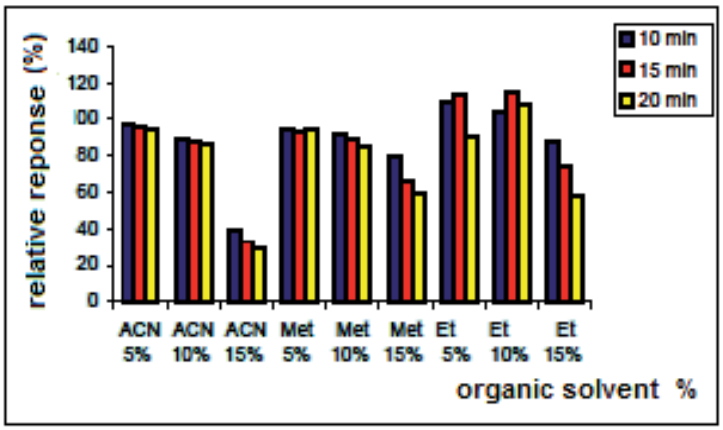


Figure 17. The effect of organic solvent (%, v/v) and incubation time on biosensor response. (ACN: acetonitrile; Met: methanol, Et: ethanol).

Working conditions: 0.1 M phosphate buffer, pH 8, 0.1 M KCl; 1 mM ATCh, applied potential: + 100 mV vs Ag / AgCl; results are the average of three independent measurements.

For the incubation time of the biosensor of 10 minutes in phosphate buffer solution containing 5% (v/v) organic solvent, the percentage change of enzyme activity is less than 10% and for this reason it was considered that the presence of organic solvent has no significant effect on AChE.

Insecticides	Concentration of insecticides	RSD
Paraoxon in PBS	5×10^{-7}	6,3% (63 ± 4% inhibition, n = 5)
Paraoxon in PBS 5% (v/v)ACN	5×10^{-7}	7,4% (43.2 ± 3% inhibition, n = 5)
Paraoxon after preconcentration	2.5×10^{-7}	11,7% (60 ± 7% inhibition, n = 5)
Diclorvos in PBS	2.5×10^{-7}	8,4% (32.3 ± 2,7% inhibition, n = 5)
Diclorvos in 5% (v/v) ACN	2.5×10^{-7}	9,5% (20 ± 1,9% inhibition, n = 5)
Diclorvos after preconcentration	2.5×10^{-7}	11% (55.6 ± 1% inhibition, n = 5)

Table 16. Reproductibility of biosensor during inhibition tests

The recovery studies of paraoxon and dichlorvos insecticides adsorbed on the column containing XAD2 were made according to the following steps:

i. Passage of the paraoxon /dichlorvos solution through the column;
ii. Elution with organic solvent of the insecticide retained on the column;
iii. Fraction collection and solution analysis.

Eluted fractions were diluted in PBS and then analyzed with AChE biosensors. It was found that on the use of ACN, the first eluted fraction was sufficient to remove the insecticides adsorbed, while larger volumes of solvent were required for ethanol (two fractions) and methanol (three fractions). Considering these results, ACN was chosen for XAD2 column elution of insecticides adsorbed. Concentration of 5% (v/v) ACN was chosen as the optimal incubation environment.

Three diagrams were obtained for inhibition of dichlorvos and paraoxon on the following working conditions:

i. Incubation of the biosensor in 0.1 M PBS, pH = 8;
ii. Incubation of the biosensor in PBS containing 5% (v/v) ACN to observe its sensitivity reduction due to organic solvent.

After the sample preconcentration on XAD2 column, the first elution fraction from the column was diluted with phosphate buffer, divided in two portions, and analyzed with the two different biosensors.

The inhibition diagrams obtained (Fig. 18 and Fig. 19) show a similar behaviour. The inhibition percentages increase with insecticide concentration until a plateau is reached. The difference between the percentages of inhibition of paraoxon and diclorvos is caused by different net toxicity of these insecticides on AChE.

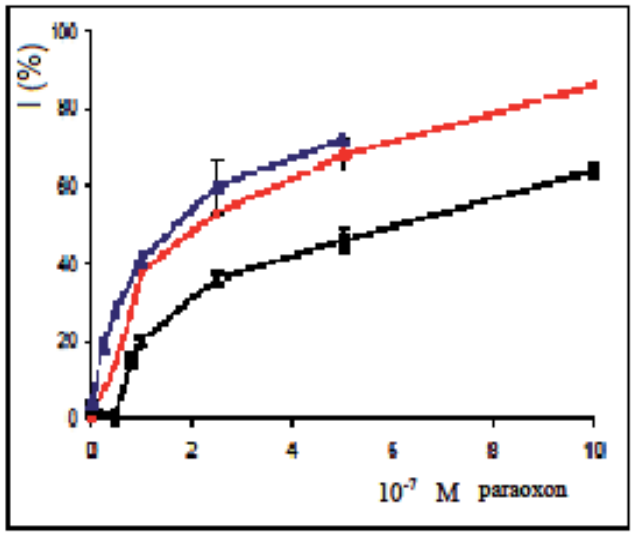


Figure 18. Inhibition curves obtained with the biosensor for paraoxon with AChE immobilized in sol-gel after 10 minutes of incubation in phosphate buffer, phosphate buffer with 5% (v/v) CAN

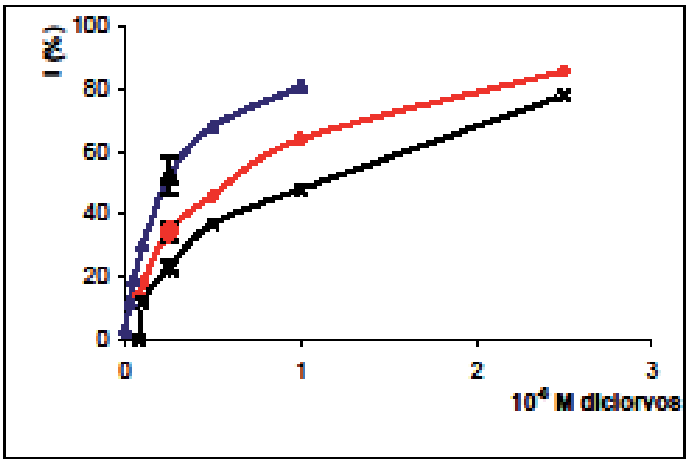


Figure 19. Inhibition curves for dichlorvos obtained with AChE biosensors immobilized in sol-gel after 10 minutes incubation in phosphate buffer, phosphate buffer with 5% (v/v) ACN.

The results are obtained from analysis of the first fraction (0.5 mL) eluted from the XAD2 column with ACN where the insecticide was preconcentrated. Fraction eluted in ACN was diluted to 10 mL with phosphate buffer.

For paraoxon, the calibration curve obtained in PBS is linear up to 10^{-7} M with a detection limit of 5 x 10^{-8} M. Small inhibitory effect of acetonitrile and reduction of inhibition measured with biosensor incubated in 5% (v/v) ACN is visible at low concentrations of insecticide. The inhibition curve obtained after passage of standard insecticide solutions through the column was linear between 2.5 x 10^{-8} (detection limit) and 2.5 x 10^{-7} M.

The results are obtained from analysis of the first fraction eluted with 0.5 mL of ACN on XAD2 column where the insecticide was preconcentrated. Fraction eluted in ACN was diluted to 10 mL with phosphate buffer.

For dichlorvos, the inhibition curve in PBS is linear from 8 x 10^{-8} (detection limit) up to 2.5 x 10^{-7} M. In case of biosensor incubation in standard solutions of dichlorvos prepared in PBS containing 5% (v/v) ACN, the graph is linear between 10^{-7} (detection limit) and 5 x 10^{-7} M.

Using preconcentration stage allows produce a calibration curve at lower concentrations the linear part of which is between 2.5 x 10^{-8} M (detection limit) and 2.5 x 10^{-7} M.

Thus the detection limit for paraoxon and dichlorvos was improved from 5x10^{-8} and 8x10^{-8}M for inhibition measurements conducted in buffer at 2.5 x 10^{-8} and 2.5 x 10^{-8} M after using preconcentration phase.

The method allows detection of organofosforotionate insecticides (P = S) by their oxidation to Oxon form (P = O) which is more toxic.

Heterogeneous oxidation of insecticides was performed using methyl parathion oxidized to methyl paraoxon. Studies performed on biosensors based on AChE inhibition by methyl paraoxon obtained by heterogeneous oxidation of methyl parathion were compared with inhibition studies performed with standard solutions of ethyl paraoxon. This comparison was performed to estimate the effectiveness of oxidation of methyl parathion and denaturating effect of the enzyme produced by the reagent used for oxidation.

Oxidation of organofosforotionate compounds was performed in heterogeneous system. Preliminary studies were conducted to investigate and find the optimal parameters able to minimize the negative effect of the oxidant on the biosensor.

6. Conclusion

The goal of sustainable development is achievable in terms of counteracting the global challenges that humanity must face. These challenges that reinforce each other are: climate change, severe restriction of species biodiversity, including the default value of genetic resources, not fully explored, degradation processes, diminishing freshwater resources, soil erosion and sustainability of life support, the universal generator of food for all livings.

Research topics addressed in this context are part of the suite of scientific research conducted in European and world community regarding the action on the environment of pesticides used in agriculture. Moreover, the research results obtained are turned into account by offering the base of the evaluation of the social and economical impact on the environment and life quality.

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Chapter 7

An Overview About Recent Advances in Sample Preparation Techniques for Pesticide Residues Analysis in Cereals and Feedstuffs

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1. Introduction

Nowadays, more than 1100 pesticides are possibly used in various combinations and at different stages of cultivation and during postharvest storage to protect crops against a range of pests and fungi and/or to provide quality preservation. Pesticide residues in cereals samples, which might pose a potential risk for human health due to their sub acute and chronic toxicity, could possibly end up in the final products of crops. Contaminants of animal feed can cause harmful health effects in the animals and may be harmful to people through secondary exposure of consumers to products deriving from these animals. Contamination of feedstuffs may include both naturally occurring and synthetic toxic compounds [1].

International regulatory agencies have placed emphasis on the control of pesticides such that shall not contain residues of individual pesticides at levels exceeding regulatory maximum residue limits (MRLs), for example, 10 $\mu g\ kg^{-1}$. To analyzed a large number of pesticides in various food commodities consistently remain a challenge for analytical chemists [2].

Pesticides can be analyzed by gas chromatography (GC) with electron capture detection, flame ionization detection, or nitrogen-phosphorus detection and/or liquid chromatography (LC) with ultraviolet, diode array, fluorescence, or electrochemical detection. However, these techniques may lack the selectivity and/or sensitivity required to meet the requirements for analysis of residues due to the complexity of food matrices. These techniques have been largely replaced by GC and LC coupled to mass spectrometric techniques, especially they using tandem mass spectrometry [3].

The aim of this chapter is to present an overview about recent advances in sample preparation techniques that were developed for the determination of pesticide residues in cereals and feedstuffs by gas or liquid chromatographic methods. The different extraction and cleanup procedures were pointed out in this chapter with several applications related to the analysis of cereals and feedstuffs.

1.1. Pesticides residues in cereals and feedstuffs

During cultivation cereals are attacked by a great variety of pests, diseases and weeds. A key challenge to the protection of current production is the emergence of new pests and diseases, in addition to the spread of current diseases. Crop protection through pesticides has made a significant contribution to growth the cereals productivity since the 1950s. However, losses due to pests globally are still high. The extensions of these losses vary between countries and crops, but one estimate suggests an overall loss of around 40 per cent. Another more recent assessment suggests losses of 26 to 29% for soybean and wheat, and 30 to 40% for maize and rice. The same study suggests that losses for wheat could be as high as 50 per cent without effective plant protection, and even higher for other crops. Improved crop protection in the face of new pests and diseases, as well as resistant strains of current diseases, will rely on a variety of approaches. The well-managed use of different classes of pesticides (herbicides, fungicides, insecticides, etc) must continue to play a key role. In face of this, particular attention should be addressed to pesticide residues due to the common use of these compounds in agriculture [4].

Plant protection products may be ingested or absorbed by livestock in three ways: (1) following direct application of the product to the animal, (2) through residues in feeding stuff, (3) as a result of treatment of their accommodation. The usual source of residues is through the legitimate use of pesticides (herbicides, insecticides and fungicides) in the production of crops used in preparation of feeds. The need for information relevant to the conduct of risk profiles or for management of residues will always remain. Published data about pesticides residues on feed are very scattered and not easy to find. The results are not necessarily published and a compilation of feed monitoring data is still in the early stages [1]. The analysis of undesirable contaminants in various food and feed samples is nowadays a problem of primary concern for quality control laboratories due to human and animal health risks associated with the accumulation of these substances. Contaminants in animal feeding stuff can cause harmful health effects in the animals and may be harmful to humans through secondary exposure of consumers to contaminants deriving from these animals. In the European Union and also in several countries, feeding stuffs are subject to legislation covering their composition, manufacture, storage, transport and usage.

1.2. Aspects of analytical methods

In the last years, one of the current trends in analytical chemistry is the method development for optimized many tools used in classical methods. Fast analysis, consumption of small amounts of samples and reagents, high sensitivity and automation are

some of the most important goals desired to be achieved. For many years a large number of research laboratories and analytical instrument manufacturing companies have been investing their efforts in this field, which includes new sample preparation methods and rapid analysis. Nowadays, improved pesticides multiresidue analysis methodologies with high sensitivity and expanded scopes, which include as many compounds and commodities as possible in a single method, are always required for checking compliance with MRLs and/or for risk assessment of consumer exposure to pesticides. Otherwise, the multiresidue method development is difficult due to the fact that compounds of different polarity, solubility and volatility have to be extracted and analyzed simultaneously [5]. In practice, multiresidue methods consist of the following basic steps [6]:

i. isolation of residues from a representative sample (extraction);
ii. separation of co-extracted matrix components (cleanup);
iii. identification and quantification of target analytes (quantitative step), and if the need is important enough, this is followed by next step;
iv. confirmation of results by an additional analysis.

The choice of sample treatment applied depends heavily on the complexity of the matrix. Water, in general, represents a less complicated matrix than air, sediment, soil or food samples. This choice is also related to the detection method. The more sensitive and detection method is used, the less stages of sample treatment will be required. Modern analytical strategies tend towards automatization and integration of sample pretreatment in the chromatographic systems as far as possible. Development of solventless (or at least with low solvent consumption) sample preparation techniques constitute a pillar of green analytical chemistry and have taken a rapid development during last year's. The great interest in this approach is due to toxicological, environmental and economical aspects [7].

For extraction, although different organic solvents, and mixtures of organic solvents, have been used to extract a wide range of compounds with different physico-chemical properties from food, the use of acetone, ethyl acetate, and acetonitrile has predominated in multiresidue methods. These solvents provide high pesticide recoveries over a wide polarity range; however, at the same time a lot of matrix components are co-extracted. To achieve required performance characteristics, cleanup techniques, are commonly employed for their removing. These procedures lead to increasing overall cost of the method, extending analysis time and requiring additional labor [6].

The difficulties of pesticide residue analysis in cereals and animal feed samples are caused by the needed of the elimination of chemically non-related main matrix components (e.g., organic matter, lipids, proteins) and, then, if required, by removal of other chemically related analytes that could interfere in the instrumental determination of the investigated compounds [8]. Feedstuffs are also burdened with large quantities of other components after extraction as animal feeds can be complex mixtures that include constituents such as grains, milling by products, added vitamins, minerals, fats, and other nutritional and energy sources. Even simpler cereal matrices contain much more co-extractants than typical matrices of high water content such as fruits and vegetables [1]. This fosters the

development of strategies to isolate/extract the pesticide fraction from the whole fatty matrix. In fact, it is very difficult to avoid the co-extraction of fatty material, even more, taking into account that some of the pesticides which are usually targeted are fat-soluble non-polar compounds (e.g. organochlorine), and tends to concentrate and remain in the fat. Since, high recoveries of most multiclass pesticides must be obtained in an ideally fat-free extract, an additional clean-up step is usually included prior to subsequent steps in the analytical process. Additionally, the exact composition of the sample is often unknown to the testing laboratory [9].

2. Sample preparation

Despite advances in the sensitivity of analytical instrumentation for the end-point determination of analytes in food samples, a pre-treatment is usually required to extract and isolate the target analytes from the food matrix, thus facilitating their determination [9]. Extraction of pesticides from food depends on their polarity and the type of matrix. Generally, it comprises homogenization of the sample with an organic solvent alone or mixed with water or pH adjusted, using an ultrasonic bath, a blender or a homogenizer [10]. In most cases, although the analytes of interest are isolated from the bulk matrix, several contaminants may also be co-extracted, as well as part of the matrix, which could interfere in the determination step of the analysis. After the extraction process, generally a clean-up procedure is carried out in order to remove the co-extracted compounds that may act as interferences during chromatographic analysis, causing problems in detection and quantitation of the analytes [11]. The clean-up step aims at the isolation of the target analytes from potential interfering co-extractives as well as discarding the extraction solvent and preparing the target analytes in an appropriate chemical form for its characterization and quantification. Therefore, pesticide residue analysis protocols involve two main stages: the isolation of the pesticides from the matrix (sample treatment) and the analytical method for the determination. Sample treatment, which involves both the extraction of the pesticides and the purification of the sample extract obtained, still remains as the bottleneck of the entire procedure, despite much progress on automation has been accomplished [9]. In food analysis, traditional methods for sample preparation are laborious, time consuming and usually involve large amounts of solvents, which are expensive, generate considerable waste, contaminate the sample and can enrich it for analytes. In addition, usually more than one clean-up stage prior to detection is required [12]. As a result, modern sample preparation procedures have been developed or improved to overcome the drawbacks of the traditional approaches. Growing concern over food safety necessitates more rapid and automated procedures to take into account the constant increase in the number of samples to be tested, so interest in procedures that are fast, accurate, precise, solventless, inexpensive and amenable to automation for on-line treatment is ongoing. Today special attention is paid to such analytical sample preparation procedures which ensure reduction of the amount of liquid solvents used or their complete elimination in the course of the analytical procedure. A great increase in interest in the so-called solventless method is the result of

both ecotoxicology (dumping residual solvents, usually highly toxic, into the environment) and economics (high purity solvents are expensive) [12].

Different studies have been described in the literature about the sample preparation and chromatographic determination of pesticide residues in food and feedstuffs and these results are described in this chapter.

2.1. Solid-liquid extraction

The first step in the pesticide residues analysis from semisolid and solid samples is usually the exhaustive extraction of the target compounds from the matrix in which they are entrapped. The essentially non-selective character of this initial treatment makes mandatory the subsequent purification of the obtained extract, first by elimination of matrix [8]. In the last decades, one of the most applied pesticide extraction technique from cereals was solid–liquid extraction (SLE). Before the SLE, solid samples are transformed into fine and homogeneous particles by mechanical grinding, mixing, rolling, agitating, chopping, crushing, macerating, mincing, pressing, or pulverizing. The homogenized solid samples are repeatedly extracted with an immiscible organic solvent, and the extracts are then centrifuged, concentrated and/or purified before the final analysis [10]. An important step in the preparation of food samples prior to final analysis is isolation and/or enrichment. The procedures consist of the transfer of analytes from the primary matrix into the secondary one with a concurrent purging of interfering substances (isolation) and increasing the analytes concentrations to a level above the detection limit for a given analytical technique (enrichment). In the case of organic contaminants, such as pesticides, in cereals samples, it is necessary to replace the solid matrix with a liquid one. For this purpose, an appropriate extraction method should be used. Conventional extraction of organic analytes from food samples usually begins with a homogenization step, followed by solvent extraction aided by shaking is based on the partitioning of analytes between liquid and solid phases [9]. When considering this technique, there are many inherent disadvantages, e.g., it is laborious and time-consuming, expensive and apt to form emulsion, it requires the evaporation of large volumes of solvents and the disposal of toxic and flammable chemicals. Moreover, a relatively large amount of matrix is required. Smaller sample sizes become important when dealing with real life problems, such as consumer complaints and alleged chemical contamination. Recent regulations pertaining to the use of organic solvents have made classical SLE unacceptable because of very large amounts of solvents used in this technique. For these reasons (to reduce the usage of solvents), many innovations can be found in analytical processes that can be applied to food preparation for extraction [13, 14]. This has resulted in the recognition that SLE can now be replaced with faster and less expensive techniques. These new approaches in pesticides residues extraction from cereals and feedstuffs samples were showed in the next reviewed sections.

2.2. QuEChERS

The QuEChERS (quick, easy, cheap, effective, rugged and safe) method was introduced by Anastassiades *et al*. [15] as a new approach to extract a wide range of pesticides from

different food matrices with high water content. This basic procedure is based on a liquid partitioning with acetonitrile followed by a dispersive solid phase extraction (d-SPE) cleanup with primary secondary amine (PSA). This procedure has been applied with success in several nonfatty (<2%) and low-fat (2–20%) food matrixes. In this method anhydrous magnesium sulphate is use to reduce water in the sample, along with either sodium chloride [16].

Since 2003, modifications to the original method to ensure efficient extraction of pH dependent compounds (by using different buffers solutions, e.g. acetate or citrate) [17, 18] or addition of water to dry samples (e.g. cereals) in order to obtain the necessary moisture have been introduced [19]. To remove matrix components in the clean-up step, modifications of the original d-SPE step by using graphitized carbon black (GCB) and C18 sorbent, SPE in cartridge or Florisil cartridges have been used. The QuEChERS method is particularly popular for determination of polar, middle polar and non-polar pesticide residues in various food matrices because of its simplicity, inexpensiveness, amenability to high throughput, and relatively high efficiency results with a minimal number of steps, enabling a laboratory to process significantly larger number of samples in a given time as compared to the earlier methods, e.g. liquid-liquid extraction (LLE) methods [7, 20]. QuEChERS offers several advantages over most conventional techniques because it does not require glassware or auxiliary equipment (e.g. vacuum manifolds), uses low volumes of solvent, generates little solvent waste and provides high recovery of analytes [21]. When compared with other sample preparation methods, it is clear that it is extremely fast and inexpensive. It has already received world wide acceptance because of its simplicity and high through put enabling a laboratory to process a high number of samples in a short period of time [20].

The Table 1 summarizes the results described below and another applications based on QuEChERS extraction method for pesticides residues determination in cereals and feedstuffs.

Walorczyk [22] employed a buffered QuEChERS method to prepare samples of cereals grain and some dry feedstuffs prior to the determination of 122 pesticides by GC-MS/MS. A 5 g finely ground sample was placed in centrifuge tube and 10 mL water were added. Later, 15 mL acetonitrile were added and the mixture shaken vigorously. Further, 0.5 g disodium hydrogen citrate sesquihydrate, 1 g trisodium citrate dihydrate, 4 g anhydrous magnesium sulphate, and 1 g sodium chloride were added, and the mixture was hand-shaken, then centrifuged. Afterwards, a 7.5 mL aliquot of the supernatant was transferred to a centrifuge tube (15 mL) containing 0.75 g anhydrous magnesium sulphate, 0.5 g C18 and 0.125 g PSA. The tube was vortexed and centrifuged. A 3 mL aliquot of the supernatant was transferred into a glass test tube, the extract was evaporated to nearly dryness under a stream of nitrogen and the residue was re-dissolved in 1.5 mL toluene prior to its injection into the GC-MS/MS system. Despite the fact that the method was found to be useful for the purpose of multiresidue screening as it permitted detection at low levels (0.01 mg kg^{-1}) for approximately 68% of the target pesticides, many recoveries and RSD values were not as good as required for validation in compliance with the criteria established by the European Union.

Analytes	Sample	Analytical technique	LOD (μg kg^{-1})	Recovery (%) (RSD %)	References
140 multiclass pesticides	wheat, bran, feedstuffs	GC-MS/MS	-	70.0-120.0 (≤ 20)	[1]
24 multiclass pesticides	wheat, bran, flour	GC-MS	2.5	70.0-120.0 (≤ 20)	[18]
122 multiclass pesticides	wheat, bran, feedstuffs	GC-MS/MS	≤ 10.0	73.0-129.0 (1.0-29.0)	[22]
80 multiclass pesticides	wheat flour	GC-MS/MS and LC-MS/MS	-	70.0-110.0 (≤ 10)	[23]
171 multiclass pesticides	wheat flour	LC-MS/MS	-	70.0-120.0 (≤ 20)	[24]
simeconazole enantiomers	wheat and rice	GC-MS/MS	0.4-0.9	101.1-93.7 (2.4-5.7)	[25]
13 phenoxyacid pesticides	rice	LC-MS/MS	≤ 13.3	45.0-104.0 (≤ 13.3)	[26]
180 multiclass pesticides	corn, oat, rice, wheat	GC-TOF-MS and UHPLC-MS/MS	-	70.0-120.0 (≤ 20)	[27]
41 multiclass pesticides	maize	GC-MS	8.0-55.0	70.0-120.0 (≤ 20)	[28]
90 multiclass pesticides	wheat	LC-MS/MS	-	70.0-120.0 (≤ 20)	[29]
42 multiclass pesticides	polish rice	LC-MS/MS	0.07-4.0	70.0-120.0 (≤ 20)	[30]
151 multiclass pesticides	barley, basmati rice, rice flour, popcorn, wheat, seven grains, and buckwheat	UHPLC-MS/MS	-	81.0-110.0 (≤ 20)	[31]
23 multiclass pesticides	cereal based infant foods	LC-MS/MS	-	60.4-125.4 (≤ 29.7)	[32]
9 organophosphate and 1 pyrethroid	maize and soy	GC-MS	0.2-6.0	105.0-52.0 (4.6-8.2)	[33]
7 neonicotinoid pesticides	brown rice, millet, oat, maize	HPLC-DAD	2.0-5.0	76.0-123.0 (0.9-12.6)	[34]

Table 1. Different methods applied for determination of pesticide residues in cereals and feedstuffs based on QuEChERS extraction method.

In a new work Walorczyk [1] improved his previous method. The most important aspects considered during the present work were (i) extension of the scope of the previous method to include as many as 144 target analytes and (ii) re-design of the GC-MS/MS acquisition method. The linearity of the calibration curves was excellent in matrix-matched standards, and yielded the coefficients of determination $r^2 \geq 0.99$ for approximately 96% of the target analytes. Average recoveries of the pesticides spiked at 0.01 mg kg^{-1} into a feed mixture and wheat grain were in the range 70–120% with associated RSD values ≤ 20% for approximately 60 and 67% of the compounds, respectively. Base on these results, the proposed approach has been proven to be highly efficient and suitable for routine determinations of multiclass pesticides in a range of cereal and related matrices. In this study, 145 samples of matrices of differing complexity including cereals grain, bran, whole ears, straw, hay, feed mixtures and other samples such as malt, starch and dry vegetables have been analyzed. A total of 15 different compounds have been detected, among which pirimiphos methyl, deltamethrin, tolylfluanid, dichlofluanid and tebuconazole were the most frequently encountered ones.

Kolberg *et al*. [18] developed and validated a multiresidue approach also based on QuEChERS method for the determination of 24 pesticides in wheat, white flour and bran using triplequadrupole GC-MS in negative chemical ionization mode. The method was validated evaluating the following parameters: linearity, limit of detection, limit of quantification, matrix effect as well as precision and accuracy, evaluating the percentage of recovery at four different spike levels. The linear range used in the calibration curves was from 1.0 to 100 μg L^{-1} for wheat and 2.0 to 200 μg L^{-1} for flour and bran, both with values of $r^2 \geq 0.99$. The recoveries had been considered satisfactory presenting values between 70 and 120% with RSD < 20% for the majority of compounds.

Koesukwiwat *et al*. [26] modified the original QuEChERS method for the analysis of phenoxy acid herbicide residues in rice samples. The new approach was based on the extraction with 5% (v/v) formic acid in acetonitrile and inclusion of citrate buffer for helped partitioning of all the analytes into the acetonitrile phase. The extract was then cleaned up by d-SPE using C18 and alumina neutral as selective sorbents. Further optimization of sample preparation and determination allowed recoveries between 45 and 104% for all 13 phenoxy acid herbicides with RSD ≤ 13.3% at 5.0 μg kg^{-1} concentration level. Limit of detections (LODs) of 0.5 μg kg^{-1} or below were attained for all 13 phenoxy acids. Quantitative analysis was done by UHPLC-MS/MS in the multiple-reaction monitoring (MRM) mode using two combinations of selected precursor ion and product ion transition for each compound. This developed method when compared with original QuEChERS method produced relatively higher recoveries of the acid herbicides with a smaller range of variation and less susceptibility to matrix effects.

Otherwise, the main disadvantage of the QuEChERS method compared to other common methods is that the 1 g mL^{-1} final extract concentration is lower than the 2–5 g mL^{-1} concentrated extract of most traditional methods. If matrix is not the limiting source of noise in the analysis, this leads to a higher LOQ for the same injection volume in the QuEChERS method [3]. This method is adaptable and can be easily tailored to cope with new matrices

through the selection of alternative sorbents. In fact the initial extract can be divided across tubes containing different sorbents to cater for problem analytes. Work in progress indicates that the developed extraction conditions will recover the majority of food contaminants [35]. Although QuEChERS has mainly been used for the determination of pesticides, some other compounds, such as pharmaceuticals, or veterinary drugs have been determined using QuEChERS in other food and environmental samples [7, 36].

2.3. Pressurized-liquid extraction (PLE)

Also known as accelerated solvent extraction (ASE), pressurized fluid extraction (PFE), pressurized hot solvent extraction (PHSE), subcritical solvent extraction (SSE) and hot water extraction (HWE), the PLE utilizes solvents that are raised to the near supercritical region, where they show better extraction properties [37]. This effect is due the decreasing of surface tension and increasing of solubility and diffusion rate into the sample showed by the solvent at high temperatures. Pressure keeps the solvent below its boiling point and, in solid samples, like cereals, forces its penetration into the pores of the sample [12].

The faster extraction process (5-10 min) is obtained by the use of high pressures (500-3000 psi) and temperatures (50-200 °C). This technique requires smaller amounts of solvent compared with traditional extraction, this is a very positive advantage because reduces the dilution of the sample [38]. The main factor that's affects de extraction time and efficiency of extraction is the temperature meanwhile the sample mass [12]. There are three ways to perform PLE: static, dynamic (flow-through) and mixed mode. In static mode, the sample is enclosed in a stainless steel vessel filled with an extraction solvent, and following extraction the remaining solvent is purged with N_2 into a collection vial. Flow-through systems continuously pump solvent through the sample, but this has the disadvantage of using larger volumes of solvent and of diluting the extract. A desiccant, such as sodium sulphate, diatomaceous earth or cellulose can be added directly to the extraction cell or sorbent materials can be used to provide in situ cleanup [39, 40]. When compared with LLE, the PLE show as main advantage the low solvent consumption, but requires highly expensive and specialized equipment. The limitation of PLE is the presence of large amounts of co-extracted lipids when working with fatty matrices, which means that post-cleanup of the extract is required to carry out lipid elimination [12].

The effect of sample matrix depends on sample composition. Food samples can differ significantly in their physical-chemical properties, type of compounds present, or granulation (particle diameter). The factor that mainly affects the extraction of trace compounds in the presence of extractable major sample components are lipids, that offers special problems in the subsequent determination by gas or liquid chromatography [41].

Carabias-Martinez *et al*. [37] developed a method based on pressurized liquid extraction and liquid chromatography–electrospray ionization mass spectrometry (LC-ESI-MS) for the determination in cereal samples of seven endocrine-disrupting compounds: bisphenol A (BPA), 4-tert-butylbenzoic acid (BBA), 4-nonylphenol (NP), 4-tert-butylphenol (t-BP), 2,4-

dichlorophenol (DCP), 2,4,5-trichlorophenol (TCP) and pentachlorophenol (PCP). Methanol was selected as the extraction solvent. The recoveries achieved for the all seven compounds were in the 81 to 104% range, with relative standard deviations of 4 to 9%. The detection limits achieved in corn breakfast cereals were in the 0.003 to 0.043 μg g^{-1} range.

A method based on pressurized liquid extraction and LC-MS/MS has been developed for determining nine benzoylureas in fruit, vegetable, cereals and animal products. Samples (5 g) were homogenized with diatomaceous earth and extracted in a 22 mL cell with 22 mL of ethyl acetate at 80 °C and 1500 psi. Method LOQs were between 0.002 and 0.01 mg kg^{-1}. Excellent linearity was achieved over a range of concentrations from 0.01 to 1 mg kg^{-1}. Validation of the method was performed in seven different commodities (milk, eggs, meat, rice, lettuce, avocado, and lemon). The recoveries ranged from 58 to 97% and the RSDs from 5 to 19% [42].

In order to extract eight acetanilide herbicides from cereal crops, Zhang *et al*. [43] evaluated the effect of four parameters for PLE efficiency (temperature, static time, static cycles and solvent). The results of final method, based on accelerated solvent extraction (ASE) and solid-phase extraction (SPE) for cleanup using graphitized carbon black/primary secondary amine (GCB/PSA), GCB, Florisil and alumina-N were compared with shake-flask extraction method. At 0.05 mg kg^{-1} spiked level, recoveries and precision values for rice, wheat and maize were from 82.3 to 115.8% and from 1.1 to 13.6%, respectively. For all the herbicides, LOD and LOQ ranged from 0.8 to 1.7 μg kg^{-1} and from 2.4 to 5.3 μg kg^{-1}, respectively.

2.4. Supercritical-fluid extraction (SFE)

From 1960, when applications of supercritical fluid extraction have been extensively examined until 90 years, few researchers investigated the use of supercritical fluids in analytical, non-chromatographic applications. After that, commercial supercritical fluid extraction (SFE) instruments have been developed and the studies began to be published using SFE as extraction technique in combination with chromatographic techniques for analytical applications for analysis of pesticide residues [44, 45].

The unique properties exhibited by supercritical fluids have been applied for the analysis of pesticide residues in solid samples SFE is selective and Less solvent consuming, thus it is environmental friendly. The critical step in the off-line SFE methods is evaporation of solvent at the end of extraction to acquire high pre-concentration factor. This procedure is time-consuming and can contaminate the environment. Another negative factor is loses or degradation of collected analytes [46].

Carbon dioxide (critical conditions = 30.9 ºC and 73.8 bar) is the most used supercritical solvent, because is cheap, environmentally friendly and safe. Supercritical CO_2 ($SCCO_2$) had high diffusivity and easily tuneable solvent strength. Another advantage is that CO_2 is gaseous at room temperature and pressure, which makes analyte recovery very simple and provides solvent-free analytes. Also, important for food and natural products sample preparation, is the ability of SFE using CO_2 to be operated at low temperatures using a non-oxidant medium,

which allows the extraction of thermally labile or easily oxidized compounds. The main drawback of $SCCO_2$ is its low polarity, and some modifiers as required to change the polarity of supercritical fluid and increase de solvent power. The modifiers can also reduce the analyte–matrix interactions improving their quantitative extraction [45].

In a typical commercial equipment, the fluid is pumped, at a pressure above its critical point, with the sample placed in an inert extraction cell. The temperature of the cell is increased to overcome the critical point of the fluid. After depressurization, the analytes are collected in a small volume of organic solvent or on a solid-phase filled cartridge (solid adsorbent trap). Extraction can be performed in three ways: static, dynamic or recirculating (mixed) mode: in the static extraction mode, the cell containing the sample is filled with the supercritical fluid, pressurized and allowed to equilibrate; using the dynamic mode, the supercritical fluid is passed through the extraction cell continuously; finally in the recirculating mode the same fluid is repeatedly pumped through the sample and, after the required number of cycles, it is pumped out to the collection system [47].

SFE is usually an efficient extraction method, primarily applicable to solid samples. However, as well as its numerous advantages (efficacy, selectivity, short extraction times, low solvent volumes) it also has serious drawbacks: difficult optimisation, high apparatus and maintenance cost, high blank and noise levels, co-extraction of lipidic content of samples [48].

With commercially equipments available SFE has been used not only for analytical applications in samples preparation, but mostly for food science, pharmaceutical and environmental science in lab or pilot scale [45]. Recently, only a few applications describing sample preparation for determination of pesticides in cereals were published.

Aguilera et al. [49] used the SFE method followed by a cleanup step with aminopropyl SPE for the analysis of 22 pesticides in white and wild rice. The authors tested different experimental conditions for extraction with CO2: temperature, volume and pressure in order to optimize the extraction method. The authors noticed that fat extraction from rice was appreciable and the use of higher pressures might also increase extraction of fat and other non-fat material in this matrix. For the final procedure validated pesticide mean recoveries obtained from rice samples, at fortification levels around 0.5 mg kg^{-1}, ranged between 74 and 98%, except for captafol and dimethoate for which mean recoveries lower than 21% were determined.

2.5. Solid-phase extraction (SPE)

Solid-phase based extraction techniques are widely applied to many matrices, like foods and include: matrix solid-phase dispersion (MSPD), solid-phase extraction (SPE), solid-phase microextraction (SPME) and stir-bar sorptive extraction (SBSE). In this technique a sorbent will retain and concentrate some target analytes from the sample solution due strong affinity between sorbent and analytes [12]. Solid-phase extraction involves the use of disposable cartridges and disks to trap analytes. As the sample solution passes through the activated sorbent bed, analytes concentrate on its surface, while the other sample

components pass through the bed or vice versa, if necessary for cleanup [50]. When compared with LLE, the advantages of SPE are: simultaneous removal of interfering substances and concentrations of analytes, multiple samples can be treated in parallel and the use of a relatively small quantity of solvent [12]. Before SPE can be applied to a solid matrix (soil, vegetables and fruits), a separate homogenization step and, often, filtration, sonication, centrifugation, and liquid/liquid cleanup are required. However, the presence of interfering substances, such as salts, humic acids, and other humic substances in water or proteins, lipids and carbohydrates in food makes the determination of polar or early-eluted pesticides, difficult or almost impossible [51].

An analytical method for the determination imazaquin residues in soybeans was proposed by Guo *et al*. [52] based on liquid/liquid partition strong anion exchange solid-phase extraction. This technique was used, in order to achieve an effective cleanup, removing the greatest number of sample matrix interferences. In this procedure, the combination between optimized chromatographic conditions and detection by ultraviolet the procedure was showed to be sensitive and reliable for determining the imazaquin residues in soybean samples. This method is characterized by recovery >88.4%, precision 6.7% RSD, and sensitivity of 0.005 mg kg^{-1}. The proposed method was successfully applied to the analysis of imazaquin residues in soybean samples grown in an experimental field after treatments of imazaquin formulation.

A sensitive and simple method for simultaneous analysis of acetochlor and propisochlor in corn and soil has been developed by Hu *et al*. [53]. The extraction of pesticides from soil and corn matrices was performed with methanol/water and acetone, respectively, followed by solid phase extraction (SPE) to remove co-extractives, prior to analysis by gas chromatography with electron capture detection (GC-ECD). Primary secondary amine (PSA) SPE cartridges (500 mg, 3 mL) were used for sample preparation. The elution was made with 5 mL petroleum ether-acetic ether (95/5, v/v) and 3 mL petroleum ether-acetic ether (95/5, v/v), respectively. The recoveries of two pesticides ranged from 73.8% to 115.5% with relative standard deviations (RSD) less than 11.1% and sensitivity of 0.01 mg kg^{-1}. The method was successfully applied to determine acetochlor and propisochlor in real corn and soil samples. The authors related that residues of acetochlor and propisochlor residues were detected (<0.01 mg kg^{-1}) in corn at harvest time with holding period of 2.5 months after treatments of the pesticides.

The use of SPE in combination with HPLC-DAD was employed to determine bispyribac-sodium residues in rice. The liquid–liquid partition and anion exchange solid phase procedures that were developed provide effective extraction and cleanup methods for analysis feasibility, with recoveries between 83.98 to 98.51% with a RSD from 0.56 to 6.36% and sensitivity of 0.01 mg kg^{-1}, with main advantages of high precision, accuracy and good selectivity. Another favorable feature is the reduction of sample processing time [54].

2.6. Matrix solid-phase dispersion (MSPD)

In 1989, Barker *et al*. [55] introduced the MSPD, as a sample preparation technique for solid or semi-solid samples. It provides both a porous structure to enable the solvent to penetrate

the matrix and extract the analytes, but also has some functionality which can retain the fat/lipids [56]. MSPD can combine the procedures of homogenization, disruption, extraction and cleanup into one simple process. In fact, it is a sample preparation strategy that consists of a manual blending of samples with a bulk dispersing agent, to produce complete disruption of the original matrix structure, thus providing an enhanced surface area for subsequent sample extraction. Usually, the blended material is then transferred and packed into a column to perform sequential extraction and eventual cleanup with an appropriate solvent or a sequence of solvents [57].

Most applications have utilized 5–10 mL of solvent to perform analyte extraction. Evidence from some studies indicates that most target analytes are eluted in the first 4 mL of extractant, provided that 0.5 g of the sample is mixed with 2 g of the solid support [9]. In general, the choice of the adsorbent used depends on the polarity of the analyte and on the possible coextracted components of the matrix. The most usual adsorbents are Florisil and C18; nevertheless, there are others (e.g., diatomaceous earth, alumina, silica and C8) that have been used for the analysis of pesticides in food [11].

The main advantages of MSPD procedure compared to other extraction techniques are as follows: (1) the analytical protocol is simplified and shortened; (2) the possibility of emulsion formation is eliminated; (3) solvent consumption is substantially reduced; and (4) the extraction efficiency of the analytes is enhanced as the entire sample is exposed to the extractant. An interesting feature of the MSPD technique is that it can be used for extracting analytes from both solid and liquid foods. The main disadvantage is the lack of automation of the procedure [9].

Tsochatzis *et al*. [58] development and validation of such a method for the determination of eight rice pesticides penoxsulam, tricyclazole, propanil, azoxystrobin, molinate, profoxydim, cyhalofop-butyl and deltamethrin) and 3,4-dichloroaniline, the main metabolite of propanil. Pesticide extraction and cleanup was performed by an optimized MSPD protocol on neutral alumina (5 g) using acetonitrile as the elution solvent. Samples were analyzed by high-performance liquid chromatography with diode array detection (HPLC-DAD). Method validation was performed by means of linearity, intra-day accuracy, inter-day precision and sensitivity. Linear regression coefficients (r^2) were always above 0.9948. Limits of detection (LOD) and quantification (LOQ) varied from 0.002 to 0.200 mg kg^{-1} and 0.006 to 0.600 mg kg^{-1}, respectively. Recoveries were investigated at three fortification levels and were found to be acceptable (74–127%) with relative standard deviations (RSD) below 12%. Application of the method for the analysis of five commercial rice grain samples showed that the pesticide levels were below the LOD.

Michel and Buszewski [58] proposed MSPD for extraction of carbendazim residue from wheat grain and determination by column-switching HPLC-DAD. For extraction, a representative portion (200 g) of sample grain was transferred into a mill, and sample material disintegrated by high speed blending. A subsample of 5 g was weighed into a mortar of ca. 10 cm of diameter, 10 g of acidic silica gel was added and ground to obtain a homogeneous mixture. The extraction column was fitted with polyethylene frit, the

powdery sample was transferred through a wide mouth polypropylene funnel (10 cm top i.d.). Mortar and pestle were rinsed with 20 mL of methanol-dichloromethane (1:5, v/v), and the rinsings were carefully poured into the column. The carbendazim residues were extracted with total volume of 120 mL eluent and collected in round-bottomed flasks. The mean recovery rate for fortified samples was 87.3% with a RSD of 2.9%. The achieved method LOQ was 0.04 μg g^{-1}. The method was applied to the determination of carbendazim residues in wheat grain samples from a treated field.

MSPD method have been published also for determination of other residues and contaminants e.g. mycotoxins in cereal samples. Rubert *et al*. [60] reported a reliable and rapid method for the determination of 14 mycotoxins in flour samples (with different cereals composition). MSPD was performed weighing 200 g which were prepared using a food processor and mixed thoroughly. Portions of 1 g were weighed and placed into a glass mortar (50 mL) and were gently blended with 1 g of C18 for 5 min using a pestle, to obtain homogeneous mixture. The homogeneous mixture was introduced into a 100×9 mm i.d. glass column, and eluted dropwise with 20 ml of acetonitrile/methanol (50/50, v/v) 1 mM ammonium formiate by applying a slight vacuum. In a total of 49 samples investigated, 9 mycotoxins were identified. Nivalenol and beauvericin were the mycotoxins found most frequently in the studied samples. The samples that presented major contamination were wheat flours and bakery preparations.

Juan *et al*. [61] determinate ochratoxin A (OTA) in organic and non-organic cereals and cereal products from Spain and Portugal. A method based on extraction with matrix solid phase dispersion (MSPD) using octylsilica (C8) followed by liquid chromatography coupled with fluorescence detection (LC–FD) was used to determine OTA from the selected samples. Recoveries of OTA from the studied samples spiked at 10 ng g^{-1} level ranged from 78 to 89% with a RSD of 3.7%. The limits of detection and quantification of this method were 0.05 and 0.19 ng g^{-1}, respectively. Furthermore, LC–FD after OTA methylation was used to confirm the identity of OTA in all positive samples. This procedure was applied to 83 organic and non-organic samples including rice, wheat, barley, rye, oats and maize from Spain and Portugal.

2.7. Gel-permeation chromatography (GPC)

In the last years polystyrene divinylbenzene type gel with mixture of elution solvents as ethyl acetate and cyclohexane has been used in pesticide residue analysis of cereals samples taking advantage of GPC to isolate pesticides from co-extractants with higher molecular weight (600–1500 g mol^{-1}). GPC is able to separate the pesticides from lipids, waxes and other low volatile larger non-polar co-extractives. Generally, in pesticide residue analysis in fatty food, addition of sulfuric acid or additional SPE cleaning (avoiding decomposition of several pesticides occurring in the former case) are desirable to offer satisfactory protection for the gas chromatographic capillary column [3].

Regarding analysis of pesticide residues in cereals and feedstuffs, Lee *et al*. [62] developed a method for the analysis of 106 compounds in cereal-based animal feed applying ethyl

acetate extraction, GPC and d-SPE cleanup steps, additionally determination by comprehensive two-dimensional gas chromatography (GC×GC) with TOF-MS detection in the full scan mode. The method was demonstrated to give recoveries between 70 and 110% and RSDs below 20% at two levels of 0.01 and 0.1 mg kg^{-1} for the majority of the studied compounds. Zhang *et al*. [63] used GPC for the determination of pesticides in unpolished rice. GPC provides ideal cleanup for high molecular weight components. Otherwise, low molecular weight components may result in serious interferences that can affect the final result.

GPC is highly effective as a post-extraction cleanup procedure in removing interferences with high molecular weight (e.g. lipids, proteins and pigments) prior to the analysis by GC or LC. GPC also increases analytical precision and accuracy. It also ex-tends the column life and lifetime of the instrument. This technique can provide very good results. However, GPC, use large columns, need long analysis times and large amounts of solvents. Some pesticides with high molecular mass (e.g. pyrethroids) need not be sufficiently separated from wide elution band of co-extractants that can result in lower recoveries [64].

3. Instrumental identification and quantification techniques

The two main analytical techniques used in food analysis are gas chromatography (GC) and liquid chromatography (LC), which allow identification and quantitation of pesticide residues in complex matrices.

GC is combined with different kinds of detection methods, mainly depending on the class of pesticides to be quantified [48]. Electron capture detection (ECD), nitrogen-phosphorus detection (NPD), flame-ionization detection (FID), flame-photometric detection (FPD) and mass-selective detection (MSD) have been employed for pesticide residue determination in cereal samples. GC-MS is also frequently used for determination pesticide residues in cereal samples and can be done by electron impact (EI), and Positive or negative chemical ionization (PCI, NCI). The Table 2 summarizes details of several methods developed for the determination of pesticide and others analytes in different cereal samples.

LC has been used for the analysis of polar and/or non-volatile and/or thermally labile pesticides for which GC conditions were not suitable [65]. Most used detectors are UV, diode array detector (DAD) and MSD, as we can see the Table 2. Traditional UV detectors and DADs are often less selective and less sensitive than GC instruments but, in recent years, the commercial availability of atmospheric pressure ionization (API), in combination with MS instruments, has increased the sensitivity by several orders of magnitude [66]. LC-MS/MS applications reported for the analysis of pesticides residues in cereal samples are performed with two different ionization techniques: electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI). Although ESI is the more often used when compared with APCI source.

Analytes	Sample	Extraction Procedure	Cleanup	Analytical technique	LOQ (μg kg^{-1})	Recovery (%) (RSD %)	References
bisphenol A (BPA), 4-tert-butylbenzoic acid (BBA), 4-nonylphenol (NP), 4-tert-butylphenol (t-BP), 2,4-dichlorophenol (DCP), 2,4,5-trichlorophenol (TCP) and pentachlorophenol (PCP)	breakfast cereals	PLE	-	LC–ESI-MS	3.0 – 43.0	81 - 104 (4 - 9)	37
benzoylureas	fruit, vegetable, cereals and animal products	PLE	-	LC-MS/MS	2.0 – 10.0	58 - 97 (5 - 19)	42
acetanilide herbicide	rice, wheat and maize	PLE	SPE	GC-ECD	2.4 - 5.3	82.3 - 115.8 (1.1 - 13.6)	43
22 pesticides	white and wild rice	SFE	SPE	GC-ECD GC-TSD	-	74 98	49
acetochlor and propisochlor	Soil and corn	methanol/water/SPE		GC-ECD	10.0	73.8 - 115.5 (< 11.1)	53
Bispyribac sodium	rice	liquid–liquid partition and anion exchange solid phase		HPLC-DAD	10.0	83.98 - 98.51 (0.56 - 6.36)	54
Penoxsulan, tricyclazole, propanil, azoxystrobin, molinate, profoxydim, cyhalofop-butyl, deltamethrin and 3,4-dichloroaniline (the main metabolite of propanil)	rice	MSPD		HPLC-DAD	6.0 - 600.0	74 – 127 (< 12)	58
carbendazim	wheat grain	MSPD		HPLC-DAD	40.0	87.3 (3.3)	59

Table 2. Different methods applied to the determination of pesticide and others analytes in different cereal samples.

4. Conclusions

Although detection equipment's are becoming more specific and sensitive, there is still a need for efficient sample preparation methods for pesticides residues analysis in cereals and feedstuffs, which are compatible with modern analytical techniques. Despite the advances in separation and detection of the chromatographic systems, cleanup remains important for obtaining reliable data. There is still a need for effective, environmentally friendly and fast methods for sample treatment and determination of pesticide residues in fatty food matrices e.g. cereals and feedstuffs. Modern sample preparation techniques should be not only simple, reliable, cheap and take into account chemical laboratory waste problems, but also

must be similar to common analytical techniques, in order to minimize errors. For these reasons, modern trends in analytical chemistry are towards the simplification and miniaturization of sample preparation, and the minimization of sample size and organic solvent used. The development of such procedures combined with modern chromatographic-mass spectrometric techniques will enable analysis at the low levels now required by legislation for many pesticides, but more importantly, result in methods which produce more reliable data to support food safety monitoring programs. This is the trend although it is impossible to develop a unique protocol covering such a wide range of compounds. Finally, multiclass multiresidue methods covering large number of pesticides are highly desirable, although the different nature, classes and physico-chemical properties of pesticides hamper the development of such methods.

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Chapter 8

Recent Developments and Applications of Microextraction Techniques for the Analysis of Pesticide Residues in Fruits and Vegetables

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Additional information is available at the end of the chapter

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1. Introduction

Analysis of pesticide residues and other contaminants in fruit and vegetable samples is becoming increasingly important due to the health hazards caused by their accumulation in human tissue. The body requires some important nutrients which can be provided by the consumption of fruits and vegetables. The purpose of any analytical study is to obtain information about substances and analytes present in the sample. Analytical process involves several steps: sampling, sample preparation, separation, quantification and data analysis [1]. Sample preparation is a very important step and indeed the bottleneck of analytical methodologies, in the analysis of fruits and vegetables for the presence of pesticide residues in fruit and vegetable samples [2].

The first step in any instrumental analysis is sample preparation, which involves the isolation or extraction of the desired analytes from the sample matrix, since they are present at trace concentration (usually μg.kg or less). This helps in the elimination of any interferences and also reduces the volume of extracts, thereby concentrating the analytes [2]. The type, nature and composition of sample and the nature and concentration of analytes to be isolated or extracted determines the choice of separation and detection method to be used, and this also dictates the type of sample preparation to be employed [3, 4], since the efficiency of any analysis is determined by the sample preparation step. The current trend of microextraction techniques is aimed at a reliable and accurate analysis of contaminants from complex samples. It is focused on the reduction of sample and solvent volume, with the automation/coupling of the sampling step to the analytical instruments, while maintaining the high throughput performance, low cost operation and improvement of the sample preparation, such as extraction, concentration, isolation of analytes, and clean-up [5, 6].

The conventional solvent-based sample preparation methods: liquid-liquid extraction (LLE), solid phase extraction (SPE), accelerated solvent extraction (ASE), matrix solid phase dispersion (MSPD) [7-11], usually require various matrix pretreatment steps, which are tedious, time consuming, contains multistep techniques, require large volumes of sample and toxic solvents which impose environmental pollution and health hazards with high operation cost [5]. Therefore it is necessary to reduce the number of sample preparation steps in order to reduce the sources of error. Microextraction techniques are recently developed sample preparation methods which are vital as well as effective and efficient ways to save time, reduce solvent use and operation cost, and can efficiently measure the trace analytes embedded with high molecular mass compounds in complex sample matrices [12].

The microextraction techniques have been developed by different researchers, to corroborate the recent advances in the development of highly sensitive and efficient analytical instrumentation. Instrumental techniques like gas and liquid chromatography and capillary electrophoresis which are compatible with the microextraction technique, and coupled to different detectors (mass spectrometry, diode array detector, ultraviolet detector, *etc*), have been developed for qualitative and quantitative analysis of pesticide residues and other contaminants from foods. Prior sample preparation is necessary in order to extract, isolate and concentrate the analytes of interest from the complex fruits and vegetables matrices, which contain high molecular mass compounds. The low cost and ease of hyphenation of these microextraction techniques to analytical instruments helps to reduce errors, due to contamination and sample losses.

In this review, recent advances in different microextraction techniques used in the analysis of pesticide residues from fruit and vegetables are discussed, with a major focus on their methodologies, advantages, limitations and future trends.

2. Solid phase microextraction (SPME)

Solid phase microextraction, a solvent-free sample preparation technique, was developed by Pawliszyn and his co-workers in 1990 [13], and its applications has been examined, optimized and automated [14, 15]. It eliminates the need for solvents and combines sampling, isolation, concentration and enrichment in one step and offers the benefit of short sample preparation step, solvent-free extraction, small sample volumes and analyte concentration from solid, liquid or gaseous samples. It employs a chemically inert fused-silica optical fiber or metal alloys (Fiber SPME) coated on the outside with a thin film of sorbent [14] and a thin film internally coated fused silica capillary column (In-tube) [5, 16], as the extraction stationary phase, containing a polymeric organic compound. The fiber SPME has been widely used for the extraction of pesticide residues in fruits and vegetable samples, and will be discussed extensively in this review.

There are two major types of extraction modes used in the analysis of pesticides residues in fruit and vegetable samples. These are the headspace SPME (HS-SPME) and direct immersion SPME (DI-SPME). [5, 17-19]. The efficiency of each extraction mode is dependent

on the sample matrix composition and the nature and volatility of the analytes. In the headspace sampling mode, the analyte is transported through a layer of gas before reaching the coating. The HS-SPME involves the exposure of the fiber to the vapour phase above a liquid or solid sample [5], where the analytes are extracted from the gas phase equilibrated with the sample matrix [20]. It could be effectively used for the extraction of analytes from complex liquid or solid samples [21]. This method helps to protect the coated fiber from the effects of any non-volatile high molecular weight compounds in the sample matrix, which binds irreversibly to the coating and often cause interference in the extraction process. In the DI-SPME, the coated fiber is completely inserted into the samples, where the transport of the analyte from the sample matrix into the extracting phase is achieved [22]. It is used for the extraction of gaseous or simple liquid samples.

The DI-SPME can also be applied for the extraction of analytes from complex samples, provided optimum dilution is achieved [18]. The HS-SPME is suitable for the extraction of volatile or semi-volatile and low-to medium-polar analytes, but the headspace volume should always be taken into consideration. The DI-SPME is used for the extraction of non-volatile analytes or analytes with low volatility and high-to-medium polar analytes [18, 21]. The advantage of HS-SPME over DI-SPME is the shorter equilibrium time, due to the higher diffusion coefficients in gaseous state, higher concentration of the analytes in the headspace prior to extraction and also allows for the variation of sample matrix properties without any effect on the fiber. Extraction mode is an important parameter which should be considered and optimized in the experimental design (described later) of SPME for a particular analyte under investigation.

There are two distinct steps in SPME; partitioning of the analytes between the extracting stationary phase and the sample matrix in DI-SPME mode and partitioning between fiber, headspace and sample matrix in the HS-SPME mode, followed by the desorption of concentrated extract [23], into analytical instruments, thermally when coupled to GC or with a mobile phase solvent when coupled to LC, and in the offline and online mode when combined with CE [12]. The technique is based on the partitioning of analyte and establishment of equilibrium between the analyte in the sample matrix and the stationary phase of the coated fused silica, which can either be liquid or solid particles suspended in a liquid polymer or a combination of both [14, 17, 24]. Recently, new SPME microextraction techniques have been developed, which make use of a microsyringe and is called in-needle SPME and with a pipette tip, known as in-tip SPME [12].

The experimental design of SPME for the analysis of pesticide residues from fruits and vegetables involves several steps and parameters which must be considered and optimized in order to achieve an efficient extraction. Parameters such as fiber type, extraction mode (described earlier), extraction and desorption time and temperature, separation and detection system, agitation method, ionic strength, sample pH, water and organic solvent content and method of calibration are considered and optimized based on the nature of analyte and constituent of sample matrix. Optimization of these parameters in the extraction of pesticide residues from food samples have also been reviewed in the literatures [5, 17, 22, 25, 26]. The first step in the development and optimization of an SPME method is the

selection of fiber coating, since the efficiency of SPME technique is dependent on the fiber coating/sample matrix distribution constant [18], because the extraction process involves the diffusion of analyte from sample matrix to the fiber/headspace, through the interface between the fiber/headspace and sample matrix.

The commercial fibers are coated with different polymeric materials (single or mixed), which extract by either absorption or adsorption. Due to their relative low recommended operating temperature (200 – 300 ^{O}C) of the commercially available fiber, the recent trend is focused on the development of new fibers using sol-gel technology, with high thermal stability (>320 ^{O}C) [27-37] and molecular imprinted polymers (MIPs) [38-42]. Ionic liquids and sol-gel prepared ionic liquid coated fibers have also been recently developed and have been used extensively for the SPME extraction of pesticide residues in water samples [43-46], but their use for the extraction of analytes from food samples is a potential research area to be explored in the future.

The PDMS is the most commonly used SPME fiber coating in fruit and vegetable analysis. Although, it is suitable for the extraction of non-polar pesticides, it has also been widely used for the extraction of more polar pesticides, after the extraction conditions have been optimized. [5]. The mixed coating: PDMS/divinylbenzene (DVB), DVB/PDMS/carboxen (CAR), carbowax (CW)/PDMS, CW/DVB and CW/template resin (TPR) extract by adsorption and possess complementary properties of each constituent polymeric coating.

The fiber SPME has been used successfully for the analysis of pesticide residues from fruit and vegetable samples. The 65 μm PA fiber was employed for the extraction of 54 multiclass pesticides from orange and peach in the DI mode [47]. The fiber coating was used for the extraction of phenyl urea pesticides from carrot, onion and potato in the DI mode [48], organophosphorus pesticides from orange, grape and lemon juice in the DI mode [49], triazole in strawberry in the DI mode [50], 14 multiclass pesticides from mango in the DI mode [51], while 80 μm PA fiber was used for the extraction of strobilurin fungicides from grape, ketchup, strawberry and tomato in the HS mode [52]. The 100 μm PDMS was used by Chai et al for the extraction of multiresidue organochlorine and organophosphorus pesticides from cabbage, tomato and guava in HS mode [53] and from strawberry, guava, pak-choi, tomato, star fruit and cucumber in HS mode [15, 54]. Organochlorine, organophosphorus and carbamate pesticides from tomato and cucumber in DI mode [55] has been employed. Organophosphorus pesticides was extracted from pear and apple in DI mode [56], strawberry, cherry and cherry juice in HS mode [57, 58], *C. coronaium* plant in DI mode [59] and 13 different vegetables in HS mode [60] using 100 μm PDMS. Multiclass pesticides were also extracted from tomato in the DI mode with 100 μm PDMS (Guillet [61].

A pre-extraction method, involving the use of matrix-assisted extraction followed by SPME extraction using the 100 μm PDMS was developed. The method was used for the extraction of organophosphorous pesticides from strawberry, tomato and pakchoi in the HS mode [62] and extraction of pyrethroid pesticides from strawberry in the DI mode [63]. The 100 μm PDMS gave better extraction efficiency, good linear range, lower detection limit and good recovery with relative standard deviation lower than 10%.

The most widely used mixed phase fiber for fruits and vegetables analysis is the 65 μm PDMS/DVB fiber. It was employed in the extraction of pyrethroid pesticides from tomato and strawberry [64], and 70 multiclass pesticides from cucumber, pepper and tomato [65]. The 60 μm PDMS/DVB fiber was employed for the extraction of 25 multiclass pesticides from tomato [61], carbamate pesticides from strawberry [66] and apple [67] and pyrethroid pesticides from cucumber and water melon and 7 multiclass pesticides from tomato [68], the 65 μm PDMS/DVB coupled to multi-dimensional GC/MS was recently used for the analysis of multiclass pesticides in peach, orange and pineapple juices [69]. The less frequently used fibers in the extraction of pesticides from fruit and vegetable samples include the 65 μm CW/DVB, used for the extraction of carbamate pesticides from apple and grape juice [70], the 50 μm CW/TPR used for the extraction of postharvest fungicides from cherry, orange and peach [71], and multiclass pesticides in lettuce [72] and activated carbon/polyvinylchloride (AC/PVC) used for the extraction of organophosphorus pesticides from grape [73]. The extractions gave good repeatability and reproducibility with better efficiency than the single phase coated fibers.

3. Liquid phase microextraction (LPME) also called Solvent Microextraction (SME)

This is also a newly developed microextraction technique, which drastically reduces the quantity of solvent used for extraction [74]. It is rapid and less expensive, and is performed between a small amount of water-immiscible solvent called the acceptor phase, and an aqueous phase (donor phase) containing the analytes of interest [75]. The acceptor phase can either be immersed directly in the sample matrix or suspended above the sample for headspace extraction [12]. The volume of the receiving phase is in microliter (droplet of organic solvent). It is also known as liquid-liquid microextraction (LLME) and can be divided into three major categories and their difference is the way the extraction solvent comes in contact with the sample matrix.

1. Single Drop Microextraction (SDME)
2. Hollow Fiber Liquid Phase Microextraction (HF-LPME)
3. Dispersive Liquid Liquid Microextraction (DLLME)

The development of the methods for the analysis of pesticides by the LPME technique as in SPME, requires the optimization of the parameters related to the donor and acceptor phases. The first and the most important is the selection of an organic solvent which is of high purity, less volatile, high boiling point, so as to prevent solvent evaporation and ensure analyte concentration. The solvents should also be water immiscible for extraction of analytes from an aqueous sample. The SDME extraction like SPME is a non-exhaustive extraction method, thus an appropriate drop volume must be selected to prevent shrinking, and volumes are usually between 0.9 and 1.6 μL. Although, the use of a large volume of solvent drop yields better instrumental response and improve extraction efficiency, they are difficult to manipulate and could be easily dislodged from the syringe. Agitation increases the extraction efficiency, by decreasing the extraction time. It also leads to an increase in

aqueous mass transfer and exposes the analytes to the extraction solvent, and should be optimized to avoid the dislodgment of the solvent drop.

Salt addition enhances extraction efficiency by reducing the solubility of the analytes in the sample, especially for the moderately polar and low molecular weight analytes. The optimization of extraction temperature is important especially in the headspace mode, but a higher temperature may affect the stability of the microdrop, and for this reason most of the reported analyses have been carried out by DI- mode at room temperature. The sample and headspace volume should be maintained constantly at minimal level, since larger sample volumes will require longer extraction time and affect the stability of the microdrop. In the headspace mode, the optimized volume should be at a level which allows the microdrop to be suspended over the stirred sample, while in the direct immersion mode, the sample volume should only be enough to avoid the sample coming in contact with the septum [3, 76, 77]. The presence of air bubbles should also be avoided in the syringe to avoid errors in the analysis.

3.1. Single drop microextraction (SDME)

SDME is a microextraction technique, which is based on the suspension of a single droplet of water-immiscible organic solvents (typically 0.5–3 μL) from the tip of a microsyringe needle in an aqueous solution [12, 75], thus drastically reducing the volume of organic solvents used. The analytes are distributed between the microdrop of the solvent at the tip of the syringe and the sample solution [78]. The analyte which is extracted by passive diffusion onto the droplet is retracted back into the syringe and injected directly into the analytical instruments (GC, HPLC or CE). The evaporation and reconstitution of analytes before injection is eliminated and the technique provides highly enriched extracts of the analytes [12].

The SDME extraction can be carried out in direct immersion (in which the droplet is suspended directly into the aqueous sample), and is most suitable for the analysis of medium polar or non-polar analytes or headspace mode (where the droplet is suspended in the headspace of the sample solution) and is suitable for the analysis of volatile or semi-volatile analytes. The technique was first reported by Liu and Dasgupta, using a 1.3 μL chloroform suspended in a large aqueous solution containing a methylene blue active substance [79], and later by Jeannot and Cantwell for the extraction of 4-methyacetophenone in aqueous solution, using 8 μL of n-octane suspended at the end of a Teflon rod [80]. The extraction technique was found to be simple, flexible and can easily be coupled to chromatographic instruments. Its coupling with GC and HPLC has been reported and reviewed extensively, while it has also been successfully coupled with ICP-MS, ET-AAS, FI-AAS, MALDI-MS, CE and MS [3, 81]

The use of a Teflon rod as a microdrop holder implies that extraction from the sample and injection of analytes into chromatographic instruments are performed separately with different apparatus [75, 82]. This limitation was overcome by the introduction of a microsyringe as the microdrop holder [83], and the organic solvent can be withdrawn after

extraction and injected directly into the GC system [81]. Another limitation is the instability of the microdrop of solvent in the DI-mode at high stirring rate and long extraction times [3]. Therefore, in the development of SDME technique, there is need for the optimization of factors such as selection of extraction solvent, solvent drop volume, agitation rate, ionic strength, extraction temperature and time, sample and headspace volume and automation [76].

Few studies have been published regarding its application for the extraction of pesticide residues from fruit and vegetable samples. The techniques was used for the analysis of 20 multiclass pesticides in apple [84], and in tomato, using toluene [85, 86], analysis of triazole pesticides in water and grape juice samples, with extraction by n-hexanol/n-hexane (50:50,v/v%) [87]. The presence of organochlorine pesticides was also investigated in vegetable samples (cabbage, cauliflower and Chinese cabbage) using SDME-GC/MS using a mixture of 1 μL p-xylene/acetone (80:20, v/v %) [88] and organophosphorus pesticides in fruit juices. [89], and in apple, pear and orange juice samples [90], using toluene as the extraction solvents. Organophophorus and organochlorine pesticides were also determined in cucumber and strawberry using toluene (1.5 μL) [91], while a mixture of octanone and undecanone was used for the extraction of oxazole fungicides from fruits samples [92]. The choice of toluene by most authors was based on the fact that it is stable, less toxic, possesses a higher extraction efficiency and its suitability for direct GC injection. All the developed methods gave comparable analytical figures of merit compared to SPME.

3.2. Hollow fiber liquid phase microextraction (HF-LPME)

HF-LPME is a multi-phased microextraction system, which was developed to enhance the stability of the organic solvent in 1999 [93, 94]. It is based on the principle of a supported liquid membrane [95], in which the organic solvent is used to fill both the wall pores and the HF lumen. It makes use of a polymeric membrane which forms a barrier between the solvent and the sample [96], and acts as a support for the small volumes of extracting solvents. It involves filling of a few microliters of solvent inside the pores and lumen of a semi-permeable polypropylene hollow fiber attached to a syringe. The HF-LPME can be carried out in either the static or dynamic mode. In the static mode, the acceptor phase is introduced in the lumen followed by the immersion of the fiber into the aqueous sample by a syringe, while in the dynamic mode the HF is attached to a syringe connected to programmable pump [75, 78].

The extraction can be carried out in two- or three phases [95]. In the two-phase mode, the aqueous sample (donor) makes contact directly with the organic solvents (acceptor) through the membrane pores, by a repeated pushing and pulling of the microsyringe plunger [97], and the mass transfer of the analytes is driven by the diffusion of the analytes from the sample matrix into the organic solvent. When the pores are prefilled with an organic solvent, which provides a supported liquid membrane, a three-phase system is formed, and the sample solution and organic solvent are separated by the hollow fiber membrane. The solvent used must be compatible with the membrane, so as to ensure that the pores in the

wall of the membrane is completely filled [75]. The technique can also be carried out in the headspace mode, but most researchers have used the direct immersion mode for efficient pre-concentration of the analytes. The limitation of this technique is the issue of carry-over [98], therefore a new membrane should be used for each extraction.

The HF-LPME membranes are capable of accommodating larger volumes of organic solvents which increases the efficiency of the extraction, while the pore acts as a filter which prevents interference of large molecular weight molecules present in the sample matrix. The technique has not been used extensively for pesticides analysis in food samples, therefore only a few studies have been reported for its applications in fruit and vegetable samples. It has been used for extraction of pesticide residues in vegetables using dihexyl ether [99], triazines in watermelon using toluene immobilized on MIP-HF [100], fungicides from orange juice using 2-octanone [101], organochlorine pesticides from tomato and strawberry using a mixture of toluene and hexane [102] and pesticides from grapes with pressurized hot water [103]. The choice of the water-immiscible solvents was based on their ease of immobilization into the HF, with low volatility and high partition coefficients.

3.3. Dispersive liquid-liquid microextraction (DLLME)

DLLME is a microextraction technique introduced by Assadi and co-workers [104] which also makes use of microliter volume of a mixture of extraction solvent and dispersive solvent. This helps to minimize dislodgement of the organic solvent drop inherent in SDME. It involves the formation of a cloudy solution when an appropriate mixture of high density water-immiscible extraction solvent and dispersive solvents is injected rapidly into an aqueous solution [104, 105], containing the analytes of interest. The hydrophobic solutes are then enriched in the extraction solvents, which are dispersed into the bulk aqueous solution and the mixture is centrifuged, thus, making DLLME a two-step extraction technique. The extractive solvent accumulates at the bottom of the extraction vessel and can be injected with or without further treatment into analytical instruments. The choice of the type and volume of dispersive solvent is as important as that of the extraction solvent, because, it helps the extraction solvent to form fine droplets in the sample matrices. The nature of the fine droplet has been found to enhance extraction efficiency, because of the abundant surface contact between the droplet and the analytes, thus the mass transfer of analytes into the extraction solvent is speeded up [75].

The use of DLLME has also been extended to the analysis of pesticide residues in fruit and vegetable samples which resulted in efficient extraction, high enrichment factors and low detection limits. The technique was used for the analysis of multi-residue pesticides in peach juice, pulps and peels using dodecan-1-ol (4 μL) and acetone (4 μL) as the extraction and dispersive solvents respectively, and the extraction was based on floating organic solvent [106]. Organophophorus pesticides were analyzed in orange juice samples using chlorobenzene (50 μL) and ultrasound assisted emulsification [107], also in banana using an ionic liquid ([BBIm][PF_6]) based extraction solvent and methanol (600 μL) [108], in tomato with ultrasound assisted solvent extraction followed by DLLME with chlorobenzene (10 – 11 μL) as

an extraction solvent with acetone (1 ml) as the dispersive solvent [109], and in water melon using chlorobenzene (27 μL) and acetonitrile (2.5 ml). Carbamate pesticides were also analyzed in banana, pineapple and tomato juice using trichloromethane (800 μL) and methanol (1.5 ml) [110] and in apple using trichloromethane (60 μL) and acetone (1 ml) [111].

Neonicotinoid insecticides was also analyzed in apple using chlorobenzene (9 μL) and acetone (1 ml) [112], and in tomato and cucumber using tricholoromethane (200 μL) and acetonitrile (2.5 ml) [113]. Multiclass pesticides were determined in banana [114], table grape and plum [115], using ionic liquid ([C_6MIm][PF_6]) based extraction solvent and methanol (714 μL) and in apple juice using tetrachloromethane (100 μL) and acetone (500 μL). It was also used for the analysis of triazophus and carbaryl from apple, grape and peach [116], fungicides from pear, grape and strawberry [117], imidacloprid from toamato [118], using tetrachloroethane and acetonitrile, cypermethrin and permethrin from pear juice using tetrachloroethene and methanol, diethofencarb and pyrimethanil from apple pulp and peel using 1-undecanol and acetonitrile as the extraction and dispersion solvents respectively. The technique was also found to yield a better enrichment factor and low detection limit.

4. Stir bar sorptive extraction (SBSE)

SBSE is also a microextraction technique similar to SPME but with a greater extraction capacity. It helps to overcome the small volume of the coated SPME fiber for a better enrichment factor. In the SBSE technique, a 10 to 40 mm long magnetic stir bar coated with 50 – 300 μL of polydimethylsiloxane (PDMS) is used as the extracting phase [12]. The extraction mechanisms are similar to those of SPME, but the enrichment factor, which is determined by the amount of extractive phase is higher. The analytes are adsorbed on a PDMS coated magnetic rod, by stirring the sample solution with the rod for a given time. The stir bar can then be thermally desorbed into GC or by organic solvent to be subsequently injected to an LC system [119, 120]. The limitation of this technique include the need for reconstitution in a solvent before chromatographic analysis, since the stir bar fiber cannot be directly injected into the split/splitless injection port of a GC [96, 120]. The tedious reconstitution step can lead to introduction of contaminants and loss of analyte. It also involves longer desorption time, due to the higher amount of stir bar coating.

The extraction process like SPME is dependent on the partition coefficients between the analyte and the coated stir bar. The PDME coated fiber is commercially available, and has widely been used, while other polymeric phases such as restricted access materials, sol-gel prepared coatings and carbon adsorbents have also been recently developed. In the SBSE method development, factors such as extraction time and temperature, pH, salt and organic solvent addition, sample volume, agitation speed and volume of acceptor phase are very important and need to be optimized [96, 120] for higher extraction efficiency, recovery and low detection limit. The technique has successfully been applied for the extraction of pesticides residues in fruit and vegetable samples. An organophosphorus pesticide (OPP) was analyzed in cucumber and potato using sol-gel coating stir bar [121]. It has also been used for the extraction of multiclass pesticides in different fruits and vegetable samples [122-

124], using PDMS coated stir bar, organochlorine and organophosphorus in grape and peach juice using poly(phthalazine ether sulfone ketone) coated stir bar [125]. A method which compared SBSE and matrix solid phase dispersion was developed for the determination of multiclass pesticides in orange using PDMS coated stir bar [126].

5. Microextraction in packed solvents (MEPS)

MEPS is a recently developed microextraction technique. It is a miniaturization of conventional SPE packed bed devices from milliliter bed volume to microliter volumes, and uses the same sorbent bed integrated into a liquid handling syringe that allows for low void volume sample manipulation [127]. It can be connected online with GC or LC without any modifications, and can be fully automated [128]. The sorbent (1-2 mg) is either inserted into the syringe (100-200 μL) barrel as a plug or between the needle and the barrel as a cartridge [127, 129]. The cartridge bed can be packed or coated to provide selective and suitable sampling conditions and make use of silica based (C2, C8 C18) and strong cation exchanger (SCX), polystyrene-divinylbenzene copolymer or molecular imprinted polymer, as the extracting phase [127, 130].

The extraction technique which can be performed on-line with GC or LC involves dilution of the sample followed by drawing the sample up and down through a conditioned sorbent solid phase plug in the syringe. The solid phase is washed with water or with other solvents in the case of water soluble analytes, to remove any interfering materials and the analytes are eluted with a microliter volume of organic solvent. The technique was recently used for the analysis of multiclass pesticides in water samples [131, 132], but till today it has not been used for fruits and vegetables analysis. It presents a good and promising microextraction technique for the analysis of a broad range of pesticides in food samples. It also requires the optimization of factors such as the nature of adsorbent, nature and volume of elution solvent, nature and volume of washing solution and number of extraction cycles [127, 128, 130], to achieve an efficient extraction for better sensitivity and low detection limits.

6. Interface to analytical instrumentation

The automation of the microextraction techniques described in this review has gone a long way in increasing the efficiency and accuracy of the extraction procedures and subsequent instrumental analysis, by preventing loss of sample and introduction of other contaminants. All the techniques except SBSE have been conveniently interfaced to chromatographic analytical instruments. At present SPME offers the best technique because of its solvent-less nature, since other microextraction techniques make use of water-immiscible solvents, and the GC technique is the most preferred analytical instrument and has been used in most of the published work in microextraction analysis. The techniques have also been successfully interfaced with HPLC and CE, but only a few papers have been reported [81, 94]. The GC analysis provides higher sensitivity, selectivity and better detection limits than LC in pesticide analysis, while the CE provides a faster alternative to the chromatographic techniques but with higher detection limit [77]

7. Limitations and future trends

The use of microextraction technique is emerging as a very reliable sample preparation method, while employing little or no solvent. The advantages over the traditional method include their simplicity of operation, rapid sampling, low cost, high recovery and enrichment factor and being environment-friendly. The major limitations of these techniques include low recommended operating temperature, low volume of fiber coatings, fiber breakage and stripping of coatings in SPME, low volume of microdrop solvent, instability of the solvent, possible loss of organic solvent in SDME, the presence of porous membrane, use of a large amount of solvent for analyte elution in HF-LPME, difficulty of the automation, use of large volume of dispersive solvent and solvent dissolution in DLLME. In SBSE, the major limitations include the need to rinse, dry and reconstitute the analyte, while in MEPS, limitations include, carry-over of analytes, low enrichment factor and the presence of small amounts of water residue on the packed sorbents. The use of more selective, efficient and versatile extraction procedure and increasing interest in overcoming the aforementioned limitations and trend towards automation will provide better integration of sampling and instrumental analysis which can be used for a wide range of analytes. The limitations can also be reduced by careful optimization of all the required parameters. The use of nanoparticles as the fiber coating (SPME and SBSE)), microdrop solvents (LPME) and packed sorbent (MEPS) is a fast growing area of microextraction technique which is yet to be fully explored.

8. Conclusion

The miniaturization of sampling procedure and the need to reduce sampling time and solvent volume has led to the development of various microextraction techniques. The introduction of ionic liquid based and sol-gel prepared ionic liquid extraction phase, which can be used for all the microextraction techniques will provide a better enrichment factor, higher recovery, low detection limit and higher extraction throughput due to their unique properties such as negligible vapour pressure, good thermal stability, and high viscosity. SPME remains the most widely used technique, especially with the introduction of ionic liquid based, molecular imprinted and sol–gel prepared fiber coatings and introduction of the metal alloy fiber. However, the microextraction technique is an emerging field of study which will provide inexpensive and solvent-less analysis of a wide range of pesticide residues and other contaminants in food samples.

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Section 2

Metabolism of Pesticides

Chapter 9

Degradation of Fenamiphos, Chlorpyrifos and Pirimiphos-Methyl in the Aquatic Environment: A Proposed Enzymatic Kinetic Model That Takes Into Account Adsorption/Desorption of the Pesticide by Colloidal and Sediment Particles

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Additional information is available at the end of the chapter

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1. Introduction

Organophosphate compounds are among the most common chemical classes used in crop and livestock protection, and account for an estimated 34% of world sales (Singh and Walker, 2006; Caceres et al., 2010). Fenamiphos (*N*-[Ethoxy-(3-methyl-4-methylsulfanylphenoxy)phosphoryl] propan-2-amine, [CAS number 22224-92-6]), chlorpyrifos (o,o-diethyl-o-3,5,6-trichloro-2-pyridyl phosphorothioate, [CAS Number 2921-88-2]) and pirimiphos-methyl (o-2-diethylamino-6-methyl pyrimidin-4-yl-o,o-dimethyl phosphorothioate, [CAS number 29232-93-7]) are among some of the commonly used organophosphate pesticides. Fenamiphos is a systematic nematicide used for the control of ecto and endo parasitic free living nematodes. Fenamiphos is applied to the soil before planting or at planting time. It can also be applied to established plants. Fenamiphos is toxic to mammals (LD_{50} = 8 mg/kg body weight male rats (USEPA, 1984)), and to fish at concentrations above 3 mg/litre. It is moderately persistent in soil, and half-life ranges of 10 to 67 days and 12 to 87 days, and an average half-life of 30 days, have been reported (Nicholls, 1994; Simon et al., 1992).

Chlorpyrifos is a non-systemic insecticide, with broad spectrum insecticidal activity (Rackie, 1993). It is used in a wide range of agricultural and specialty pest control scenarios to control insects and other arthropod pests, e.g., corn and cotton agriculture, termite control, citrus horticulture. Chlorpyrifos is toxic to mammals (LD_{50} = 163 mg/kg body weight male rats (USEPA, 1984)), and acts as a cholinesterase inhibitor. Chlorpyrifos is a very persistent

pesticide, and a half-life range of 30 to 120 days has been reported (Nicholls, 1994). Murray et al. (2001) studied the persistence of chlorpyrifos applied for termite control on six Australian soils at initial chlorpyrifos concentrations of 1000, 100 and 10 mg/kg and reported half-lives of 385, 155, and 41 days for the three concentrations respectively for all six soils. Soil pH had no effect on the rate of degradation at all concentrations studied. Hui-ming and Guo-nian (2003) reported half-lives of 278, 91.8 and 79.2 days for similar experiments with chlorpyrifos. Bondarenko and Gan (2004) studied the degradation and sorption of chlorpyrifos in urban stream sediments from southern California, USA, and reported half-lives of 14 - 24 days and 58 – 223 days under aerobic and anaerobic conditions respectively. Half-lives of 4.28, 0.58 and 1.35 days were reported by Zhang et al. (2011) for the dissipation of chlorpyrifos from rice plants, water and soil respectively under paddy field conditions. Gilani et al. (2010) reported 100% recovery of chlorpyrifos after 6 months and 1 year from tap water and irrigation water treated with different fertilizers.

Pirimiphos-methyl is a post-harvest insecticide used on stored corn and sorghum grain and seed, and incorporated into cattle ear tags. It is used for the control of various insects, e.g., mealy bugs and mites, various types of beetles, grain weevils, grain borers and moths. Pirimiphos-methyl can cause cholinesterase inhibition in humans (USEPA, 2002a). Use of pirimiphos-methyl as an insecticide for warehouses, stored grain, and vegetable crops will results in its release into the environment. If released into water, pirimiphos-methyl is expected to adsorb to suspended solids and sediments. Pirimiphos methyl hydrolyses rapidly at acidic pH, with half-lives of 7.3 days at pH 5, 79.0 days at pH 7, and 54.0 - 62.0 days at pH 9 (USEPA, 2002b). Patakioutas et al. (2002) studied the degradation of pirimiphos-methyl in top soils of untilled and tilled soil, and reported half-lives of 16.7 and 9.2 days respectively.

Pesticides find their way into surface and ground water as a result of agricultural land drainage or industrial waste discharges. Organophosphate pesticides are highly toxic to fresh water invertebrates (Caceres et al., 2007), hence there is need to understand the factors that determine the persistence of these pesticides in the aquatic environment. The persistence of pesticides in the aquatic environment is usually expressed in terms of the half-lives of the pesticides in the environment, in accordance with the conventional pseudo first order kinetics approach to the study of pesticide persistence in the environment. Half-life data that have been obtained for fenamiphos, chlorpyrifos and pirimiphos-methyl under different environmental conditions are summarized in Table 1. It is apparent from this brief survey that half-life data reported in the literature are highly variable, whereas in true first order kinetics, a constant value should be obtained for the half-life, irrespective of the actual environmental conditions prevailing. Wania and Mackay (1999) suggested that techniques for modeling the persistence of pesticides should take into account spatial, temporal and climatic variability, as well as concepts such as equilibrium partitioning. It is apparent therefore that more studies are required to elucidate further the kinetics of the degradation of pesticides in the aquatic environment.

Pesticide	Environment	Half-life (days)	Reference
Fenamiphos	Soil	10 - 67	Nicholls, 1994
	Soil	12 - 87	Simon et al., 1992
Chlorpyrifos	Soil	385, 155, 41[a]	Murray et al., 2001
	Soil	30 - 120	Nicholls, 1994
	Soil	278, 91.8, 79.2[a]	Hui-ming & Guonian, 2004.
	Sediment	14 – 24 (aerobic)	Bondarenko & Gan, 2004
	Sediment	58 -223 (anaerobic)	Bondarenko & Gan, 2004
	Rice plants	4.28	Zhang et al., 2011
	Water	0.58	Zhang et al., 2011
	Soil	1.35	Zhang et al., 2011
	Tap water	>180	Gilani et al., 2010.
	Irrigation water	>365	Gilani et al., 2010.
Pirimiphos-methyl	Water	7.5, 79.0,59 – 62[a]	USEPA, 2002.
	Soil (untilled)	16.7	Patakioutas et al., 2002.
	Soil (tilled)	9.2	Patakioutas et al., 2002.

[a]Depending on pH.

Table 1. Persistence data for Fenamiphos, Chlorpyrifos and Pirimiphos-methyl from the literature.

Zaranyika and Nyandoro (1993) studied the kinetics of the degradation of glyphosate [N-(phosphonomethyl) glycine] in the aquatic environment and observed two linear rates of degradation. The results were explained in terms of a steady state enzymatic kinetic model, which takes into account microbial degradation of both free and colloidal-particle-adsorbed glyphosate. According to this model the rate of degradation of glyphosate was given by

$$dP/dt = k_2[G_B] + k_6[GC_B] \quad (1)$$

where G denotes glyphosate, GC denotes glyphosate-colloidal-particle complex, the subscript B denotes microbial bound, and k_6 and k_2 are the rate constants for the degradation of the colloidal-particle adsorbed and unadsorbed glyphosate respectively, and P denotes products. It was further shown that, provided the concentration of glyphosate in the medium was in excess of the microflora that can bind the pesticide, then a steady state obtains and the rate equation becomes:

$$dP/dt = k_2' + k_6' \quad (2)$$

These experiments were conducted using river water and sediment in order to simulate as closely as possible conditions to be found in the natural aquatic environment. Biphasic linear degradation rates have also been observed for the degradation of endosulfan I and endosulfan II in experimental microcosm aquatic ecosystems (Zaranyika et al., 2010).

The aim of the present work was to carry out similar semi-field experiments with pirimiphos-methyl, chlorpyrifos and fenamiphos in order to determine whether the degradation of the pesticide in the aquatic environment can be interpreted in terms of steady state kinetics. The

experiments were conducted using river water and sediment contained in a plastic drums covered with clear perforated plastic and exposed to sunlight. The rate of degradation of the pesticide was monitored in the water as well as the sediment phase of the experiment.

2. Experimental methodology

2.1. Equipment

A microprocessor controlled Perkin Elmer Autosystem Gas Chromatograph equipped with a built-in Auto sampler, a split/splitless injector, and a nitrogen-phosphorus detector (NPD) (Perkin Elmer, Norwalk, CT, USA), was used in conjunction with a BPX-5- 95% phenyl polydiphenylene-silicone capillary column 30m x 0.25 mm id , film thickness 0.25 μm (SGE Analytical Science, Melbourne, Australia); white plastic tanks, 200 L capacity; 3.7 mL Pyrex glass sample vials with hollow caps and Teflon-lined septa (Supelco SA, Switzerland); A Buchi Rotary Evaporator Model R-124 (Buchi Labortechnik AG, Switzerland), equipped with a water bath was used to concentrate the sample extracts to near dryness; and a Stuart Scientific model SF1 flask mechanical shaker (Stuart Scientific, Redhill, UK).

2.2. Materials

The following were used: pesticide residue analysis grade diethyl ether, ethyl acetate, hexane and acetone (Fischer Scientific, Loughorough, U.K.). Other materials used include glass wool, Florisil (60 – 100 mesh, [Fluka Chemie AG, Switzerland]); anhydrous sodium sulphate (99.8% purity, Acros Organics, New Jersey, USA); Whatman 541 filter paper; double distilled water; river water and sediment collected from Kutsaga Dam on the Hunyani river near the Tobacco Research Board Kutsaga Research Station, Harare, Zimbabwe; high purity nitrogen carrier gas; fenamiphos (99.5% pure), chlorpyrifos and pirimiphos-methyl (99.8% pure) reference standards, supplied by Dr. Ehrenstnfer, D86 199, Ausberg, Germany; Nemacur (containing 400 g/L fenamiphos), Dursban (containing 480 g/L chlorpyrifos), and Shumba (containing 40 g/100mL pirimiphos methyl) (Agricura (Pvt) Ltd., Zimbabwe).

2.3. Procedure

Volumes of 100 L each of Kutsaga dam and distilled water were charged into two separate 200 L tanks and the levels were marked. About 1.93 kg of sediment was added into the tank containing dam water. Volumes of 37. 5 mL of nemacur, 31.25 mL of dursban, and 37. 5 mL of Shumba, meant to give 150 μg/mL each fenamiphos, chlorpyrifos and pirimiphos-methyl respectively, were added into each of the tanks. The contents were thoroughly mixed. Samples were taken at zero time immediately after the system had settled. The tanks were covered with transparent perforated polyethylene and left exposed to sunlight at the roof of the Pesticide Analysis Laboratory Building, Kutsaga Research Station. Thereafter samples of water and sediment were collected periodically for a period of 90 days, each time compensating for evaporation 24 hours prior to sampling. Water and sediment samples were taken with minimal disturbance of the system. The new level was marked after each sampling, then the system was stirred and left to settle.

Once collected all samples were stored in the freezer in plastic bottles with screw caps until required for analysis. All the samples were thawed and mixed thoroughly prior to analysis.

2.4. Extraction, cleanup and concentration

Water samples were analysed by GC-NPD using a BPX-5, phenyl-polydiphenylene silicon capillary column following liquid-liquid extraction (Greeve and Goewie, 1985; Mansour et al., 1998). Water samples were analyzed in duplicate. 100ml of water samples were extracted 3 times with 50 ml portions of diethyl ether using a separatory funnel, and collected over anhydrous sodium sulphate. The combined extracts were concentrated to near dryness using the rotary evaporator maintained at 40^0C in the water bath, and then redissolved in 2.0 ml acetone for subsequent clean-up prior to analysis by GC-NPD.

Sediment samples were analysed following liquid-solid extraction (Mansour et al., 1998). Sediment samples were extracted after the excess water in the sample had been removed by suction from a Buchner funnel through a Whatman No. 1 filter paper, and then air dried for three hours. The moisture content of the air dried sediment samples was determined after thoroughly mixing the sample. About 20 g accurately weighed of dried sediment sample was weighed and placed in a 250ml flask, and 100 ml of acetone were added and the flask stoppered. The mixture was then shaken on the mechanical shaker for 30 min., and then filtered through anhydrous sodium sulphate. The sample was then quantitatively transferred to the rotary evaporator maintained at 40^oC, and the crude extract was concentrated to near dryness and then redissolved in 2 ml acetone for subsequent clean-up prior to GC-NPD analysis.

For clean-up the concentrated extract was transferred to a chromatographic column plugged with glass wool and containing 5g of Florisil and 1g of anhydrous Na_2SO_4 pre-cleaned by eluting with 10% diethyl ether in hexane. Care was taken not let the column run dry. The column was then eluted twice using 100 mL each of 5% diethyl ether in hexane and 10% diethyl ether in hexane. The two fractions were collected and concentrated to near dryness and then redissolved in 2 ml acetone for subsequent GC-NPD analysis (Tse et al., 2004).

2.5. Gas chromatography

Gas chromatographic conditions employed are given in Table 2. One microliter of the concentrated extract was injected each time. Pirimiphos-methyl, chlorpyrifos and fenamiphos eluted at 3.37, 3.70 and 5.19 min. respectively in the GC-NPD chromatogram, see Fig. 1. Quantification was done by the external standard technique. Recoveries of 85±1 %, 86±2 and 87±2% , and of 95±2 %, 95±2 and 70±2% were obtained when dam water and sediment samples spiked with 5 ng/g and 20 ng/g respectively pirimiphos-methyl, chlorpyrifos and fenamiphos respectively, were extracted and determined as described above. Pirimiphos-methyl, chlorpyrifos and fenamiphos were not detected when blank determinations were done on the sediment and dam water samples. The results obtained are shown in Table 3. Table 4 shows the material balance calculations for the initial and final distribution of chlorpyrifos, pirimiphos-methyl and fenamiphos in the microcosm.

GC compartment	Parameter	Setting
Column	Initial temperature	195°C
	Initial hold time	2.0 min.
	Final temperature	235°C
	Temperature program	10°C/min.
	Final hold time	9.0 min
	N_2 carrier gas flow rate	5.2 ml/min.
Injector	Temperature	230°C
	Mode	Splitless
Detector	Type	NPD
	Temperature	300°C
	Range	1
	Auto zero	on
	Time constant	200
Detector gas flows	Air	179.0 ml/min.
	Hydrogen	4.4 ml/min.

Table 2. Gas chromatographic conditions employed.

t (days)	Chlorpyrifos			Pirimiphos-methyl		Fenamiphos		
	$C_{t(DW)}$	$C_{t(LW)}$	$C_{t(LS)}$	$C_{t(LW)}$	$C_{t(LS)}$	$C_{t(DW)}$	$C_{t(LW)}$	$C_{t(LS)}$
BC	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
0	149.8	117.7	1280	117.0	930	156.0	80.1	108
3	149.9	115.5	930	116.1	740	156.0	67.1	99
6	149.1	113.3	680	101.6	nr	155.1	63.0	67
9	149.0	111.3	710	94.7	560	150.4	42.9	59
15	148.9	109.2	360	85.4	374	149.3	32.0	42
21	148.0	106.3	180	75.1	260	149.1	15.9	43
26	147.0	106.3	172	72.1	255	149.1	16.0	29
34	147.0	105.4	173	68.2	240	148.0	14.8	21
50	147.0	106.4	168	66.6	220	148.0	12.9	18
64	145.9	105.2	164	64.9	190	147.9	11.0	12
72	145.9	105.1	163	60.6	187	147.8	10.7	11
90	145.9	105.1	164	60.2	191	147.8	10.7	10

Table 3. Concentration (μg/g) of chlorpyrifos, pirimiphos-methyl and fenamiphos in distilled waster, Kutsaga dam water and sediment phases of the experiment as a function of time, t (C_t = concentration (μg/g) at time t; DW = distilled water; LW = lake water; LS = Lake sediment; BC = before charging; nr = no result).

Pesticide	Phase	Initial concentration		Final concentration	
		Analysis[a]	Total[b]	Analysis[a]	Total[b]
Chlorpyrifos	Water	117.7	11.77	105.1	10.1
	Sediment	1280	2.27	164	0.317
	Container[c]		0.76		0.76
	Deg. loss[d]				3.623
	Total		14.90		14.80
Pirimiphos-methyl	Water	117.0	11.70	60.2	6.02
	Sediment	930	1.79	191	0.369
	Container[c]		1.51		1.51
	Deg. Loss[d]				7.101
	Total		15.0		15.0
Fenamiphos	Water	80.1	8.01	10.7	1.070
	Sediment	108	0.21	10	0.019
	Container[c]		6.78		6.78
	Deg. Loss[d]				7.131
	Total		15.0		15.0

[a]µg/g/day (see Table 2); [b]g/vol (or mass) of phase; [c]Adsorbed to walls of the container; no change assumed during period of the experiment. [d]Loss due degradation (see Table 5).

Table 4. Material balance calculations: Initial and final distribution of chlorpyrifos, pirimiphos-methyl and fenamiphos in the microcosm.

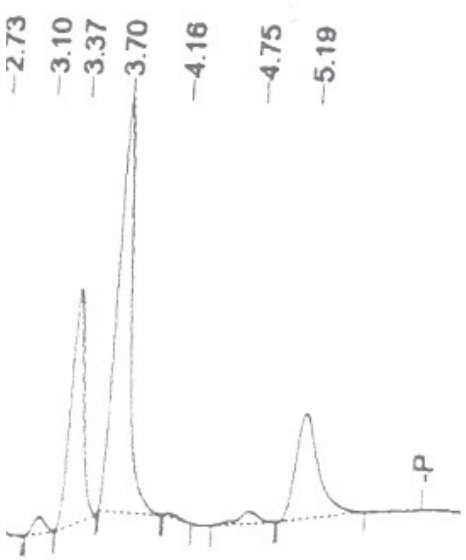

Figure 1. Typical chromatogram for the sediment sample collected on day 9 showing the retention time for pirimiphos methyl, chlorpyrifos and fenamiphos at 3.37, 3.70 and 5.19 min. respectively.

Figure 2 shows the persistence curves for chlorpyrifos and fenamiphos in the water phase of the experiment and control. The loss in the pesticide after a given time period t in days, i.e., $C_t - C_o$, was calculated and plotted as a function of t in Figs. 3 to 5. The slopes of the linear portions of the curves in Fig. 3 to 5 were obtained using regression analysis, and give the respective rates of degradation, which are summarized in Tables 5.

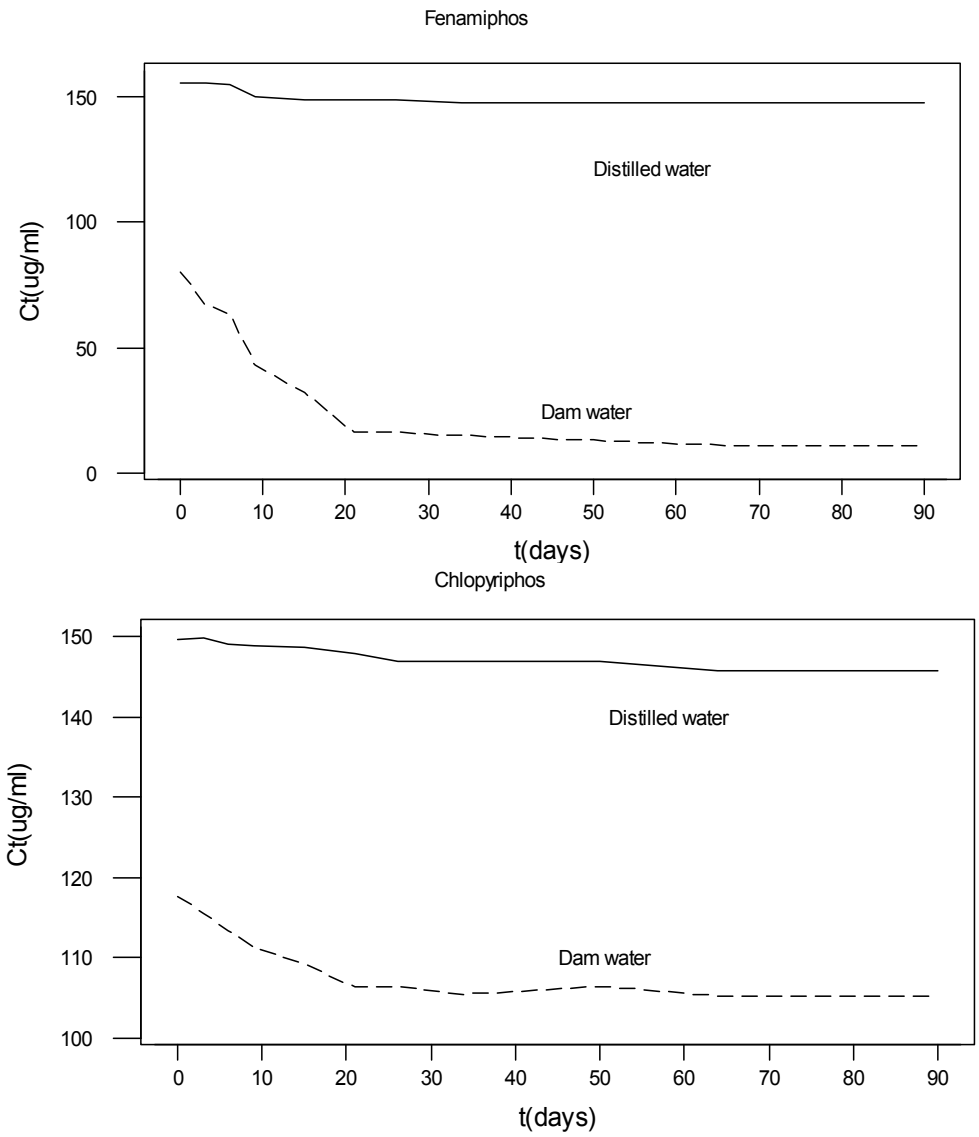

Figure 2. Persistence of chlorpyrifos and fenamiphos in distilled water and Kutsaga dam water

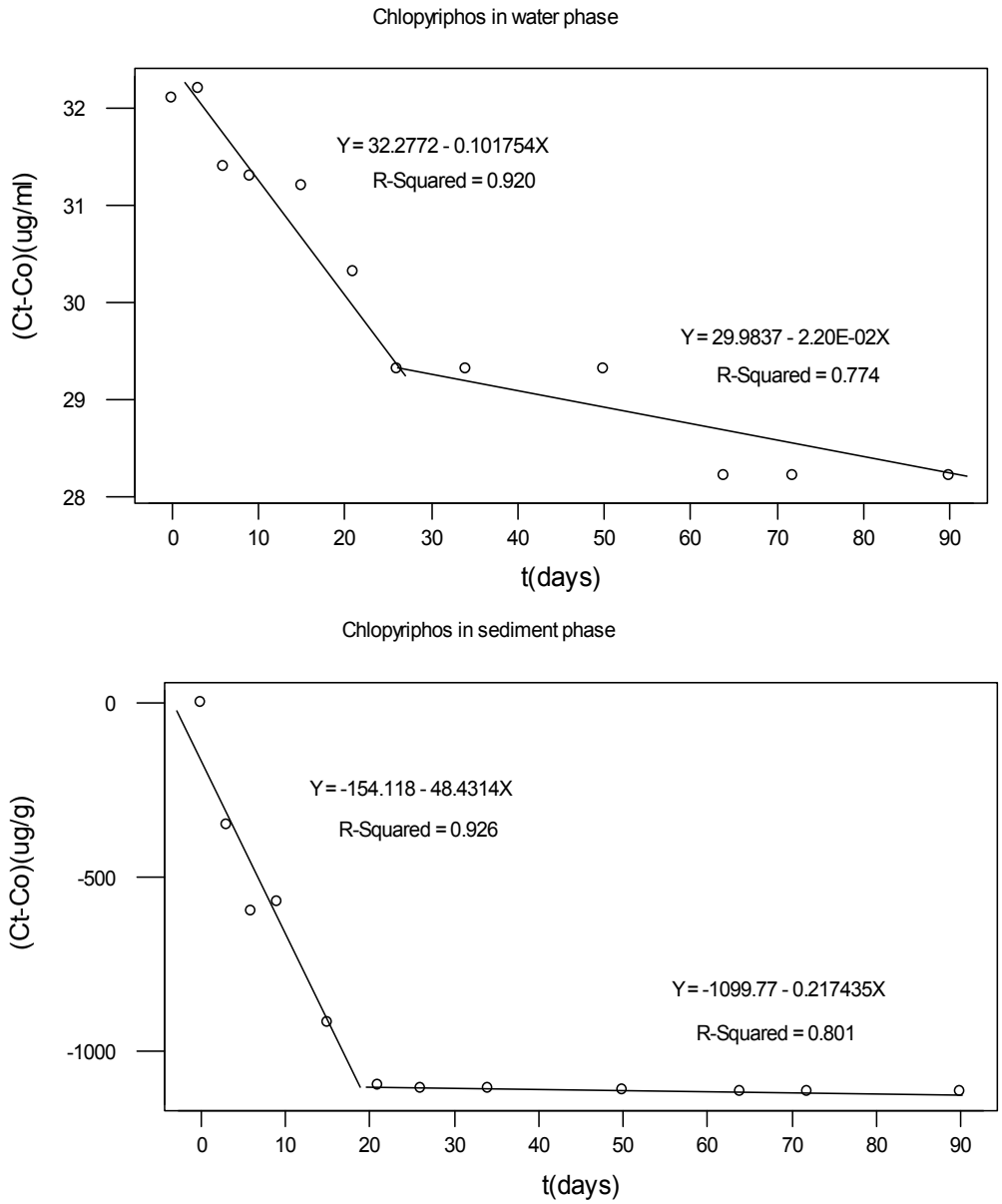

Figure 3. Rates of degradation of chlorpyrifos in the water phase and sediment phase of Kutsaga dam

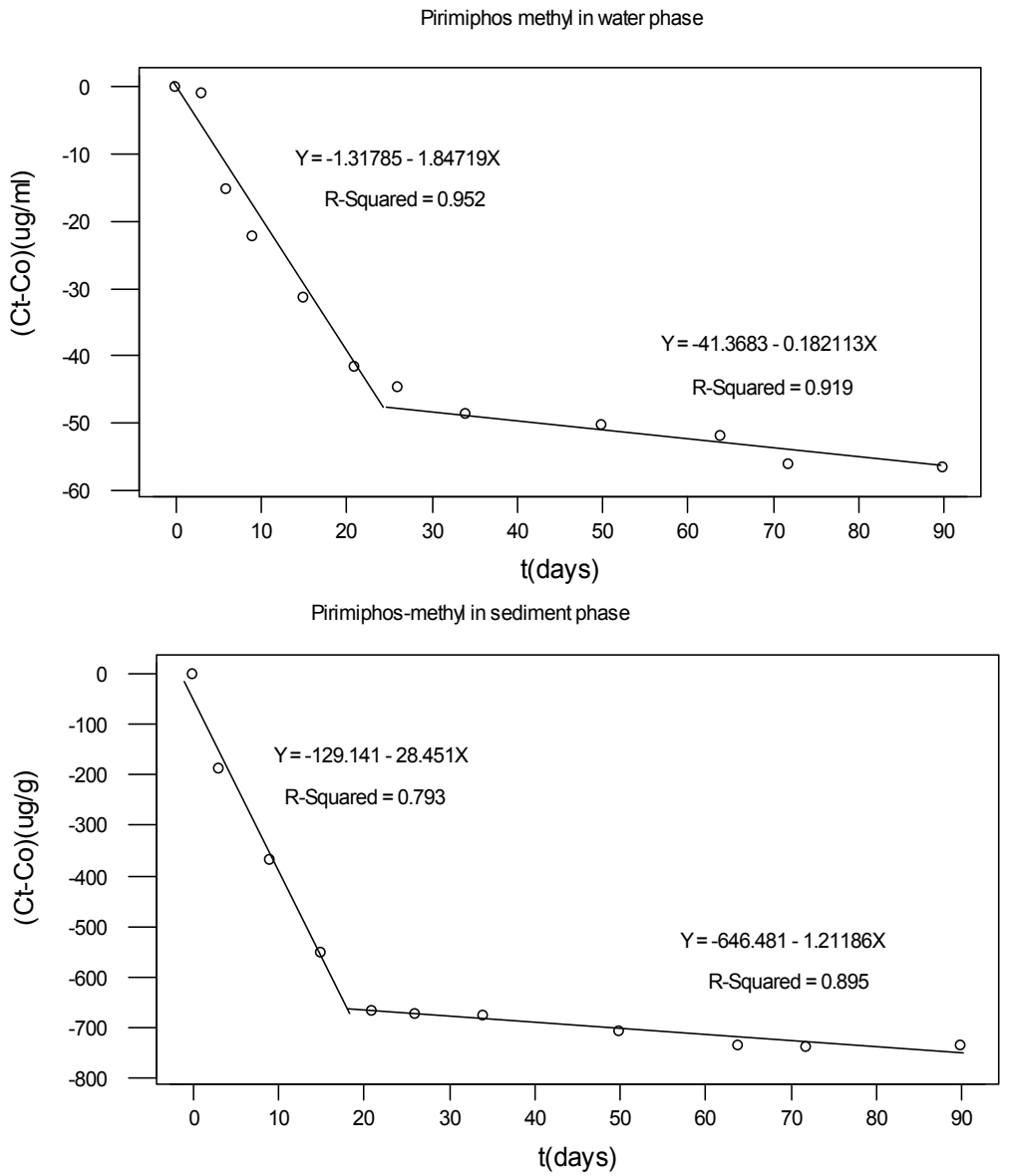

Figure 4. Rates of degradation of pirimiphos-methyl in the water phase and sediment phase of Kutsaga dam

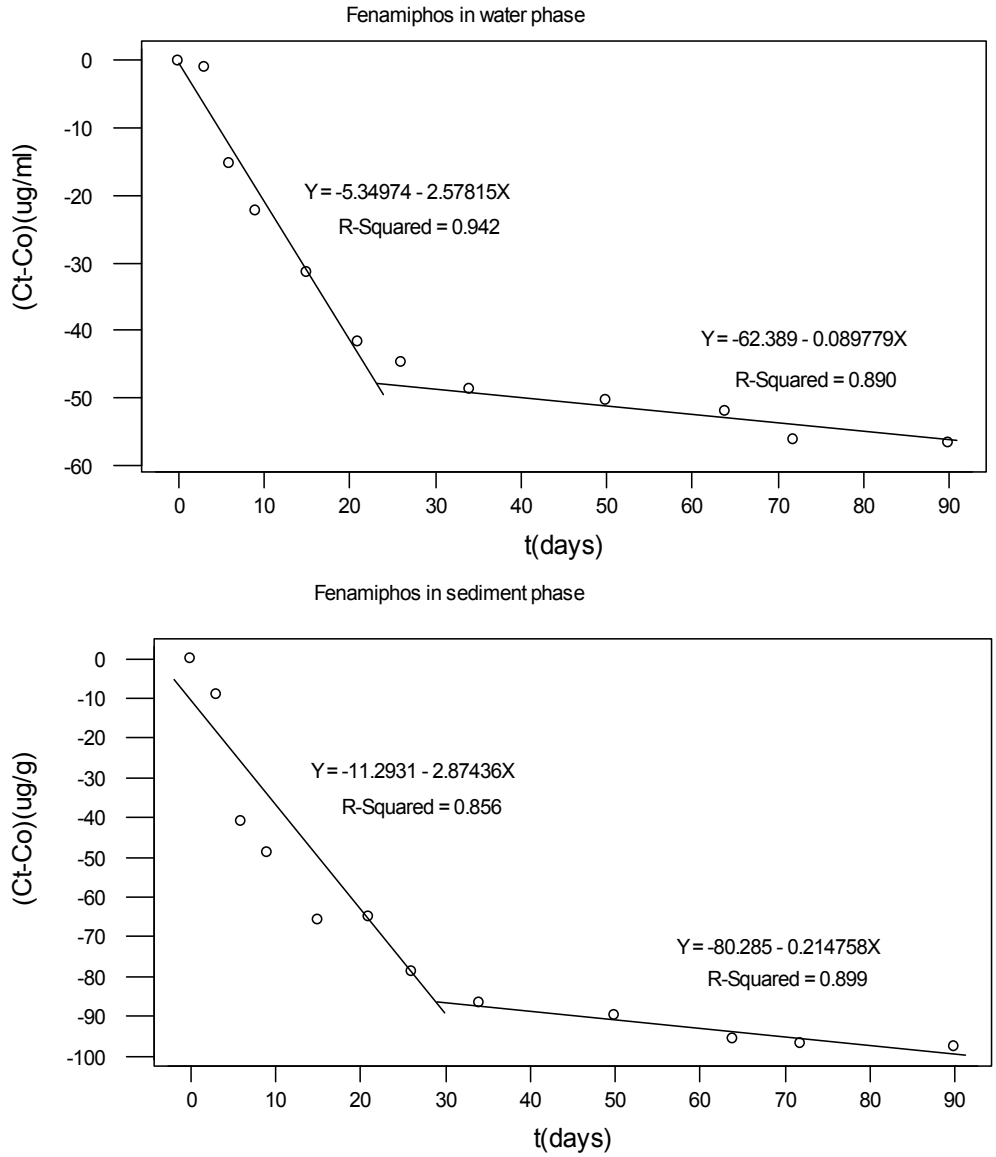

Figure 5. Rates of degradation of fenamiphos in the water phase and sediment phase of Kutsaga dam

Rate of degradation (μg/g / day)				Inference
Description	Chorpyrifos	Pirimiphos-methyl	Fenamiphos	
Fast, water phase	0.102	1.847	2.578	Free, in solution
Slow, water phase	0.022	0.182	0.098	Adsobed, in solution
Fast, sediment phase	48.43	28.48	2.874	Adsorbed onto sediment particle surface
Slow, sediment phase	0.217	1.212	0.215	Adsorbed onto sediment colloidal particles.

Table 5. Rates of degradation (μg/g / day) of chlorpyrifos, pirimiphos-methyl and fenamiphos in the sediment and water phases of the dam-water-and-sediment experiment.

3. Results and discussion

A heavy green algal growth developed in the Dam water plus sediment experiment after 6 days. The algal growth disappeared after 64 days. No algal growth was observed in the distilled water experiment. The results of material balance calculations based on day zero analysis data for the dam water-plus-sediment experiments in Table 4 show that (a) of the 14.9g chlorpyrifos charged into the experimental container, 11.77g and 2.27g respectively were found in the water and sediment phases, while the balance of 0.76g is attributed to adsorption by the walls of the container, (b) of the 15g pirimiphos-methyl charged into the experimental container, 11.7g and 1.79g respectively were found in the water and sediment phases, while the balance of 1.51g is attributed to adsorption by the walls of the container, and (c) of the 15g fenamiphos charged into the experimental container, 8.01g and 0.21g respectively were found in the water and sediment phases, while the balance of 6.78g is attributed to adsorption by the walls of the container. The amount of each pesticide lost by degradation over the 90 day period of study is shown in Table 6.

3.1. Contribution from chemical and photochemical degradation

Comparison of the data for the degradation of chlorpyrifos and fenamiphos in distilled water and the water phase of the river water plus sediment experiment, in Table 3 and Fig. 2 shows that, whereas in the distilled water experiment 2.6% and 5.3% degradation is achieved in 90 days respectively for chlorpyrifos and fenamiphos, in the water phase of the dam water plus sediment experiment 25.8% and 86.75% degradation respectively is obtained in the same period. This suggests that chemical degradation and photochemical degradation are minor routes for the degradation of chlorpyrifos and fenamiphos, if cognizance is taken of the fact that the 2.6% and 5.3% degradation observed in the distilled water experiments also contain a contribution from microbial degradation as a result of microbial contamination from the atmosphere, as no preservative was added to the distilled water control.

Pesticide	Speciation[a]	Rate of degradation (μg/g/day)	Persistence (days)	Phase volume/mass	Pesticide mass in speciation form (g).
Chlorpyrifos	Free, in solution (WP)	0.102	26	100 000 L	0.265
	CP adsorbed (WP)	0.022	>90	100 000 L	0.198
	SPS adsorbed (SP)	48.43	20	1930 g	1.869
	CP adsorbed (SP)	0.217	>90	1930 g	0.038
	Total				2.371
Pirimiphos-methyl	Free, in WP	1.847	25	100 000 L	4.618
	CP adsorbed (WP)	0.182	>90	100 000 L	1.638
	SPS adsorbed (SP)	28.48	20	1930 g	1.099
	CP adsorbed (SP)	1.212	>90	1930 g	0.211
	Total				7.565
Fenamiphos	Free, in WP	2.578	24	100 000 L	6.187
	CP adsorbed (WP)	0.098	>90	100 000 L	0.882
	SPS adsorbed (SP)	2.874	30	1930 g	0.111
	CP adsorbed (SP)	0.215	>90	1930 g	0.037
	Total				7.238

[a]WP = water phase; SP = sediment phase; CP = colloidal particle; SPS = sediment particle surface.

Table 6. Speciation and mass of the different speciation forms of chlorpyrifos, pirimiphos methyl and fenamiphos detected by the experiment.

3.2. Microbial degradation

From Table 3, it is apparent that 52.6%, 25.8% and 86.75% degradation was achieved in 90 days respectively for pirimiphos-methyl, chlorpyrifos and fenamiphos in the experiment. As explained above, most of this degradation is attributed to microbial degradation. From Figures 3 to 5, it is apparent that the degradation of the three insecticides in the water and sediment phases, can be resolved into two linear rates: an initial fast rate of degradation, followed by a slower rate. The rapid degradation in the water phase lasted for about 25, 26 and 24 days respectively for pirimiphos-methyl, chlorpyrifos and fenamiphos. In the sediment phase, the rapid degradation lasted for about 20 days for pirimiphos-methyl and

chlorpyrifos, and 30 days for fenamiphos. The slower rate of degradation lasted up to the end of the experiment period for all three pesticides.

The fact that both the fast and the slower rates of degradation of pirimiphos-methyl, chlorpyrifos and fenamiphos in the water phase, as well as the sediment phase, of the experiment are linear, points to steady state kinetics, and hence microbial degradation, whereby the observed rates correspond to the plateau in the Michelis-Menten curve (Zaranyika and Nyandoro, 1993; Zaranyika et al., 2010). The difference between the fast linear rate and the slow linear rate of degradation in the water phase of the experiment is attributed to adsorption of the pesticide by colloidal particles (Nomura and Hilton, 1977; Zaranyika and Nyandoro, 1993; Zaranyika et al., 2010).

Linear rates for biodegradation were reported previously by Siddique et al. (2003) when the biodegradation of α- and β-endosulfan by Fusanium ventricosum and a Pandoraea species was studied using zero order kinetics.

Table 4 summarizes the different rates of degradation obtained in the experimental ecosystem. The fast and slow rates of degradation in the water phase are attributed to degradation of free and colloidal particle adsorbed insecticide respectively, as has been discussed above. The existence of free and particle bound insecticide in water samples was previously demonstrated by Lee and Skemitt (1998). These workers developed a small-volume filtration method for the filtration of small volumes of water for separation of dissolved pesticide residues, with which they were able to demonstrate the existence of free and particle bound insecticide in water samples of moderate or low turbidity, using immunoanalysis. These workers were also able to show that in highly turbid water, antibodies were unable to recognize particle bound endosulfan as well as other pesticides. This is consistent with the microbial degradation mechanism being proposed above. If we assume that microorganisms will only bind the pesticide in the desorbed state, then the rate of insecticide desorption will be rate limiting for the colloidal particle adsorbed pesticide (Briggs, 1981; Nicholls, 1994).

3.3. Speciation trends of pirimiphos-methyl, chlorpyrifos and fenamiphos in the microcosm

Persistence data in Table 4 are presented in Fig. 6 in bar graph form. Comparison of the slow rates in the water phase and the slow rates in the sediment phase in Fig. 6 (B), shows that the slow rates in the water and sediment phases follow a similar pattern, i.e., degradation rates follow the order: chlorpyrifos < pirimiphos-methyl > fenamiphos. The similarity in the pattern of the slow rates in the water phase and sediment phase, suggests that the slow rates arise from the degradation of similar speciation forms. For the water phase, the slow rate can only be attributed to degradation of pesticide molecules adsorbed by colloidal particles within the water phase of the experiment, hence we conclude that the slow rate in the sediment also results from degradation of colloidal particle adsorbed pesticide.

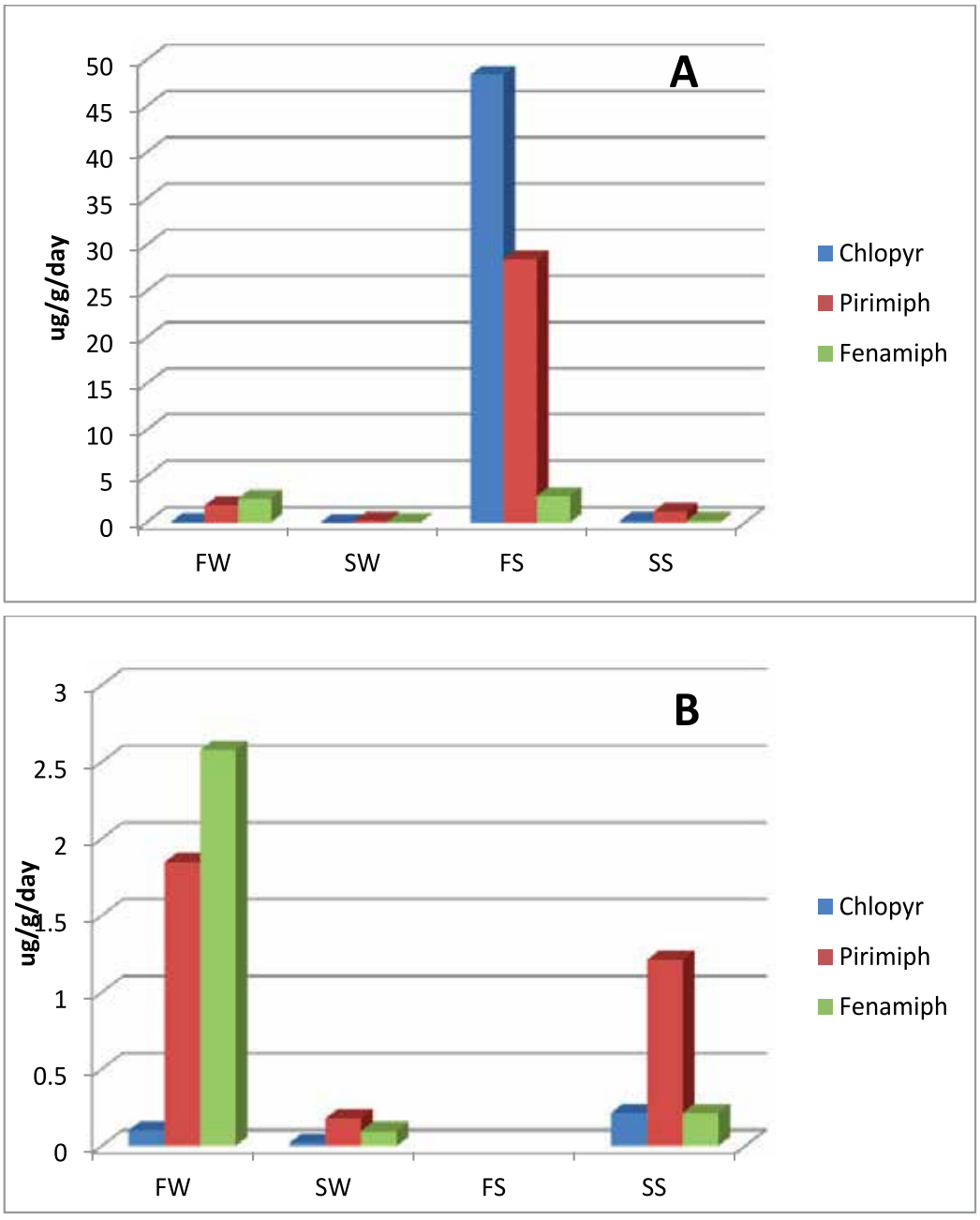

Figure 6. Persistence pattern of free and colloidal and/or sediment particle adsorbed insecticide: (Chlorpyr = chlorpyrifos; Pirimiph = pirimiphos-methyl; Fenamiph = fenamiphos; FW = fast degradation in water phase; SW = slow degradation in water phase; FS = fast degradation in sediment phase; SS = slow degradation in sediment phase; A: All 4 speciation forms; B: FS speciation form removed)..

It is recognized that the major adsorption interactions which bind small molecules in soil environments involve soil particles of colloidal dimensions (Burchill et al., 1981). Zaranyika and Mandizha (1998) proposed an adsorption/desorption equilibrium model whereby each colloidal particle, C, is associated with n pesticide molecules, X, thus:

$$nX + C \rightleftharpoons CX_n \quad (3)$$

and used a modified Freundlich isotherm to determine the value of n for the adsorption of amitraz [N'-(2,4-dimethylphenyl)-N-(((2,4,-dimethylphenyl)imino)methyl)-N-methyl-methanimidamide][33089-61-1] by a river sediment. A value of n of 0.26±0.03 was obtained, suggesting that a single molecule of amitraz was associated with 4 colloidal particles. The most likely structure of the adsorption complex in this case is one in which each phenyl ring of amitraz is sandwiched between two colloidal particles, and can be represented by XC_n, where n is the number of colloidal particles associated with each amitraz molecule. Different structures of such pesticide-colloidal particle complexes are expected, depending on the nature of the pesticide molecule and nature of the colloidal particles encountered in the ecosystem. Although equation 3 assumes a single-step desorption, in practice desorption of a pesticide molecule from such XC_n pesticide-colloidal-particle adsorption complexes in which $n > 1$ involves several steps, hence degradation of the pesticide should be very slow. Such adsorption complexes are to be expected in the bulk water phase, as well as in the sediment phase pore water.

A comparison of the pattern of fast rates in the water phase in Fig. 6(B), and the pattern of fast rates in the sediment in Fig. 6(A), shows that the pattern of rates is reversed in the sediment phase. This suggests a difference in the speciation of the pesticide in the water phase and sediment phase. Whereas the fast rate in the water phase is attributed to degradation of free pesticide molecules in solution, the degradation of the free pesticide in the pore water of the sediment appears to be influenced by the sediment. The pore water is in close contact with sediment particles, and a plausible explanation for the apparent difference in speciation of pirimiphos-methyl, chlorpyrifos and fenamiphos in sediment pore water is that molecules of pesticide in the pore water are involved in surface adsorption equilibria involving sediment particles, thus:

$$\vdash S + X \rightleftharpoons \vdash S\text{-}X \quad (4)$$

where $\vdash S$ is a surface adsorption site on a sediment particle. The rate of degradation will be limited by the rate of desorption of pesticide molecules from such surface adsorption complexes. These surface adsorption complexes are essentially 1:1 monolayer complexes, hence desorption is very fast. However we would expect rates of degradation to be slower than in the water phase because of these sediment particle adsorption equilibria. The much higher rates of degradation of the free pesticide in the sediment pore water has been attributed to greater microbial populations in the sediment. (Zaranyika and Nyandoro, 1993; Zaranyika et al., 2010).

3.4. Proposed degradation kinetic model

The degradation trends above are similar to those obtained by Zaranyika and Nyandoro (1993) for glyphosate , and Zaranyika et al. (2010) for endosulfan I and endosulfan II, as explained above. In terms of the speciation forms discussed above, data in Table 4 suggest that the degradation of pirimiphos-methyl, chlorpyrifos and fenamiphos occurs according to the steps shown in Table 7.

3.4.1. Fast degradation in water phase.

From Steps 2, it can be shown that

$$\frac{dP}{dt} = \frac{k_2 k_3 [X_B][E]}{k_{-2} + k_3} = k_E [X_B] \tag{5}$$

where k_E is the apparent rate constant for the enzymatic degradation of the microbial bound insecticide.

Step	Water phase		Sediment phase	
	Reaction[a]	Rate constant	Reaction[a]	Rate constant
1(a)	$X + M \rightarrow X_B$	k_1	$X + M \rightarrow X_B$	k_1
	$X_B \rightarrow X + M$	k_{-1}	$X_B \rightarrow X + M$	k_{-1}
2	$X_B + E \rightarrow XE$	k_2	$X_B + E \rightarrow XE$	k_2
	$XE \rightarrow X_B + E$	k_{-2}	$XE \rightarrow X_B + E$	k_{-2}
	$XE \rightarrow P + E$	k_3	$XE \rightarrow P + E$	k_3
1(b)	$X + nC \rightarrow XC_n$	k_4	$X + nC \rightarrow XC_n$	k_4
	$XC_n \rightarrow X + nC$	k_{-4}	$XC_n \rightarrow X + nC$	k_{-4}
1(c)			$X + S \rightarrow XS$	k_5
			$XS \rightarrow X + S$	k_{-5}

[a]X = insecticide; M = microorganism; X**E** = insecticide-enzyme complex; **E** = enzyme;
P = products; Subscript B = "microbial-bound"; C = colloidal particle; XC = insecticide-colloidal particle complex; S = sediment particle; XS= insecticide sediment particle complex.

Table 7. Degradation of chlorpyrifos, pirimiphos-methyl and fenamiphos in the aquatic environment: Proposed kinetic model.

From steps 1(a) , it can be shown that

$$[X_B] = \frac{k_1}{k_{-1}} [X][M] \tag{6}$$

Within the organism [E] >> [X_B], hence [E] = 1, and when [X] is in large excess of [M], [X] = 1, hence eq. 5 reduces to eq. 7:

$$\frac{dP}{dt} = k_E\left(\frac{k_1}{k_{-1}}\right)[M] = k'_E \tag{7}$$

where k'_E is the apparent linear rate of degradation of the pesticide in the water solution phase of the experiment. It is apparent from eq. 7 that k'_E is a function of the pesticide-micro-organism binding equilibrium constant, $K_B = k_1/k_{-1}$. The value of K_B will depend on the structure and properties of the pesticide, and micro organism type.

3.4.2. Slow degradation in water phase and sediment phase

From Steps 1(a) and 2, assuming [E] = 1, it can be shown that

$$\frac{dP}{dt} = \frac{k_1 k_2 k_3 [X][M]}{k_{-1}(k_{-2} + k_3)} \tag{8}$$

From Step 1(b)

$$[X] = \frac{k_{-4}[XC_n]}{k_4[C]^n}$$

hence eq. 8 becomes

$$\frac{dP}{dt} = \frac{k_1 k_2 k_3 k_{-4}[XC_n]_w[M]_w}{(k_{-2} + k_3)k_{-1}k_4[C]_w^n} = k_{Cw}[XC_n]_w \tag{9}$$

and

$$\frac{dP}{dt} = \frac{k_1 k_2 k_3 k_{-4}[XC_n]_s[M]_s}{(k_{-2} + k_3)k_{-1}k_4[C]_s^n} = k_{Cs}[XC_n]_s \tag{10}$$

where the subscripts w and s denote concentrations in the water phase and sediment phase respectively, and k_C is the apparent rate constant for the degradation of colloidal particle adsorbed pesticide. When [C] is in large excess of [X], [XC_n] is constant and assuming [M] is constant, Eqs. 9 and 10 reduce to

$$\frac{dP}{dt} = k'_{Cw} \tag{11}$$

and

$$\frac{dP}{dt} = k'_{Cs} \tag{12}$$

where k'_{Cw} and k'_{Cs} are the linear rates of degradation of the colloidal particle adsorbed pesticide in the water phase and sediment phase, respectively, of the experiment.

3.4.3. Fast degradation in sediment phase

From Step 1(c)

$$[X] = \frac{k_{-5}[XS]}{k_5[S]}$$

hence eq. 8 becomes

$$\frac{dP}{dt} = \frac{k_1 k_2 k_3 k_{-5}[XS][M]}{(k_{-2} + k_3)k_{-1}k_5[S]} = k_S[XS] \tag{13}$$

where ks is the apparent rate constant for the degradation of sediment particle adsorbed pesticide. When [S] is in large excess of [X], [*X*S] is constant and assuming [M] is constant, Eq. 13 reduces to

$$\frac{dP}{dt} = k'_S \tag{14}$$

where k'_S is the linear rate of degradation of the sediment particle adsorbed pesticide in the sediment phase of the experiment.

3.4.4. Overall rates of degradation in the water phase and sediment phase

From Table 4, the overall rate of degradation in the water phase of the experiment is given by the sum of Eq. 5 and Eq. 9, or Eq. 7 and Eq. 11, i.e.,

$$\frac{dP}{dt} = k_E[X_B]_w + k_C[XC_n]_w \tag{15}$$

or

$$\frac{dP}{dt} = k'_{E(W)} + k'_{C(W)} \tag{16}$$

whereas the overall rate of degradation in the sediment phase of the experiment is given by Eq. 13 and Eq. 10 or Eq. 14 and Eq. 12, i.e.,

$$\frac{dP}{dt} = k_S[XS] \ + k_C[XC_n]_{(S)} \tag{17}$$

or

$$\frac{dP}{dt} = k'_S + k'_{C(S)} \tag{18}$$

In the Introduction section we noted that persistence data for pesticides in the aquatic environment reported in the literature in terms of half-lives of the pesticide, in accordance with the conventional pseudo first order kinetics approach, are highly variable, whereas in true first order kinetics, a constant value should be obtained for the half-life irrespective of the actual environmental conditions prevailing. It is apparent from Eqs. 8, 9, 10 and 13 above that the linear rates of degradation obtained in terms of the steady state kinetic model presented above, depend on the type and density of the microbial organisms responsible for the degradation. The actual rates of degradation observed will also depend on the composition of the water and sediment, temperature and pH, in as much as these will affect the populations of the different microorganisms in the study medium. Thus variable rates of degradation are expected depending on the specific environmental conditions prevailing.

3.5. Possible pollution remediation strategies

Equations 8, 9 and 11 define the factors that affect the rate of degradation of pirimiphos-methyl, chlorpyrifos and fenamiphos in the aquatic environment. Any remediation measures for the abatement of pirimiphos-methyl, chlorpyrifos and fenamiphos pollution of aquatic ecosystems, must be designed to (a) maximize k_1, k_2, k_3, k_{-4}, k_{-5} and the density of microorganisms capable of degrading the pesticide, and (b) minimize k_4 and k_5, the rate constants for the adsorption of the pesticide by colloidal and sediment particles in the ecosystem. The rate constants k_1, k_2 and k_3 can be maximized by proper selection of the microorganism(s). Under such conditions, $k_3 \gg k_{-2}$, and assuming C and S are in large excess, eqs. 9 and 13 reduce to:

$$k_{C(\max)} = k_2 \left(\frac{k_1}{k_{-1}} \right) \left(\frac{k_{-4}}{k_4} \right) [M] \tag{19}$$

And

$$k_{S(\max)} = k_2 \left(\frac{k_1}{k_{-1}} \right) \left(\frac{k_{-5}}{k_5} \right) [M] \tag{20}$$

Many attempts are being made to find the optimal pH and the best microorganisms for the degradation of specific pesticides (Singh BK et al., 2003, 2006, Megharaj et al., 2003; Caceres et al., 2008; Cabrera et al., 2010; Yang et al., 2005; Salama et al., 1999). Equations 19 and 20 suggest that even after k_1, k_2, k_3 and [M] have been maximized, the rate of desorption of the pesticide can still be rate limiting.

Figures 3 to 5 show that the degradation of pirimiphos-methyl, chlorpyrifos and fenamiphos in the aquatic environment proceeds via biphasic linear rates in the water phase as well as the sediment phase. Similar results were reported previously for endosulfan I, endosulfan II and glyphosate, although only one linear rate was obtained for glyphosate in the sediment phase. This suggests that the kinetic model proposed above will also apply in the cases of endosulfan I, endosulfan II and glyphosate, and the comments made above regarding possible remediation strategies for pollution of aquatic ecosystem with pirimiphos-methyl, chlorpyrifos and fenamiphos, should also apply in the case of pollution of aquatic ecosystems with endosulfan and glyphosate.

4. Conclusions

From the foregoing discussion, we conclude that pirimiphos-methyl, chlorpyrifos and fenamiphos exist in two adsorption speciation forms in the aquatic environment. The adsorption forms consist of (a) a sediment particle surface adsorbed form which is highly labile, and in equilibrium with pirimiphos-methyl, chlorpyrifos and fenamiphos in solution in the sediment phase pore water; and (b) a slow degradation colloidal particle adsorbed form, found in the water phase as well as the sediment phase. The degradation of all speciation forms proceeds via linear rates, and is primarily due to microbial decomposition.

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Chapter 10

Heptachlor and Its Metabolite: Accumulation and Degradation in Sediment

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Additional information is available at the end of the chapter

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1. Introduction

1.1. Organochlorine pesticide and river delta

Chemical pesticides are substances that kill or control an uninvited organism, which all share the common property of blocking a vital metabolic process of the target to which they are toxic [1]. Practically pesticides have been a concern since their introduction, because of the potential impact on human health through chemical contamination and their accumulation in food chain and environment.

Organic pesticides were developed during and after World War II to displaced inorganic and metal organic substances such as sulfur, arsenic, sodium fluoride and boric acid, which used by society for thousands of year. Some of these pesticides were OCPs which are organic compounds that contain chlorine. These OCPs were preferred because of their effective against the target pests even if only small amount was applied to the environment. Neither small amounts of chemicals should enter the environment; however, the organic substances are generally much less toxic to human than the inorganic and metal organic compounds.

Many OCPs share several remarkable properties together with stability against decomposition or degradation in the environment and their very low solubility properties in water, unless oxygen or nitrogen is also present in the molecules. In addition, their high ability to dissolve in hydrocarbon-like environments, fatty material in living matter for example. And the important one is OCPs is relatively high toxicity to insects, but low toxicity to humans [1]. OCPs can enter the environment from direct application and runoff, disposal of contaminated wastes into landfills, emissions from waste incinerators, and releases from manufacturing plants that produce these chemicals. Some OCPs are volatile or can adhere to soil or particles in the air. In aquatic systems, OCPs are adsorbed onto sediments in water that can then bioaccumulate in fish and other marine mammals.

Massive volumes of OCPs have been widely used for more than two decades as the backbone of a pest control strategy. Their lipophilic nature and stability contribute to their high bioaccumulation potential and long persistence. Organochlorine compounds have become an interesting object of study all over the world and are monitored continuously because of their proven toxicity to human beings, animal and plant life, and also food chain. Residues of OCPs were detected in almost all environmental compartments including water bodies, food chain, as well as in humans. Soil is the main repository of these OCPs [2]. The contamination of soil and sediments with persistent OCPs may be related to point sources such as industrial discharges and waste plant effluents, but more frequently, it is attributed to diffuse sources, precipitation, agricultural runoff and particle transport.

Because of hydrophobic characteristics, OCPs are least soluble in water but show a high affinity for different surface area, and those with organic content show the highest adsorption capacity. The organochlorines bound with particulate matter ultimately sink, thereby enhancing the concentration of the pollutant in the bottom sediment. Therefore, sediment acts as one of the main sinks and potential sources for OCPs in the environment [3]. Various reports have already confirmed the contamination with OCPs from different parts of the world. Chang and Doong [4], reported the contamination of BHCs, aldrin, α- and β-endosulfan, dieldrin, endrin and DDTs in estuaries surface sediments of Taiwan. In the same year, Pandit et al. [5] also reported the distribution of BHCs and DDTs in coastal marine of Mumbai. In 2005, Wure and Obbard [6] studied the distribution of organochlorine compounds, BHCs and DDTs, in the sea surface microlayer, water column and sediment of Singapore's coastal environment. Additionally, Cheevaporn and Menasveta [7] reviewed and reported the water pollution in the Gulf of Thailand, which included some OCPs such as BHCs, DDTs, aldrin, dieldrin, α-endosulfan, heptachlor and heptachlor epoxide.

Although the major sink of organochlorine compounds is the sediment, such compounds can bioaccumulate in the biota, especially for the benthic organisms and then enter the food chain. Menone et al. [8] reported that the burrowing crabs, *Chasmagnathus granulatus*, showed bioaccumulation of OCPs from sediments. They suggested that the pesticide levels are more related to the physico-chemical characteristics (e.g. total organic carbon and particle size distribution) of the sediment where the pesticides remain. Boonyatumanond et al. [9] reported the concentration of OCPs compounds in green mussel, *Cara viridis*, the bioindicator for water assessment, in coastal waters along the Gulf of Thailand.

Agricultural pesticides most often are applied as liquids sprayed on the crop or the soil or as a seed treatment. An application of pesticide, depending on crop stage, formulation, intended target, application technique, and weather conditions is distributed between soil, plant foliage or crop residues, and losses due to drift. Chemicals used in the formulation of the pesticide can change the agronomic effects such as effectiveness and phytotoxicity of the formulated product. Chemicals also affect the environmental impact, as dispersion patterns may be altered and the functional activity period of the active ingredient may be lengthened or its degradation delayed. The pesticides that reach the soil or plant material in the target area begin to disappear by degradation or dispersion. Pesticides may volatilize into the air,

runoff or reach into surface water and groundwater, accumulate by plant or soil organisms or stay in the soil [10].

Human and ecological system exposure to micro-organic contaminants is related to their distribution between the various compartments of the environment (atmosphere, soils, surface fresh waters, ground waters, and oceans), to their transport between compartments, and to their availability for uptake and bioaccumulation by organisms. All the compartments have their importance but each has its own significance. The most obvious concern relating to the pollutants in the freshwater compartment is the possibility of adverse effects upon the freshwater ecosystem. In addition, micro-organics in rivers can be washed out to the seashore and so freshwater can also act as a major source of the pollutants to the oceans, where they may result in adverse ecological effects, particularly in the coastal zone. Human exposure can then occur through eating both freshwater and saltwater fish and other seafood. Within the freshwater compartment, it is particularly important to consider the sediment phase, because the largely hydrophobic nature of many micro-organic pollutants, which are known to associate strongly with natural sediments and dissolved organic matter. Sediments therefore have the strong potential to influence micro-organic pollutants fate in bed sediment, both freshwater and coastal marine.

2. Sources and transport of pesticides in freshwater sedimentary environments

The sources and transport of OCPs to and within freshwater sedimentary environments are obviously a crucial factor in determining the concentrations and the distribution of contaminants in freshwaters [11]. After applied to the soil zone, many pesticides transport to sedimentary systems, through leaching, surface run-off, spray drift, soil erosion and volatilization. A complex range of factors determines the fate of pesticides applied to agricultural land. These include the method of application of the active ingredient, and the weather conditions such as temperature, wind direction and speed, and rainfall following the application. The antecedent conditions, topography, soil type, farming practice and crop grown on the land, also affect water movement and dispersal of pesticides. These factors are reported to be more important than the physico-chemical parameters of the compound [12].

Other major routes of these organics compounds into freshwater sedimentary environments include direct discharges from industry, treated effluent discharge from both domestic and industrial sources, storm-water discharge from storm drain and aerial deposition. When wastewaters are treated before discharge to rivers and lakes, the concentration of a range of pesticide in the effluents is likely to be lower than in direct discharges because of possible sorption to solids and degradation during waste treatment. A huge range of the compounds is used and produced in industrial processes and may be discharged either from treatment plants or direct into freshwater.

In some situations, industrial effluents are a very complex mixture of the OCPs and it is impossible to know the entire chemical composition of even the commercial products used

in manufacturing processes. This is because many commercial formulations are not pure chemicals but contain by-products of the production process. This, together with the metabolites formed during sewage treatment, leads to a wide range of compounds delivered to freshwater from a single commercial-product source. Hence any investigation of a point-source discharge containing micro-organic compounds must consider not only compounds that have been designed to be toxic, such as pesticide, but a wide range of commercial and natural products (possibly containing impurities not essential for the product application) that may have partially degraded in the pipeline or in the wastewater treatment process. Moreover, storm-water discharges, overflow from roadside and other urban drains during heavy rainfall events, can be sources of a range of micro-organic compounds to freshwaters.

3. The association of pesticides with sedimentary material and dissolved organic matter

In freshwater, micro-organic contaminants from OCPs can exist in a variety of forms: as a freely dissolved phase, as a colloidal phase or associated with sedimentary material. In terms of the fate and effects of micro-organics between these various phases is a central issue. It strongly influences bed and suspended-sediment concentrations, freely dissolved concentrations (both in pore-waters and in the overlying water) and colloidally bound concentrations, and therefore also have a strong influence on sediment and water quality [11]. Natural sediments are complex mixtures of inorganic mineral offer environments, which may be favorable for micro-organics to become associated with. The association of micro-organics with sediments and soils is known as sorption, a general term, which covers two types of process, adsorption and absorption. Outstanding to the compositional complexity of sediments, sorption is not, however, the result of a single process, but may result from both adsorption and adsorption in/on a range of matrices.

Behavior of pesticides in soil, suspended sediment and bed sediment were determined by several processes including degradation by soil microorganism, chemical degradation (e.g. hydrolysis), sorption and binding by organic and mineral soil components, uptake by plant roots, volatilization and the diluting effects of water flow process.

Losses of pesticide in the soil via microbiological and chemical pathways are collectively termed degradation. Rate of degradation in soil usually increases with temperature and with soil water content [13]. Half-lives can be very long in dry soil. Degradation is often described satisfactorily by simple exponential decay, which assumes that the amount degraded per unit time is directly proportional to the amount present. The rate of degradation can be characterized by a half-life (DT_{50}). As pesticide degradation products (metabolites) may have environmentally undesirable characteristics, the evaluation of the environmental fate of a pesticide should also take the fate of its major metabolites into consideration. However, the pesticide properties of the chemicals remain unaffected.

Field-measured half-lives generally are shorter than those measured under controlled laboratory conditions because of first, multiple degradation pathways operating under field

conditions, resulting in more rapid degradation. The last reason is losses by volatilization and photodecomposition. Both processes are occurring in the field and not in the laboratory field-measured soil; half-lives may also include dissipation losses by runoff, leaching and uptake by plants, which are not degradation pathway.

The sorption of pesticide on soil reduces its mobility, with the extent of reduction dependent upon the physical and chemical properties of the soil as well as the molecular characteristics of the pesticide. Since soil organic matter is the primary soil constituent responsible for sorption of non-ionic pesticides, a sorption constant based on only the organic carbon present (K_{oc}, $dm^3\ kg^{-1}$) can be used to assess pesticide mobility, $K_{oc} = K_d/oc$. Where K_d is a measure of the extent of pesticide sorption by the soil, and oc is the fraction of the organic C present in the soil [14]. A sorption constant expressed in this manner is dependent only on the pesticide and is essentially independent of soil type. However, in soils with extremely low or high organic C and for ionizable or ionic pesticides the use of K_{oc} to estimate K_d may be subject to large error.

A significant proportion, typically ranging from 20 to 70%, of a pesticide or its degradation products may remain in the soil as a persistent residue bound to the soil colloids [15]. In this bound state, these compounds are difficult to extract and characterize, and tend to lose their biological activities. Many pesticides formerly believed to be readily degraded and lost from the soil were later shown to form these bound residues. Although no firm evidence is available, concern has been expressed that these bound residues may be released and be taken up by crop plants or leach into groundwater.

In any water body there are areas of bed-sediment erosion and of sediment accumulation, largely determined by flow and wave patterns. Fine suspended sediment particles, weather from external influxes to the water body or from resuspended of bed sediments will be deposited, in the long term, in accumulation zones. In general, fine silty sediments will also have a higher organic C content than coarse sandy sediments. Because of these and because of their higher surface area to volume ratio, fine sediments are likely to contain higher concentrations of micro-organic compounds, on a weight for weight basis. The overall effect is that away from direct sources, bed-sediments in accumulation zones are likely to have a much higher loading of micro-organic compounds than bed sediments in other areas [11].

The effects of flow rate can also be very important in the transport of micro-organics in freshwaters. In relatively slow-flowing rivers such as the Rivers Aire and Calder in Yorkshire, bed and suspended sediments have been found to contain relatively high concentrations of a range of pesticides, notably including the synthetic pyrethroids [16]. In contrast, some fast-flowing rivers have high self-purification ability against pollution, with high water-discharge and sediment loads. This is the case for a number of rivers in some catchments in western China, where in spite of heavy industrial discharges of HCHs, DDT and PCBs, relatively low concentrations have been found in surficial river sediment (depth <3 cm) [17] compared with the concentrations found in other sediment surveys [18]. Where contaminated river have high purification ability because of rapid flushing and bed-scouring, compounds that are not found in river sediments may accumulate in coastal zones, estuaries and lakes.

Spatial variations in suspended-sediment-bound concentrations, as well as in bed sediment concentrations, can occur because of different sedimentation and flow regimes. Spatial variations in suspended-sediment-bound concentrations can also reflect source differences [16]. There is also likely to be a dilution of suspended sediments carrying contaminants by clean suspended material as the distance away from the source increases [19]. Temporal variations in non-tidal stretches of rivers usually result from changes in flow levels into and through the river, often varying with season [16]. Moreover, temporal variations can be complex if the source of the micro-organic compound is flow-related as in the case of resuspension of contaminated bed sediments, runoff from storm drains and erosion of contaminated soils, for example due to flooding. Otherwise, high flow rates giving rise to increased sediment transport will act to dilute suspended-sediment micro-organic compound concentrations.

4. Degradation of organochlorine pesticides in freshwater sedimentary environment

Degradation of OCPs contaminants in freshwater environments can occur either in the water column, or in the sediment bed following deposition. For pesticides in particular, compound half-lives and stabilities under various abiotic conditions are available from manufactures' information. However, the situation is complicated in natural waters by the presence of varied microbial populations, suspended sediments, dissolved ions, dissolved organic matter and the sediment bed. Sediment, in particular, has the potential to strongly influence degradation, because many micro-organic compounds are known to associate strongly with sediments. In addition, microbial populations are also known to be largely associated with surfaces in the environment rather than living free in solution.

Those OCPs contaminants can undergo degradation, either abiotically, through purely chemical or photochemical pathways, or biologically, with the microorganism assistant. Degradation can be complete, with compounds being mineralized to form CO_2 and other simple chemical species, or partial, with the resultant degradation products more closely resembling the parent species. Different degradation pathways can occur simultaneously, with the relative importance of the various pathways being dependent upon the compound and the environmental conditions. Even if it is difficult to generalize because of the multitude of compound and types of environment, biotic degradation pathways are often found to be more important in the degradation of micro-organic contaminants. In particular, microbial pathways usually lead to complete mineralization of microorganism compounds.

5. Abiotic degradation pathways

The two most important types of abiotic chemical degradation reactions are hydrolysis, usually either acid or based-catalyzed and redox reactions. Hydrolysis can be a significant

degradation pathway for compounds containing ester, ether or amide functional groups, s-triazine and carbamate for example, and for substituted haloalkanes. In terms of chemical oxidation, the major oxidants present in the environment are O_2, and Fe (III) and Mn (III) and (IV) oxides, with susceptible compounds including anilines and polyalkylated phenols. Whilst reduction is known to be a degradation route for a wide range of micro-organic compounds under appropriate environmental conditions, it is often difficult to identify the chemical reducing agents responsible. It is generally thought that low abundance but highly reactive species such as quinoid-type compounds, Fe porphyrines and some transition metal ions may be involved [11].

In terms of abiotic photochemical degradation or photolysis, there are few micro-organic contaminants including highly conjugated species such as polyene, nitroaromatics and some polyaromatic compounds, that will degrade directly following absorption of natural light. Indirect photolysis, in which dissolved natural organic matter becomes electronically excited after absorbing natural light, resulting in the production of a wide range of highly reactive species such as the hydroxyl radical (OH), which then may react with micro-organic pollutants. It is thought to be the more significant photolysis pathway for micro-organic compounds, with alkyl-substituted phenols, anilines, mercaptans, furans and other dienes being particularly susceptible [11].

6. Biotic degradation

Microbial populations are responsible for the vast majority of biotic transformations of micro-organics in the environment. Microorganisms involved in five basic types of transformation including, microbial metabolism, in which the compound serves as a growth substrate, which leads to mineralization and co-metabolism, in which the compound is transformed by metabolic processes, but does not serve as an energy source. The third transformation type is polymerization or conjugation, in which microbial reactions result in the polymerization of the compound with natural organic matter. The last two transformation types are accumulation, in which the compound is incorporated into the tissues of the organism itself, but not for growth, and secondary effect transformations, whereby transformation results from changes in redox potential or pH as a result of microbial reactions.

7. The study area

The study area is Mae Klong River basin, which is located in the central plain of Thailand. The climate in Thailand can be divided into 3 seasons including winter (October-February), summer (February-May) and rainy season (May-October). Approximate annual rainfall levels are up to 1,100 mm in the rainy season (85% in May to September). The temperatures are between 25-40^{o}C. The humidity is around 56-79% [20].

The Mae Klong River basin is one of three major river basins in the Chao Phraya Delta, which are an important agricultural area in rice production and other agronomic in

Thailand for some centuries. The other two major river basins are the Chao Phraya and Tha Chin River basins. The Mae Klong River basin covers a total area of 30,800 km^2 in the west central of Thailand with the very flat delta. It is periodically flooded in rainy season (mostly during August to November) [21]. The most important farming activities in this region are rice paddies and orchards including coconut, lime, lychee and mango. The main waterway in this basin is Mae Klong River which ends at the Gulf of Thailand in Samut Songkhram Province, and has the length of 132 km. The river receives water from both the irrigation canal systems and natural tributaries, which flow through cultivated areas where OCPs are intensively used to improve crop yields [7,22]. The discharge of untreated waste from agriculture is a major source of OCPs contamination in this area, and has been a major concern to citizens and officials for more than two decades [7,20,22]. Some organochlorine pesticides accumulation and their degradation along Mae Klong River basin and some of its tributaries will be discussed in this chapter.

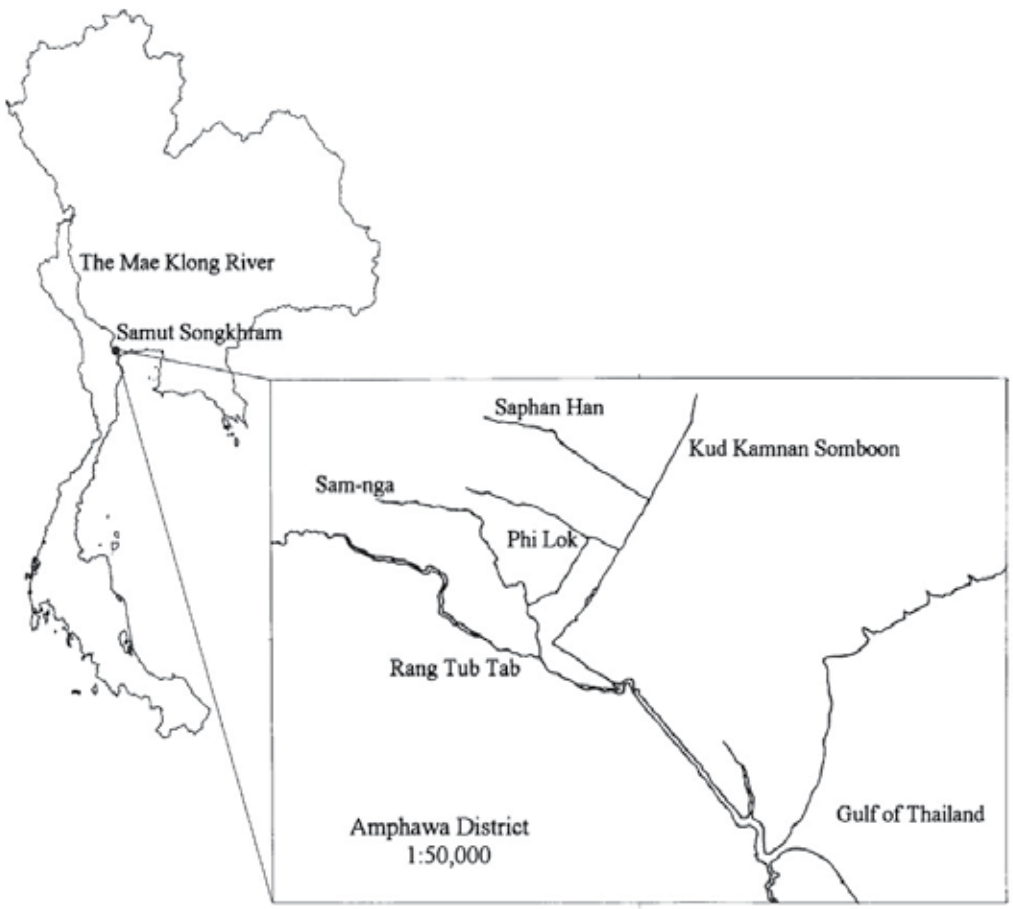


Figure 1. Map of Thailand showing the five tributaries of the Mae Klong River in the study area (Poolpak et al., 2008)

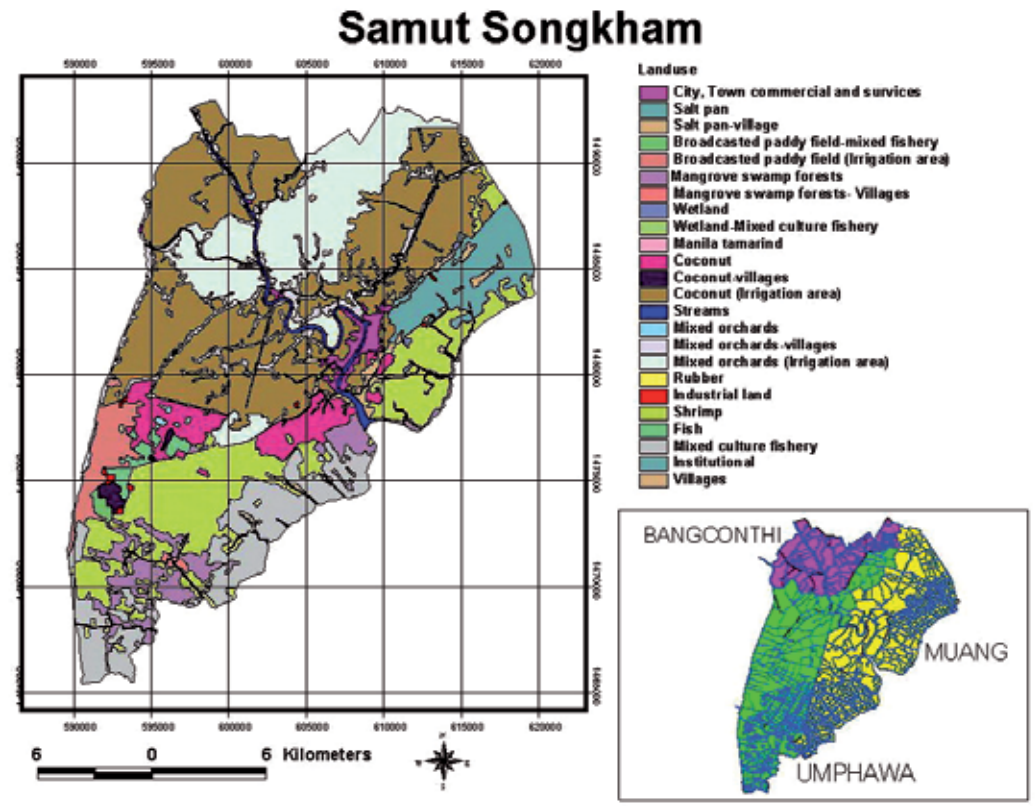


Figure 2. The land use in Mae Klong Basin in the area of Samut Songkram Province with the major land use is agricultural area (Poolpak et al., 2008)

The five sampling sites were on the Mae Klong River tributaries at Tumbon Prag Nam Dang, Amphawa, Samut Songkram Province (Figure 1). These tributaries are also major sources for household and industrial water supply in Samut Songkhram Province. As showed in Figure 2, the areas surrounding the sampling sites are a traditional rice-cultivation area in which farmers cultivate rice twice per year. The first crop (rainy-season rice) cultivation typically starts in August and harvested in November or December. An irrigated dry-season crop is planted in April or May and ends in July or August. Five sites were chosen in such a way as to include hot spots of pollution discharges along the Mae Klong River such as industrial regions, paddy fields and orchards for the first year. For the second year of monitoring, sediment samples were collected from Rang Tub Tab canal (site R) and Phi Lok canal (site P), which is an important crossing point of several tributaries in study area. Site R is also the tributary connected to the Gulf of Thailand. Sediment samples were collected monthly for a period of two years, during August 2003 to February 2005. The study areas and sampling locations along the selected tributaries were illustrated in figure 3. Furthermore, the basic physico-chemical properties of natural surface sediment samples within the area are shown in Table 1.

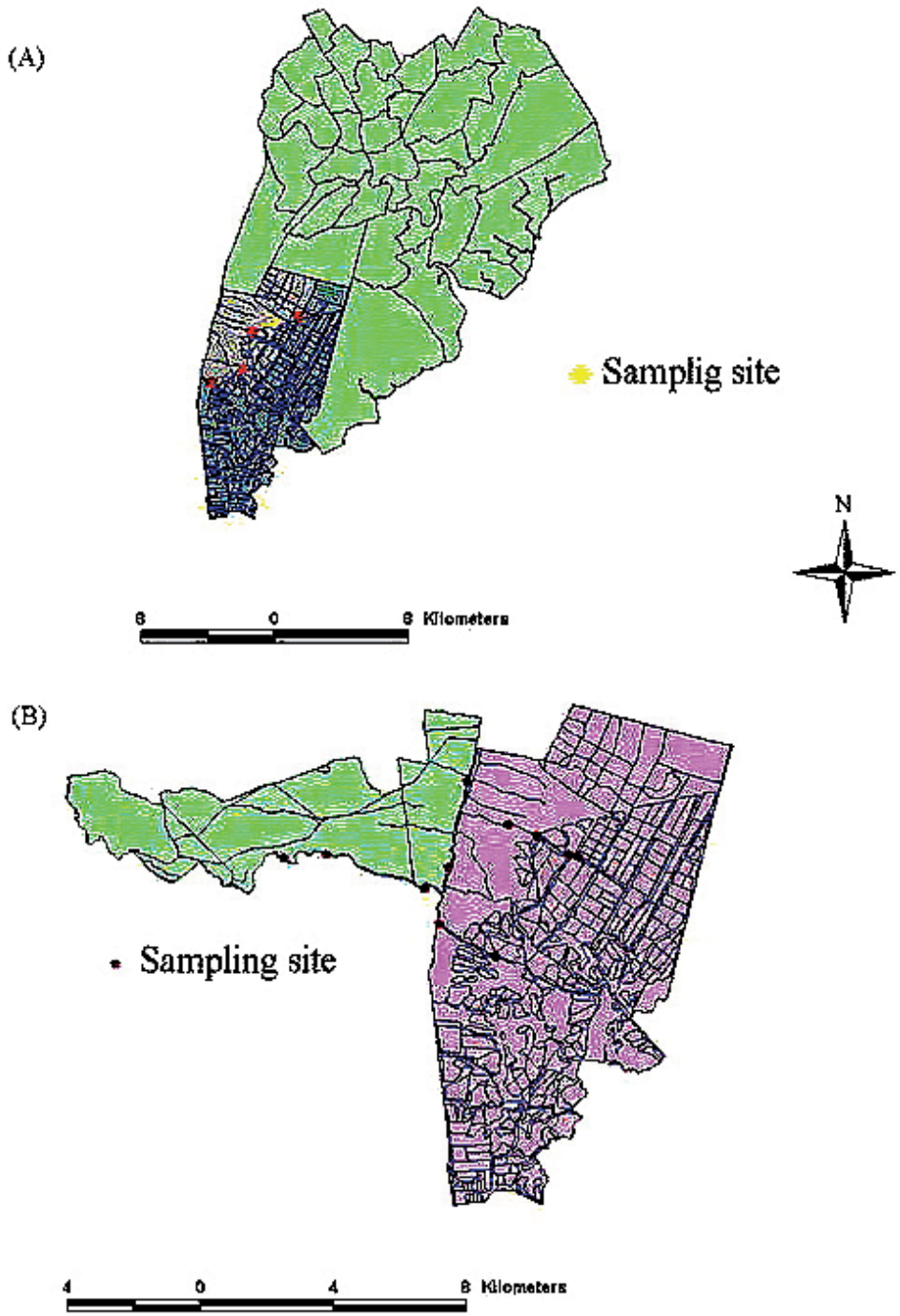


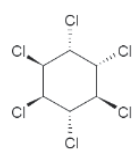
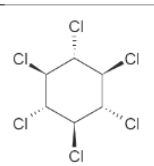
Figure 3. The study areas and sampling locations along the selected tributaries during August 2003 to July 2004 (A) and March 2004 to February 2005 (B) (Poolpak et al., 2008)

Sampling year	Sampling sites	Geographical coordinate	Water content (%)	pH	Sand (%)	Silt (%)	Clay (%)
2003	Kud Kamnan Somboon (K)	(598863N, 1475923E)	50.79	5.9	20	24	56
	Saphan Han (SP)	(598450N, 1475000E)	64.15	5.8	30	21	49
	Phi Lok (P)	(595992N, 1475052E)	56.88	5.7	25	23	52
	Sam-nga (SN)	(595235N, 1472125E)	64.41	5.6	32	20	48
	Rang Tub Tab (R)	(593496N, 1472125E)	61.79	5.8	29	30	41
2004	Phi Lok (P)	(595986N, 1472125E; 594718N, 1471496E; 595133N, 1475347E)	59.34	5.7	24	23	53
	Rang Tub Tab (R)	(588357N, 1474380E; 589628N, 1474503E; 592581N, 1473464E; 593310N, 1474090E; 593059N, 1472441E; 593899N, 1476626E)	42.44	5.8	29	30	41

Table 1. Sampling areas and codes of the tributaries of Mae Klong River, their coordinates and the basic physicochemical properties of natural surface sediment samples within the area

8. OCPs accumulation and their distribution in study area

The organichlorine compounds in this investigation were hexachlorocyclohexane (technical HCH including α-HCH, γ-HCH, β-HCH, δ-HCH), heptachlor and its epoxide, α-, β-endosulfan and endosulfan sulfate, DDT, DDE, and DDD. They were chosen in the subject of their persistence and their importance in agricultural activities. The chemical structures and some properties of the selected OCPs were presented in table 2 and 3.

Name	Molecular formula	Molecular weight	Chemical structure
α-HCH	$C_6H_6Cl_6$	290.83	
β-HCH	$C_6H_6Cl_6$	290.83	

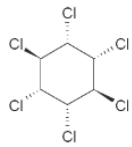
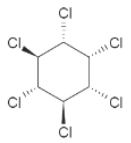
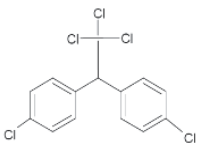
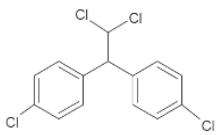
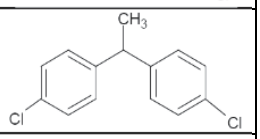
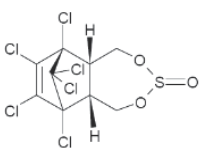
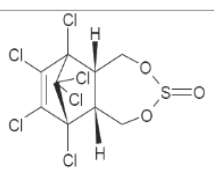
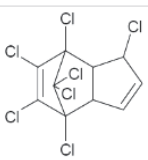
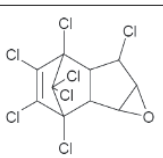
Name	Molecular formula	Molecular weight	Chemical structure
γ-HCH	$C_6H_6Cl_6$	290.83	
δ-HCH	$C_6H_6Cl_6$	290.83	
DDT	$C_{14}H_9Cl_5$	354.49	
DDD	$C_{14}H_{10}Cl_4$	320.05	
DDE	$C_{14}H_{12}Cl_2$	318.03	
α-Endosulfan	$C_9H_6Cl_6O_3S$	406.93	
β-Endosulfan	$C_9H_6Cl_6O_3S$	406.93	
Heptachlor	$C_{10}H_5Cl_7$	373.32	
Heptachlor epoxide	$C_{10}H_5Cl_7O$	389.32	

Table 2. Organochlorine compound structures and their molecular properties

Substance	Melting Pt[a]	Boiling Pt[a]	Vapor pressure[b]	Water solubility[c]	Log P	Henry's law law constant[d]	Atmospheric OH rate constant[d]
α-HCH	159.5	228	4.50E-05	2	3.80	1.22E-05	1.40E-13
β-HCH	314.5	60 at 5.80E-01 mmHg	3.60E-06	0.24	3.78	4.40E-07	5.73E-13
γ-HCH	112.5	323.4	4.20E-05	7.3	3.72	5.14E-06	1.90E-13
δ-HCH	141.5	60 at 3.40E-01 mmHg	3.52E-05	10	4.14	4.29E-07	5.73E-07
DDT	108.5	NA	1.60E-07	0.0055	6.91	8.32E-06	3.44E-12
DDD	109.5	350	1.35E-06	0.09	6.02	6.60E-06	4.34E-12
DDE	56	320	6.00E-06	0.04	6.51	4.16E-05	7.43E-12
α-Endosulfan	NA	NA	3.00E-06	0.51	3.83	7.09E-06	8.17E-12
β -Endosulfan	NA	NA	6.00E-07	0.45	3.83	3.91E07	8.17E-12
Endosulfan sulfate	181-182	NA	2.80E-07	0.48	3.66	3.25E-07	8.17E-12
Heptachlor	95.5	310	4.00E-04	0.18	6.10	2.94E-04	6.11E-11
Heptachlor epoxide	160	NA	1.95E-05	0.2	4.98	2.10E-05	5.17E-12

NA indicates that the property is not applicable
[a] at 1 atmosphere, ºC
[b] at 25ºC, mmHg
[c] at 25ºC, mg/L
[d] at 25ºC

Table 3. Physical and physico-chemical properties of some organochlorine pesticides

The variations of the OCPs levels for different rice-cropping seasons in surface sediments from the five tributaries during August 2003 and July 2004 are summarized in Table 4, whereas Tables 5 show the mean concentration of OCPs residues in the sediment samples taken along site P and site R during March 2004–February 2005. Despite the official ban and restriction on the usage of some OCPs, pesticide residues were detected in all sampling sites [20].

The range of total OCPs concentration in the sediments from five tributaries were 0.09-96.48 μg/g dw at site K, 0.01-110.92 μg/g dw at site SP, 0.06-102.05 μg/g dw at site P, 0.11-84.00 μg/g dw at site SN and 0.02-274.16 μg/g dw at site R. In the second year of observation, sites P and R were then chosen for further monitoring. Total residue concentrations of OCPs ranged from 0.15 to 99.60 at site P and 0.14 to 129.82 at site R. Comparing with the others, site R was found to be the most OCPs contaminated site in this study.

The higher concentration of OCPs at five selected tributaries indicated the high irrigation of these compounds in and around the basin. These OCPs are believed to have originated from various pesticide-rich sources, mainly agricultural areas and household. The natural processes of leaching and runoff and irrigation are likely to enhance their transfer to the main course of these tributaries, especially during the rice cultivation season [20].

The relatively high pesticide levels at site R can be explained from the input of pollutants in discharge from agricultural fields. This tributary was connected to and received water from other tributaries so it may contain pesticides from extraneous areas as well as from the immediate surroundings.

OCPs residues in dry-season rice cultivation periods were higher than during the wet season in both years. This meant that some OCPs could be released from the surrounding areas to the tributaries in the irrigation from paddy fields. Moreover, the still water in dry season is one of the major basis. Decreased OCPs concentrations in the rainy-season cultivation can be explained in term of lower upstream discharge and dilution effects. Where non-agricultural activities in cool season infer to the low levels of the chemicals found in this sampling season [20].

Pesticides	Rainy –season cultivation[a]					Non-rice cultivation[b]					Dry-season rice cutivation[c]				
	K	SP	P	SN	R	K	SP	P	SN	R	K	SP	P	SN	R
α-HCH	0.05 (0.06)	0.25 (0.18)	0.12 (0.10)	0.25 (0.18)	0.33 (0.08)	0.28 (0.05)	0.28 (0.20)	0.26 (0.11)	0.25 (0.14)	0.30 (0.13)	0.26 (0.09)	0.39 (0.07)	0.35 (0.00)	0.41 (0.04)	0.33 (0.08)
β-HCH	0.11 (0.02)	0.18 (0.02)	0.13 (0.04)	0.17 (0.02)	0.21 (0.08)	0.17 (0.06)	0.02 (0.02)	0.17 (0.03)	0.18 (0.03)	0.23 (0.07)	0.21 (0.10)	0.18 (0.02)	0.15 (0.02)	0.19 (0.03)	0.33 (0.19)
γ-HCH	0.21 (0.05)	0.35 (0.00)	0.26 (0.05)	0.32 (0.06)	0.30 (0.05)	0.23 (0.04)	0.35 (0.00)	0.29 (0.00)	0.28 (0.09)	0.33 (0.00)	0.23 (0.04)	0.35 (0.00)	0.29 (0.00)	0.32 (0.06)	0.31 (0.06)
δ-HCH	0.54 (0.24)	0.91 (0.41)	0.76 (0.72)	0.68 (0.22)	3.31 (1.32)	0.86 (0.81)	1.28 (1.51)	1.15 (1.13)	1.67 (2.49)	1.99 (1.01)	2.64 (1.08)	2.89 (2.13)	1.96 (1.35)	1.85 (1.22)	2.95 (0.95)
Heptachlor	0.06 (0.07)	0.15 (0.10)	0.21 (0.19)	0.13 (0.10)	0.19 (0.14)	0.04 (0.04)	n/a	0.09 (0.07)	n/a	0.25 (0.19)	0.10 (0.04)	0.13 (0.05)	0.15 (0.11)	0.21 (0.12)	0.61 (0.91)
Heptachlor epoxide	11.61 (4.39)	13.99 (5.96)	16.52 (20.78)	9.23 (7.87)	63.22 (24.72)	20.41 (25.67)	20.82 (27.51)	29.79 (38.96)	33.01 (56.12)	58.77 (49.73)	64.46 (29.63)	76.11 (44.67)	55.74 (19.57)	41.76 (26.84)	152.17 (55.50)
Endosulfan I	0.12 (0.00)	0.16 (0.00)	0.13 (0.00)	0.16 (0.00)	0.15 (0.00)	0.11 (0.02)	0.15 (0.03)	0.13 (0.00)	0.16 (0.00)	0.16 (0.02)	0.10 (0.02)	0.17 (0.02)	0.11 (0.03)	0.16 (0.00)	0.12 (0.05)
Endosulfan II	0.03 (0.02)	n/a	0.05 (0.04)	0.01 (0.03)	0.05 (0.04)	0.03 (0.03)	0.01 (0.03)	0.04 (0.02)	0.01 (0.03)	0.14 (0.25)	0.03 (0.07)	n/a	0.05 (0.04)	0.09 (0.06)	0.07 (0.05)
Endosulfan sulfate	0.07 (0.06)	0.13 (0.03)	0.08 (0.05)	0.15 (0.04)	0.15 (0.03)	0.11 (0.05)	0.08 (0.08)	0.17 (0.08)	0.07 (0.10)	0.38 (0.46)	0.15 (0.01)	0.21 (0.07)	0.19 (0.02)	0.19 (0.02)	0.58 (0.51)
p,p′-DDE	0.08 (0.03)	0.04 (0.08)	0.09 (0.06)	0.06 (0.08)	0.11 (0.04)	0.09 (0.08)	0.17 (0.10)	0.13 (0.09)	0.12 (0.14)	0.16 (0.07)	0.10 (0.03)	0.21 (0.04)	0.12 (0.06)	0.18 (0.08)	0.16 (0.07)
p,p′-DDD	0.01 (0.02)	0.06 (0.12)	0.03 (0.03)	0.02 (0.03)	0.02 (0.03)	0.13 (0.05)	0.03 (0.04)	0.03 (0.03)	0.03 (0.06)	n/a	0.14 (0.10)	0.13 (0.27)	n/a	0.11 (0.18)	n/a
p,p′-DDT	0.13 (0.08)	0.17 (0.12)	0.14 (0.13)	0.26 (0.11)	0.08 (0.05)	0.26 (0.07)	0.31 (0.13)	0.25 (0.05)	0.38 (0.05)	0.20 (0.14)	0.37 (0.11)	0.35 (0.08)	0.22 (0.10)	0.37 (0.03)	0.63 (0.74)

n/a means no available

[a] August 2003-November 2003

[b] December 2003-March 2004

[c] April 2004-July 2004

Table 4. Mean and standard deviation (S.D.) of OCPs (μg g-1 dw) detected in the surface sediment in five tributaries of the Mae Klong river during August 2003-July 2004 (n=60/cultivation period)

Pesticides	Dry–season cultivation[a]		Rainy-season rice cultivation[b]		Non-rice cutivation[c]	
	P	R	P	R	P	R
α-HCH	0.21 (0.15)	0.28 (0.15)	0.32 (0.06)	0.02 (0.03)	n/a	0.33 (0.08)
β-HCH	0.93 (0.83)	3.66 (3.29)	0.12 (0.02)	0.09 (0.02)	0.11 (0.02)	0.08 (0.19)
γ-HCH	0.18 (0.08)	0.21 (0.12)	0.08 (0.13)	0.05 (0.08)	0.02 (0.02)	0.01 (0.06)
δ-HCH	5.50 (3.32)	10.09 (6.31)	1.08 (1.28)	0.82 (0.94)	0.64 (0.23)	0.43 (0.95)
Heptachlor	0.53 (0.59)	2.16 (2.61)	0.04 (0.09)	0.04 (0.04)	0.04 (0.03)	0.02 (0.02)
Heptachlor epoxide	72.43 (30.90)	110.39 (56.79)	19.51 (28.43)	14.84 (21.82)	7.66 (3.41)	4.59 (1.50)
Endosulfan I	0.07 (0.05)	0.18 (0.12)	0.10 (0.05)	0.06 (0.02)	0.02 (0.01)	0.01 (0.05)
Endosulfan II	0.07 (0.04)	0.10 (0.03)	0.06 (0.04)	0.07 (0.03)	0.02 (0.01)	n/a
Endosulfan sulfate	0.17 (0.07)	4.62 (7.47)	0.04 (0.02)	0.05 (0.03)	0.01 (0.01)	n/a
p,p′-DDE	0.66 (0.12)	0.11 (0.05)	0.03 (0.01)	0.03 (0.03)	0.03 (0.01)	n/a
p,p′-DDD	1.03 (0.63)	0.17 (0.09)	0.02 (0.02)	0.02 (0.01)	0.01 (0.01)	n/a
p,p′-DDT	1.05 (0.85)	2.99 (2.59)	0.05 (0.04)	0.05 (0.02)	0.02 (0.02)	n/a

n/a means no available
[a] March 2004-June 2004
[b] July 2004-October 2004
[c] November 2004-Febuary 2005

Table 5. Mean and standard deviation (S.D.) of OCPs (μg g-1 dw) detected in the surface sediment at site P and R during March 2004-Febuary 2005 (n=36/cultivation period)

Even though usage of some imported pesticides such as DDT was officially banned in 1983, there is the official report presented that these OCPs were parts of organochlorine insecticides active ingredients not included in the ban list. That is why they were still detected in sediment samples along with their metabolites [22-24]. Additionally, the appearance of some OCPs such as endosulfan and heptachlor in the sediment may indicate continued usage of these pesticides despite the official ban. Illegal smuggling of these pesticides from the neighborhood countries has been reported several times in local newspapers. Furthermore, some pesticides are still used in some developing countries around the tropical belt [25,26] which demonstrates the degradation persistence of these substances, even in tropical environment [27].

Heptachlor epoxide was found at the highest concentration in sediments from station R. The enzymatic epoxidation of heptachlor to heptachlor epoxide in soil and animals is most likely responsible for the significant greater incidence of heptachlor epoxide than heptachlor [28]. This chemical was also extensively used for termite control in Thailand in the past, and additionally, was used in seed treatments and control of soil insects such as ants. Although the usage of heptachlor was officially banned in 1988, the observed high residue concentrations of heptachlor epoxide at all sampling sites could come either from persistence in the environment or from continued use reflecting either illegal smuggling or use of old stocks [20]. Heptachlor and its epoxide were also detected in both water samples

[23] and agricultural soil samples [24] from the Chao Phraya Basin. Furthermore, the other unlikely sources of heptachlor and its metabolite may be chlordane which is widely used against termite infestation and could be released into the environment primarily from its application as an insecticide [9]. It has the main components including α- and γ-chlordane, γ-nonachlor and heptachlor and generally metabolized in the environment and in the human body to oxychlordane and may be heptachlor epoxide found in the absence of either oxychlordane or γ-nonachlor [29].

In the case of endosulfan, the concentration of its α-isomer (the main component of the commercial product) was higher than β-endosulfan (a secondary component) at all sampling sites during the two years. Total endosulfan showed values in the range >0.001-1.34 μg/g dw in 2003, with 0.05-0.20 μg/g dw for the α-isomer and only >0.001-0.51 μg/g dw for the β-isomer. Endosulfan sulfate, an endosulfan degradation by-product, was found in large concentration in the sediment samples (range >0.001 to 1.34 μg/g dw). The total endosulfan in 2004 was 22.60 μg/g dw with the highest proportion as endosulfan sulfate (>0.001-15.72 μg/g dw) [20].

Endosulfan was extensively used to control golden apple snails in rice fields in this area and was officially banned in 2004. Endosulfan and its by-products, endosulfan sulfate, were found in relatively high concentrations, both during the dry and rainy-rice cultivation seasons. These data reflects the wide use of endosulfan in the surrounding agricultural areas over a long period [20]. Moreover, during warehouse storage, β-endosulfan can be slowly converted to α-endosulfan, which has higher toxic insecticidal properties than the β-isomer [30].

For some OCPs, chemical transformation and microbial decomposition may occur, e.g. DDT is transformed to DDE and δ-HCH to α-HCH. However, complete mineralization of organochlorine compounds does usually either not takes place, or is an extremely slow process [3]. Composition of DDT metabolites and HCH isomers were examined in detail because their differing composition in the environment could indicate different sources of contamination [31].

9. DDTs

In the first year of monitoring, levels of DDTs were found elsewhere, for example, between 0.08 and 1.83 μg/g dw in sediment from site R. The ratios of (DDE+DDD)/∑DDT in surface sediments ranged from 0.21 to 0.61 μg/g dw in 2003 and from 0.10 to 1.00 μg/g dw during 2004 (Figure 4 and 5). Most values were less than 0.5 for the first year, whereas they were greater than 0.5 for the second year. As to the individual DDTs, the concentrations in surface sediment were generally found in the order of DDT>DDE>DDD in most sampling sites and sampling seasons (Figure 6). The same trend was also reported for the second year (Figure 7) except for sites P and R (non-rice cultivation areas), at which DDE showed the highest percentage. In addition, DDE was usually present at a higher concentration than DDD [20].

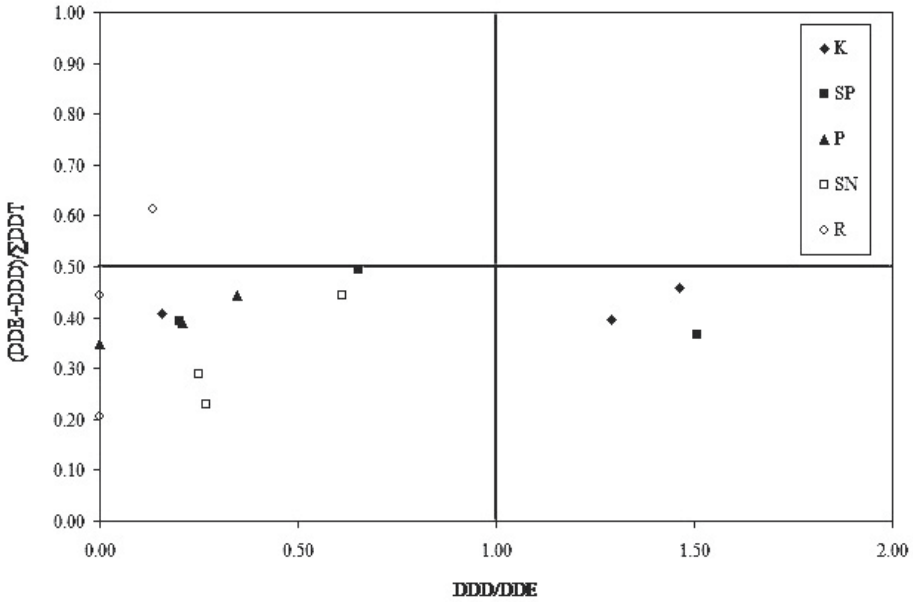


Figure 4. Relationship between (DDE+DDD)/∑DDT and DDD/DDE in surface sediments during August 2003 to July 2004 (Poolpak et al., 2008)

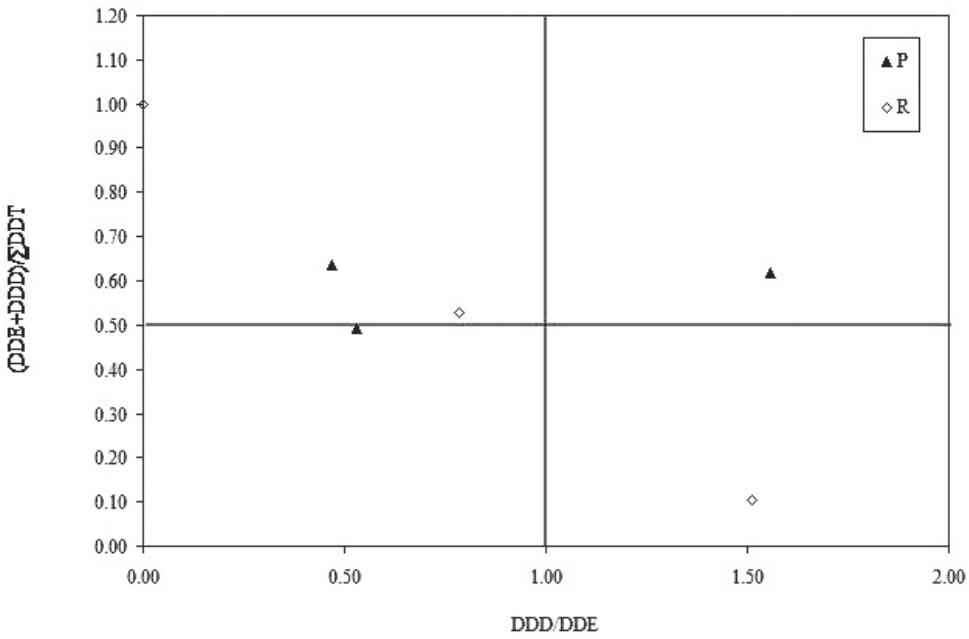


Figure 5. Relationship between (DDE+DDD)/∑DDT and DDD/DDE in surface sediments during March 2004 to February 2005 (Poolpak et al., 2008)

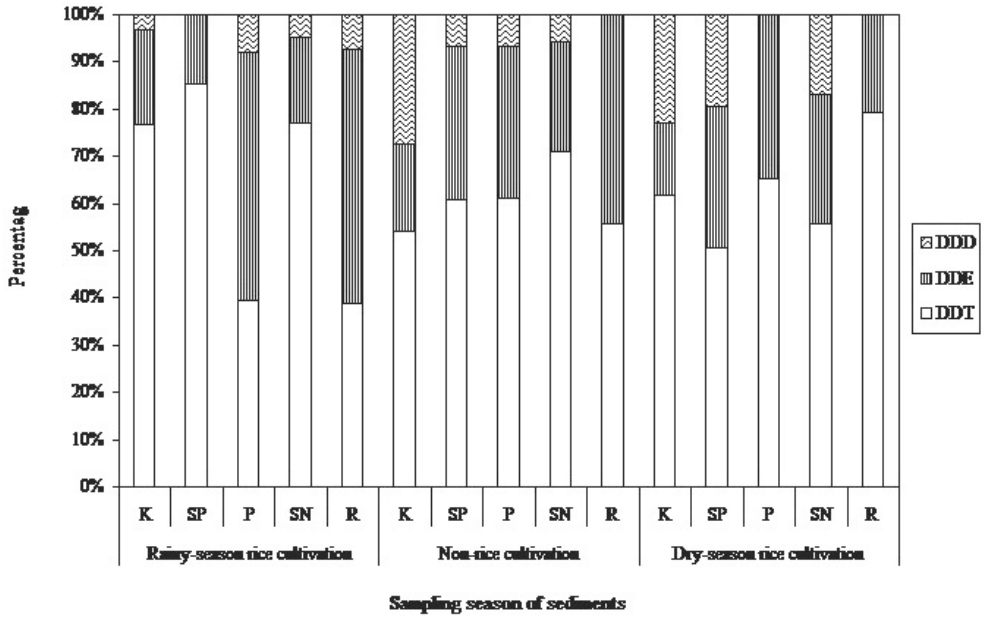


Figure 6. Composition of DDTs in surface sediments, during August 2003 to July 2004 (Poolpak et al., 2008)

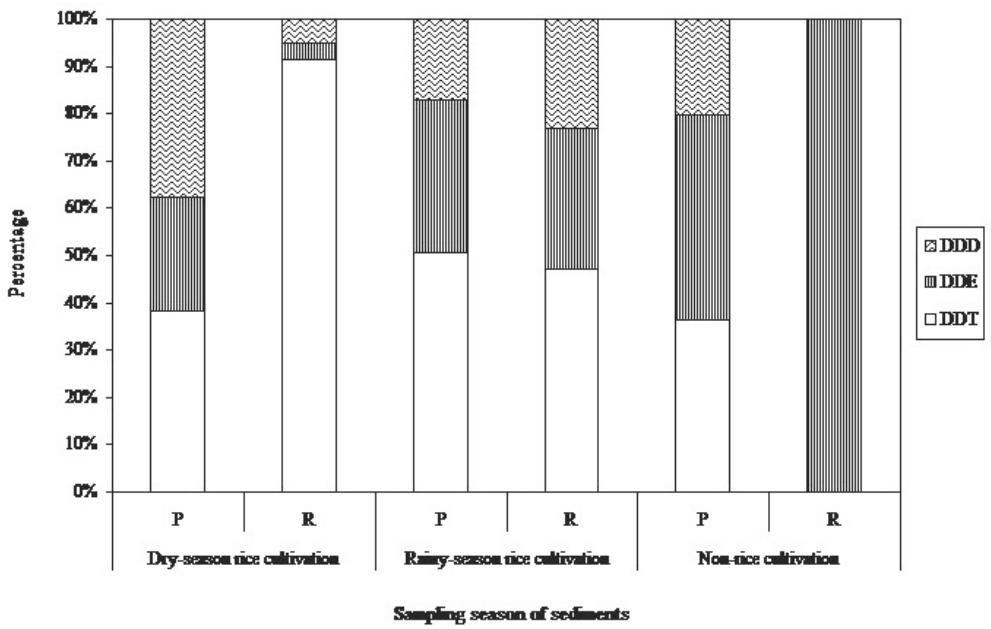


Figure 7. Composition of DDTs in surface sediments, during March 2004 to February 2005 (Poolpak et al., 2008)

The result showed that DDE was usually present in a higher concentration than DDD, suggesting that the most DDT was transformed into DDE [32]. The higher occurrence of DDD and DDE are the results of DDT degradation and the higher stabilities of the metabolites [33-35]. The metabolite DDE is produced from DDT under aerobic conditions such as that occurs in upland soils. On the other hand, DDT is converted to DDD with a half-life of a few days under anaerobic conditions such as in the aquatic sediment [3]. Microbial degradation of DDTs is generally slow resulting in environmental persistence of these compounds [36]. The relative concentrations of the parent DDT and its metabolites can indicate whether DDTs input are from the former or present uses [26,34,37].

Data from the relative concentrations of the parent DDT and its metabolites (DDE and DDD) shows that most values were less than 0.5 for the first year (2003), whereas they were greater than 0.5 for the second year (2004) [20]. The ratios of (DDE+DDD)/∑DDT > 0.5 are reported to indicate long-term weathering [26,38-41]. This suggested that the DDT compounds in sediments collected in 2003 were from the current use whereas the 2004 values which were mostly > 0.5 indicated that degraded metabolites formed a significant proportion of total DDT compounds and the contamination of the area has started long time ago. Since the DDD/DDE ratios were less than the unity at both sampling sites, this indicates that the sediment samples were dominated by the products of aerobic degradation and the contamination of the area is fairly recent. It also indicates that the ongoing use of DDT and its metabolites were derived under aerobic conditions before transported by surface runoff to the waterways sediment [20]. This may be explicable by the relatively higher transportability of DDE than the other form in the atmosphere [18].

10. HCHs

The concentrations of HCH isomers detected in this study in 2003 were in the sequence β-HCH<α-HCH<γ-HCH<δ-HCH at most sampling sites, where the order in 2004 was α-HCH≤γ-HCH<β-HCH<δ-HCH. In addition, β-HCH represented the lowest proportion (7.61% of the total HCHs compared to 11.2% and 12.0% for α- and γ-isomers, respectively during 2003 (Figure 8). On the other hand, the decreased proportion of α-HCH and the increased proportion of β-HCH in 2004 were detected (Figure 9). Among HCH isomers analyzed, γ-HCH (lindane) contributed 12% and then 2.22% of the total HCH concentrations in sediment samples in 2003 and 2004, respectively [20].

HCH residues are among the most widely distributed and frequently detected organochlorine contaminants in the environment [36]. Technical HCH was formerly used as a broad-spectrum pesticide for agricultural purpose in Thailand until it was banned in 1980 [9] whereas the γ-isomer, commonly known as lindane, (the only isomer with insecticidal activity) was used for controlling agricultural and medical pests [43] and banned in 2001. Generally, the most common isomers of HCHs in the environment are α-HCH, β-HCH and γ-HCH [44]. Whereas there were high concentrations of δ-HCH in sediments at most sampling sites in the present study, the results are similar to the levels detected at Beijing Guanting reservoir, where the possible reasons of contamination are still unclear [44]. The

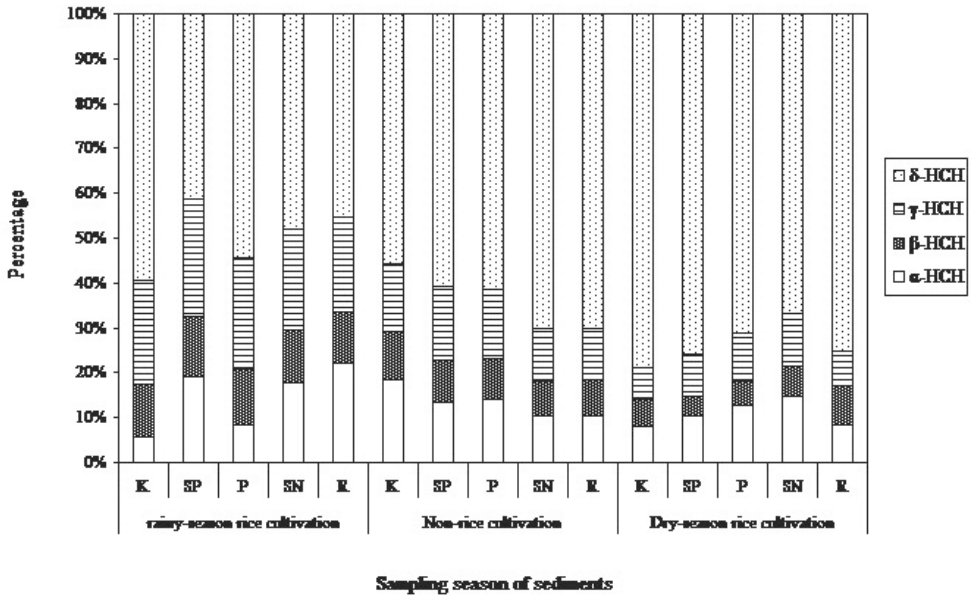


Figure 8. Composition of HCHs in surface sediments, during August 2003 to July 2004 (Poolpak et al., 2008)

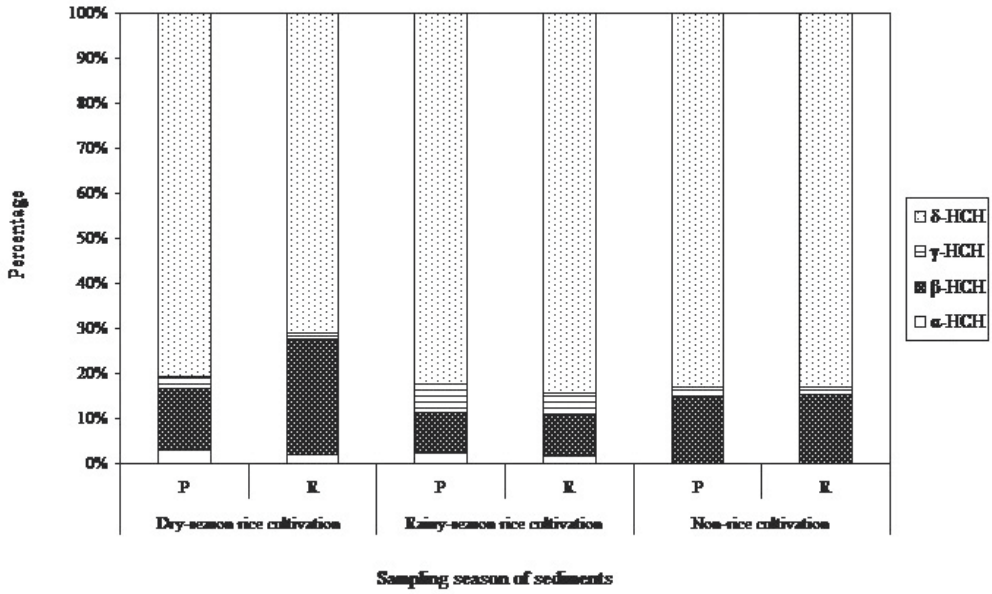


Figure 9. Composition of HCHs in surface sediments, during March 2004 to February 2005 (Poolpak et al., 2008)

typical technical HCH generally contains all four isomers and their physico-chemical properties are different. The most stable β-HCH, relatively resistant to microbial degradation due to its lowest water solubility and vapor pressure, represents a good indicator of contamination [31,33,43,45]. Many studies have reported that β-isomer was dominant in sediments from river or estuary environment after long-term migration and transformation [38,46,47]. In addition, α-isomer is typically predominant in ambient air as well as ocean waters and can be converted into the β-isomer in the environment [31,42].

The sequence of HCHs in this study can be explained by the resistance to chemical degradation of these isomers in this environmental condition and the recent use of technical HCH in this area [45]. Various technical HCH formulations have different amounts of the α-, β-, and γ-isomers; however, the long range atmospheric transport favors α-HCH, the most volatile isomer [48]. On the other hand, the decrease in proportion of α-HCH and the increase in proportion of β-HCH in 2004 implied that the HCHs contamination in the sediment samples might have originated from a relatively distant source or long-term accumulation. Additionally, this data indicates the use of technical HCHs rather than lindane (γ-HCH) and may be explained by the degradation of γ-HCH in this environmental condition because γ-HCH is degraded by microorganisms [49,50] and photochemically isomerized to the α-isomer [45,51]. Although, the predominance of α-isomer in some environmental samples reflects the recent use of technical HCH [52], the higher concentration of α-isomer than γ-isomer in this study may establish the evidence of lindane usage in the past. Since there is no historical data concerning HCHs contamination in this central plain, thus the data presented here can be used to establish a baseline for the future monitoring and management of pesticides in this area [20].

11. Degradation of heptachlor by soil microcosm

According to Poolpak [53], the biodegradation of heptachlor and its oxidative metabolite, epoxide in the microcosm were selected in the mean of their highest contamination in the filed study. The effect of initial concentrations and kinetic study of heptachlor, biodegradation in the sediment, effect of additional carbon sources, soil:water ratio and temperature on degradation of heptachlor were studied to assess its degradation in the fresh water environment. Sediments in degradation study were collected from Rang Tub Tab canal to determine the biodegradation behavior of the indigenous microorganisms in studied site.

12. The effects of initial concentrations of heptachlor on microcosm activities

The effects of different initial concentrations of heptachlor on the degradation and rate of metabolism of soil microcosms were performed in the liquid culture at the heptachlor concentrations of 50, 100 and 150 μg/mL at temperature of 30°C, 150 rpm for 7 days. The decline of different concentrations of heptachlor and the transformation of heptachlor epoxide, the oxidative metabolite, were examined. The liquid cultures without heptachlor but with 1% yeast extract were used as control. Degradation of heptachlor and heptachlor epoxide formation at various initial concentrations was illustrated in figure 10.

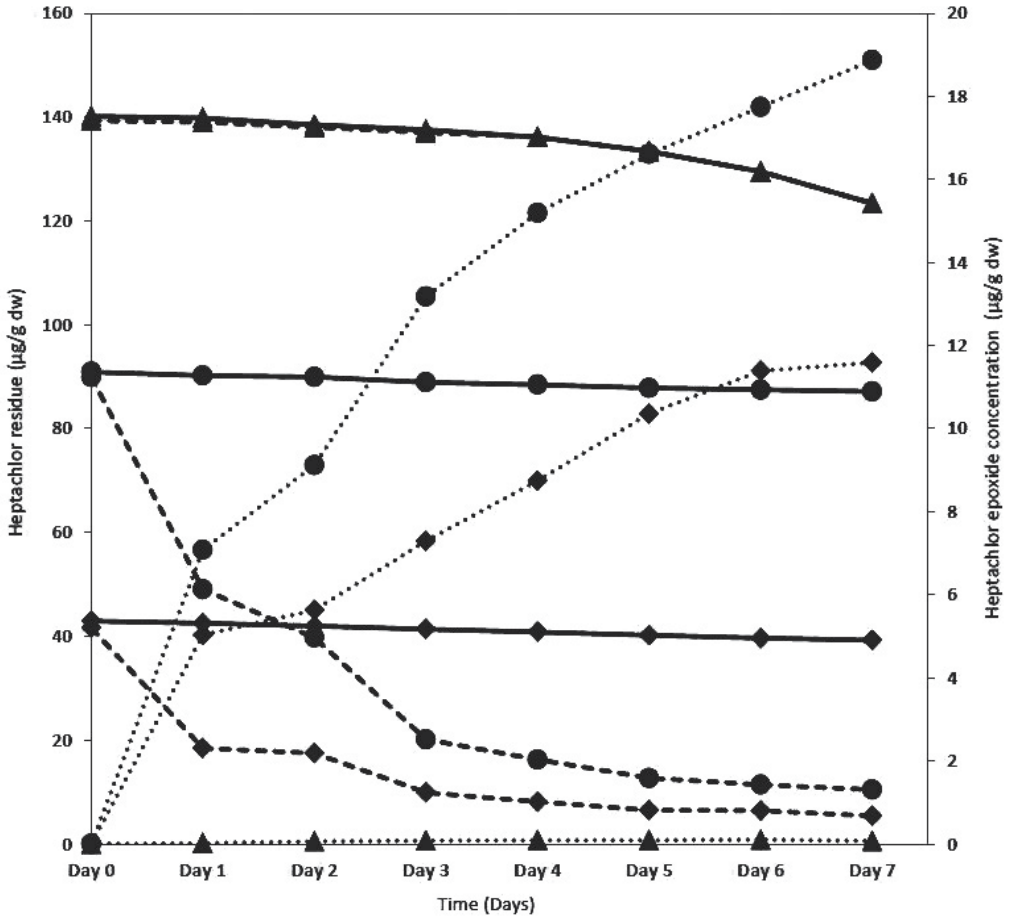


Figure 10. Degradation of heptachlor and heptachlor epoxide formation at various initial concentrations (Heptachlor residue at ◆ 50 µg/mg heptachlor (abiotic); ● 100 µg/mg heptachlor (abiotic); ▲ 150 µg/mg heptachlor (abiotic); --◆-- 50 µg/mg heptachlor; --●--100 µg/mg heptachlor; and --▲-- 150 µg/mg heptachlor. Heptachlor epoxide concentration at ••◆•• 50 µg/mg heptachlor; ••●•• 100 µg/mg heptachlor; and ••▲•• 150 µg/mg heptachlor) (Modified from Poolpak, 2008)

The highest degradation efficiencies were observed in 100 µg/mL heptachlor as 88.36%, whereas at the concentration of 150 µg/mL heptachlor, the efficiencies were lower. Accordingly, 100 µg/mL heptachlor were conditioned in further experiments. Transformation of heptachlor to heptachlor epoxide in liquid culture was evaluated and showed that its metabolite was mostly found in 50 µg/mL heptachlor treatments or 27.85% of parent heptachlor with the positive values of redox potential suggesting the oxidative activities of microorganisms. Lu et al. [54] reported the transformation of heptachlor into 1-hydroxychlordene and 1-hydroxychlordene epoxide was relatively rapid but small proportion of heptachlor epoxide was formed which suggested that heptachlor epoxide formed environmentally largely *in vivo* by microsomal multifunction oxidase action.

Growth of microorganism represented inhibition of higher heptachlor concentrations, which may decrease the degradation efficiencies of the chemical in this study. Similar results were presented in degradation of DDT by *serratia marcescens* [55] and biodegradation of heptachlor and DDT by sediment microbial [56]. In addition, Chiu et al. [56] reported that 100 μg/mL heptachlor and DDT were completely inhibited anaerobic microorganisms and no metabolite was found.

pH and redox potential of different concentrations of heptachlor in liquid culture were measured to evaluate the relationship between growth and metabolic activities of the microcosms and pesticide concentrations. The culture pH was slightly decreased to an acidic range in early treatments before slightly increased to neutral at the end of the experiment in all treatments except for the liquid culture at 150 μg/mL heptachlor concentration where the pH was decreased to acidic range until the end of the experiment. This phenomenon may be due to the metabolic activities of the growing microorganisms. The decreased of pH in liquid culture in biodegradation experiment were also reported [56,58]. In addition, the redox potential values of the liquid culture were positive earlier then decreased to the negative value at the end of the experiment.

The effects of abiotic factors such as temperature, pH and organic matter on degradation of heptachlor in sediment were also determined at 30°C for 28 days. Heptachlor was degraded in sediment only 19.62% in abiotic control compared to 51.17% in biotic control. Furthermore, its metabolite, heptachlor epoxide was not detected under abiotic condition, whereas its concentration under biotic condition was 21.13±1.62 μg/g dw or 25.58% of the parent heptachlor. Because of heptachlor epoxide can be degraded by oxidative reaction by organisms, as a result abiotic control presented no concentration of heptachlor epoxide. Additionally, heptachlor epoxide concentration in this studied was 25.58% of the parent heptachlor which higher than that reported by Bidleman et al. [59] who suggested that heptachlor epoxide accounted for about 20% of the original heptachlor. They also suggested heptachlor degradation in soil proceeded at by at least two routes, 1-hydroxy chlordane and the more persistent, epoxide which subsequently volatilized. Consequently, the oxidation product of indigenous microbial in this study was heptachlor epoxide. This result is relatively similar to the field survey data which the major contaminant in the tributaries of the Mae Klong River was heptachlor epoxide [20].

The pH values were slightly acidic in biotic than abiotic condition. The redox potentials were positive in both conditions, except for later incubation, when those of biotic condition showed the negative value. Microorganisms were increased in number when the incubation time was increased. The positive redox potential was related to the microbial metabolism employing oxidative activities. DeLaune et al. [60] reported that soil generally had a redox range from -250 to +700 mV. In the flooded soil, oxygen is consumed and disappears at a redox potential of approximately +350 mV. Accordingly, aerobic soils represent a redox range of +350 to +700 mV, and anaerobic soil a range of -250 to +350 mV.

13. Effects of additional carbon source on heptachlor degradation

The additional carbon sources other than the target compounds may influence the degradation rates in the system, so the effects of additional carbon sources including sodium succinate, sodium acetate and glucose supplements at 1% concentration were performed and compared with abiotic control (no additional carbon source) to observe heptachlor degradation and heptachlor epoxide evolution.

Higher heptachlor residue in the experiment amended with 1% glucose, without additional carbon sources and 1% succinate (40.33±2.06, 40.33±1.79 and 39.84±0.77 μg/g dw, respectively), where 1% sodium acetate showed the least heptachlor (37.13±0.39 μg/g dw) as showed in figure 11. The results illustrated that without carbon supplement, the degradation efficiency was at its highest, whereas 1% of sodium succinate and sodium acetate addition increased the efficiency of degradation. In contrast, many studied reported the inhibition of the degradation of pesticides by microbial when the presence of the more favorable carbon sources has been found [55,56,61]. Moreover, increasing organic contents by adding carbon sources made sediment microorganisms more vigorous in consuming oxygen and turned the incubating condition into anaerobic condition [62].

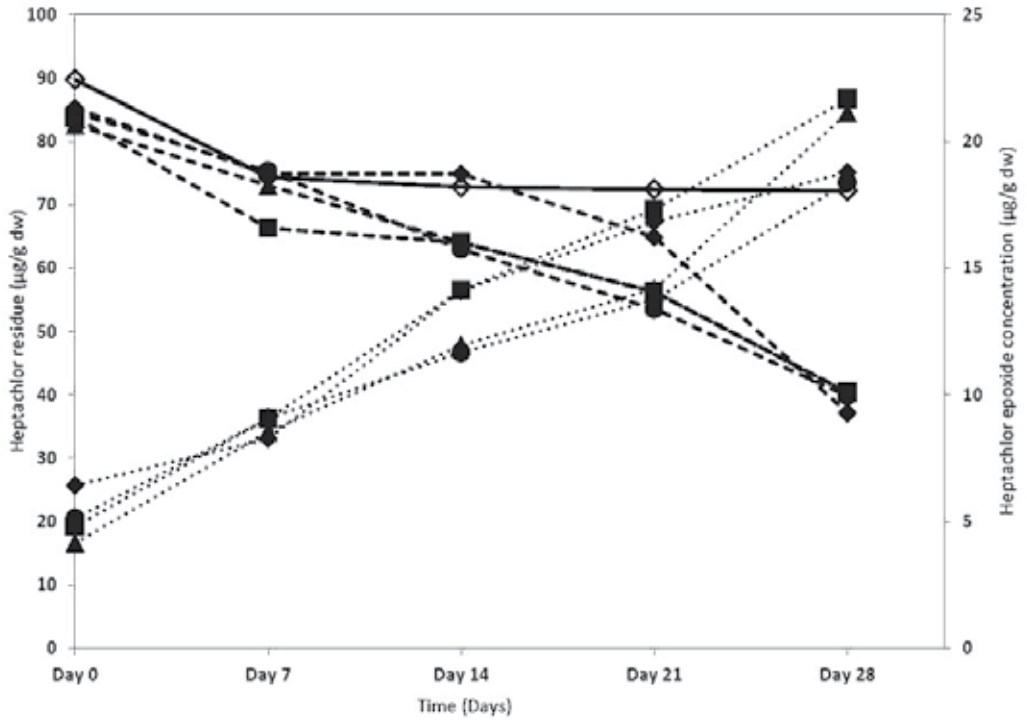


Figure 11. Degradation of heptachlor and heptachlor epoxide formation at various additional carbon sources (Heptachlor residue at ◆ abiotic control; --■-- w/o carbon source; --▲-- 1% glucose; --●-- 1% sodium succinate; and --◆-- 1% sodium acetate. Heptachlor epoxide concentration at ••■•• w/o carbon source; ••▲•• 1% glucose; ••●•• 1% sodium succinate; and ••◆•• 1% sodium acetate)
(Modified from Poolpak, 2008)

Heptachlor epoxide appeared in experiments with adding sodium succinate (21.73% of the parent heptachlor) and sodium acetate (22.02% of the parent heptachlor) was lower than the other two (25.87% and 25.58% for glucose added and without carbon source added, respectively). By means of heptachlor epoxide concentrations decreased when carbon sources were added, the degradation product of heptachlor was no longer heptachlor epoxide. The anaerobic degradation of heptachlor by study of Chiu et al. [56] indicated that sodium acetate increased the anaerobic degradation of heptachlor in river sediment better than glucose and chlordane was produced as the product of degradation. Accordingly, heptachlor could be metabolized to chlordane and 1-hydroxychlordene-2,3-epoychlordene under anaerobic condition [63].

Microorganisms grew well with 1% glucose as compared to other carbon sources. With sodium succinatqe added, the pH was increased with the increase in incubation time whereas the number of microorganisms and redox potential values were decreased. The same trend was observed in sodium acetate addition experiment, less microorganism and redox potential values were found.

14. Effect of soil:water ratio on the heptachlor degradation

The effect of water volume on heptachlor degradation in sediment, the 1:1, 1:2 and 1:4 soil:water (w/v) ratio experiments were determined and the biodegradation effects were presented in figure 12. When the soil:water ratio was decreased, the degradation rate was decreased. Together with higher heptachlor residues in the 1:1 soil:water ratio (47.89±1.52 μg/g dw), heptachlor epoxide concentration was also decreased when soil:water ratio was increased to 1:1 w/v (18.81% of the parent heptachlor). The degradation efficiencies of heptachlor were also decreased when more sediment was added. Degradation of α- and γ-HCH in the soil slurry by Siddique et al. [57] showed that the soil:water ratio significantly decreased the rate of degradation at 4 weeks of chemical incubation. In addition, the study of Castro and Yoshida [64] found that heptachlor had degraded readily in flooded soil than under upland conditions.

The fate of heptachlor in the environment is influenced by environmental factors including soil/water ratio. Decreasing the soil:water ratio decreased the degradation of heptachlor in the sediment, which might due to the adsorption of heptachlor to the soil particles, decreasing their bioavailability to the microorganisms. Furthermore, the conditions in sediments are principally controlled by the diffusion of dissolved oxygen from the overlying water into the sediment bed, which aerobic bacteria dominate, and oxidation reactions both abiotic and biotic relatively easy occurred [11].

The decrease in heptachlor epoxide productivity when soil:water ratio decreased, suggested that less oxidative metabolism by bacterial activities with the confirmation by negative redox potential. The redox profile is expected to have the greatest effect on OCPs degradation in bed sediments, since compounds will be degraded at different rates and may be degraded through different pathways under different redox conditions. In general, the degradation rates in soils and sediments have been observed to be significantly faster under

aerobic conditions, atrazine degradation in oxidizing conditions [60] and HCH degradation by microbial consortium [61,65]. This studied shows the same trend as previous studies as redox potential since 1:4 soil:water ratio were more positive than the others. Moreover the degradation efficiency of this soil:water ratio was better than other treatments.

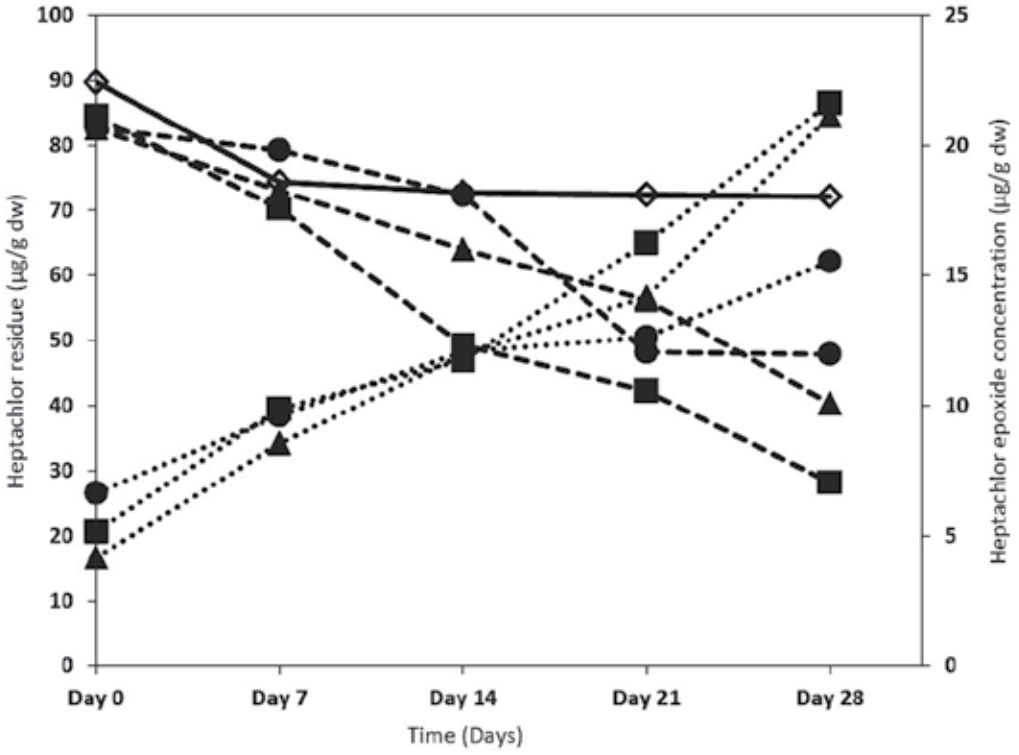


Figure 12. Degradation of heptachlor and heptachlor epoxide formation at various soil:water ratio (Heptachlor residue at ◆ abiotic control; --■-- 1:4 soil:water ratio; --▲-- 1:2 soil:water ratio; and --●-- 1:1 soil:water ratio. Heptachlor epoxide concentration at ••■•• 1:4 soil:water ratio; ••▲•• 1:2 soil:water ratio; and ••●•• 1:1 soil:water ratio) (Modified from Poolpak, 2008).

Even though degradation was slow under reducing or anaerobic condition, the anaerobic degradation of chlordane [66], atrazine [60] and endosulfan [67] were observed.

15. Effect of incubation temperature on heptachlor degradation

The influence of incubation temperatures on heptachlor degradation and heptachlor epoxide transformation was studied at 20°C and 30°C to evaluate the degradation efficiencies of the microcosm. A smaller amount heptachlor residue was presented at 30°C (40.33±2.06 µg/g dw) than that of 20°C (65.70±2.41 µg/g dw). Heptachlor epoxide concentration was decreased when the temperature was decreased, 25.58% and 14.75% of parent heptachlor at 30° C and 20°C, respectively (figure 13). Furthermore, the higher percentage of biodegradation at 30°C was also better than that of 20°C (51.17% and 26.30%, respectively).

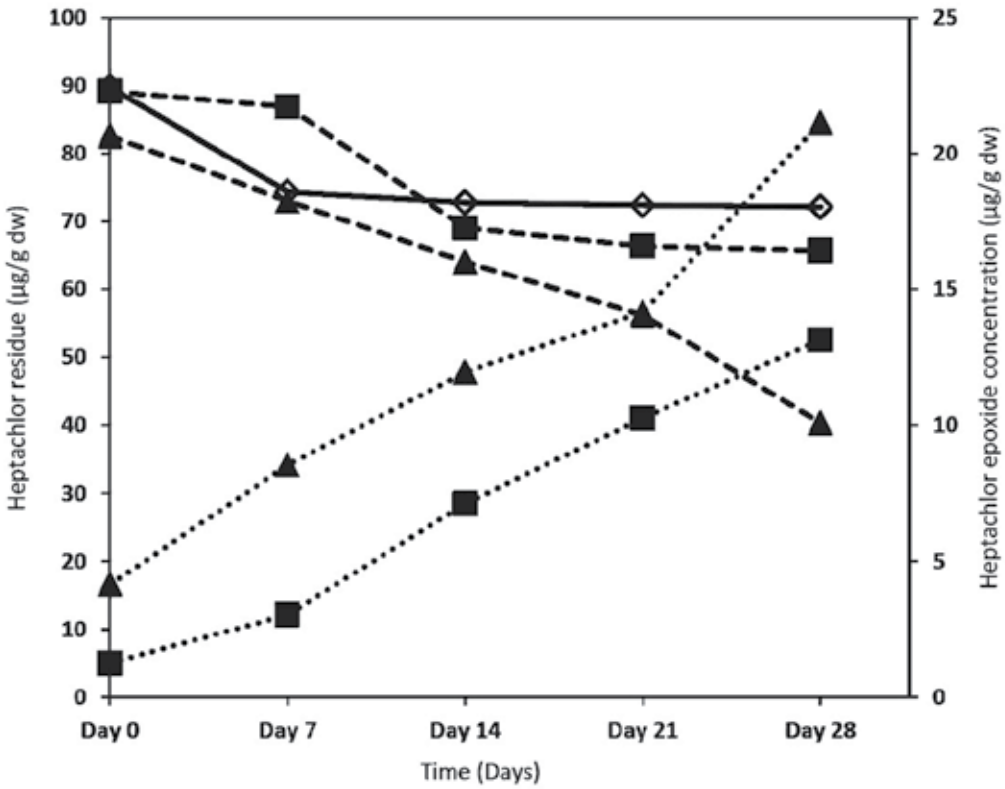


Figure 13. Degradation of heptachlor and heptachlor epoxide formation at various temperature (Heptachlor residue at ◆ abiotic control; --■-- 20°C; and --▲-- 30°C. Heptachlor epoxide concentration at ••■•• 20°C; and ••▲•• 30°C) (Modified from Poolpak, 2008).

Many studies reported that the temperature of 30°C was suitable for chemical biodegradation including biodegradation of HCH isomer in soil slurry [57] and DDT and heptachlor degradation in river sediment of Taiwan [56]. Moreover, biodegradation of endosulfan by soil bacteria [68], abiotic degradation of endosulfan in a clay soil [70] and degradation of DDT by soil bacteria [54] yielded the maximum values at temperature of 30°C. However, microorganisms have the ability to degraded pesticides in a wide range of temperatures. For example, *Serratia marcescens* DT-1P can degrade DDT at the temperature from 4°C up to 50°C [55].

16. Rate of heptachlor degradation

In liquid culture and sediment, the degradation rate at the heptachlor concentration of 100 µg/g soil dw by indigenous microorganism fitted well with the second-order reaction and first order-reaction, respectively. The constant heptachlor degradation rate in liquid culture with the degradation rate constant, k = 0.0125 µg/mL·day. The degradation acted as the

concentrations of one second-order reactant, or two first-order reactants. Heptachlor degradation in sediment hold the degradation rate constant (k) of 3.106×10^4 μg/g dw·day and acted as the first-order reaction. A first-order reaction depended on the concentration of only one reactant. Other reactants could be present but in zero-order reaction.

The reaction rate of heptachlor biodegradation in both liquid culture and sediment was slightly decreased when the incubation time was increased. This may be due to the decrease in bacterial number when the incubation time was increased. Additionally, the conditions of the experiment were unfavorable for metabolic activities for microorganisms.

Since the degradation rate in liquid culture fits to the second-order reaction, it indicates that not only bacterial activities affected heptachlor degradation but also other factors should be involved as well. Even though, degradation of sorbed pesticides in the soil is a complicated process affected by many factors, the degradation rate of heptachlor in sediment fitted well with first-order reaction. This suggests that microbial degradation of pesticide was the major factor in sediment degradation. Ghadiri et al. [70] reported the degradation rates of aldrin, dieldrin, endrin and chlordane were first-order, the same as rate of atrazine degradation in reactors studied by Ghosh and Philip [71].

17. Dominant microorganism in heptachlor degradation experiment

In all experiment of heptachlor biodegradation by soil microorganisms collected from Rang Tub Tab canal. Three indigenous microorganisms, which dominated in the biodegradation experiment in both liquid culture and sediment, were identified by 16S rDNA sequencing as *Bacillus subtilis* RS-01, *Bacillus cereus* RS-02 and *Pseudomonas putida* RS-03. Although, the numbers of bacteria were low at earlier incubation, it was increased when the incubation time increased. Additionally, degradation rate increased when the number of bacteria was increased.

Despite the fact these three bacteria were dominant in the experiment during heptachlor degradation; it is difficult to interpret the role that the bacteria play in the transformation process of heptachlor in view of the fact that the physiological actions of these isolates are still unknown. However, biodegradation of endosulfan contaminated soil and water in laboratory scale reactors by *B. circulans*-I and II enriched from contaminated soil of endosulfan processing industry has been reported by Kumar and Philip [71]. In addition, *P. fluorescencens*, *B. cereus* and *Bacillus* sp. were the consortium successful for *ex-situ* biodegradation of chlorobenzenes in soil [73]. Ambrosoli et al. [74] studied the degradation of carbofulan by bacterial population; the results showed that genera *Pseudomonas* and *Bacillu*s rapidly degraded carbofuran in soil as carbon source. Furthermore, heptachlor epoxide concentrations in all biotic experiments represented oxidative metabolism of these bacterial population. The evidences suggest that these three bacteria might be responsible for the heptachlor degradation in sediment. The proposed pathways of heptachlor metabolism in this study were summarized in Figure 14.

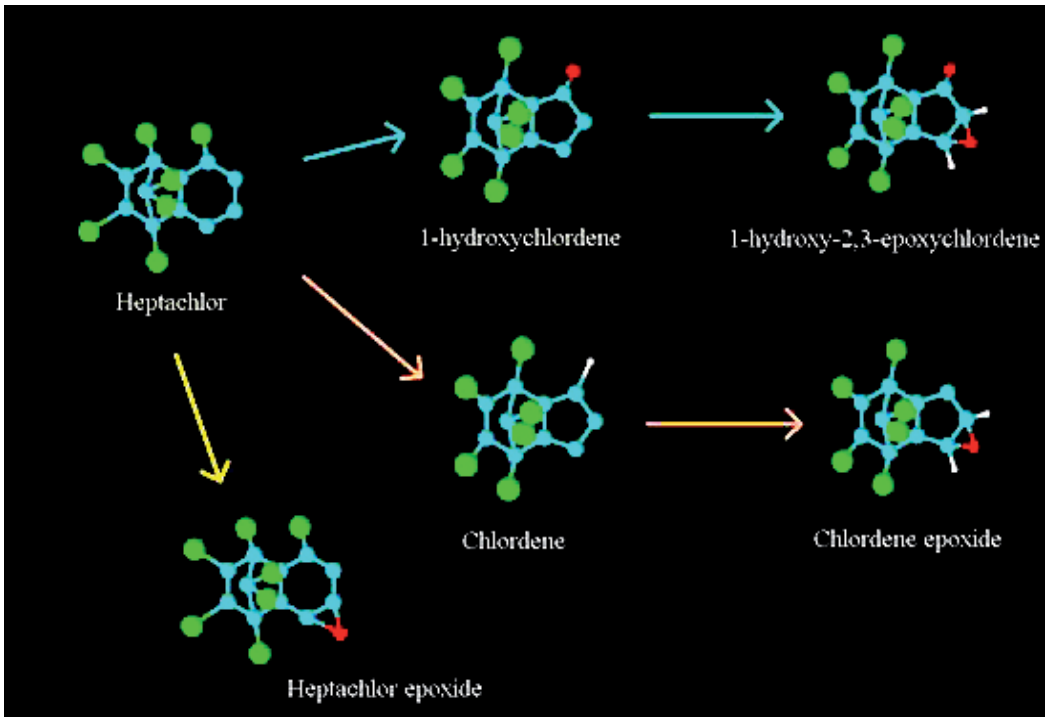


Figure 14. Pathways of heptachlor metabolism by indigenous microorganisms (T. Poolpak, 2008; modified from Lal and Saxena, 1982).

The first route of heptachlor degradation is the chemically hydrolysis of heptachlor to 1-hydroxychlordene in soil, and microbial attack on this product results in the production of 1-hydroxy-2,3-epoxychlordene. The second is microbial dechlorination of heptachlor produce chlordene, which undergoes microbial epoxidation to form the corresponding chlordene epoxide. The last route of biodegradation is oxidation of heptachlor by microorganism converts heptachlor to its epoxide by mixed-function oxidase system [63].

18. Conclusion

The surface sediment in the tributaries of Mae Klong River is polluted by the OCPs and relatively high levels of these compounds were observed in most study areas which summer was the season that highest OCPs residues were found in both sampling years. Heptachlor epoxide presented the highest concentration among detected OCPs. In addition, ∑DDT and HCHs were found in slightly elevated levels representing a recent input of these two OCPs into the study area.

For heptachlor biodegradation in liquid cultures, the highest degradation efficiency (88.36%) was observed in 100 μg/mL heptachlor where at 150 μg/mL heptachlor, the efficiency was lower. Moreover, growth of microorganism was inhibited at higher heptachlor concentration; as a result the degradation efficiency was decreased. In addition, heptachlor

epoxide, the oxidative product of heptachlor by microorganism was found at the highest concentration in the 50 μg/mL heptachlor treatments. This evidence enhanced the idea of inhibition of bacterial growth at high concentration of heptachlor.

In abiotic condition of heptachlor biodegradation and the transformation to heptachlor epoxide in sediment, there was no detection of heptachlor epoxide. Confirms microorganisms play a major role in heptachlor oxidation to heptachlor epoxide. In contrast, heptachlor degradation was detected in biotic degradation; heptachlor epoxide concentration in this study was 25.58% of the parent heptachlor.

A number of external parameters including additional carbon sources affected the degradation rates in this study. Without carbon supplement, the degradation efficiency was high. Moreover, when 1% of sodium succinate and sodium acetate was added, the efficiency of degradation was even higher. However, heptachlor epoxide concentrations decreased when these carbon sources were added and the degradation product of heptachlor was changed to chlordane instead of heptachlor epoxide.

For soil:water ratio experiments, as the soil:water ratio was decreased, the degradation rate of heptachlor decreased. At 1:4 soil:water ratio, the redox potential values were more positive and higher degradation efficiency was observed. Also both degradation efficiency and transformation of heptachlor to epoxide at 20°C were lower than those at 30°C, these may be due to 30°C was the most favorable temperature for bacterial growth and heptachlor degradation.

The degradation rate in liquid culture fitted to the second-order reaction where the degradation rate of heptachlor in sediment fitted well to the first-order reaction. Decrease of reaction rate as incubation time increased was seen in both liquid culture and sediment. This may be as a result of the decrease in bacterial number and the shortage of food source toward time.

Three dominant bacteria were isolated and identified as *B. subtilis* RS-01, *B. cereus* RS-02 and *P. putida* RS-03. Degradation rate of heptachlor and heptachlor epoxide evolution increased when the number of bacteria was increased. Although these three bacteria were dominant in the experiment during heptachlor degradation, it is difficult to interpret the role that the bacteria play in the transformation process of heptachlor in view of the fact that the physiological actions of these isolates are still unknown. Nonetheless, heptachlor epoxide, oxidative products from microorganism metabolism, presented in biotic treatments, suggests that these bacteria might be responsible for the heptachlor degradation in sediment.

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Chapter 11

Biodegradation and Bioremediation of Organic Pesticides

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Additional information is available at the end of the chapter

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1. Introduction

Pesticides can be used to control or to manage pest populations at a tolerable level. The suffix "-cide" literally means "kill", therefore, the term pesticide refers to a chemical substance that kills pests. It is incorrect to assume that the term pesticide refers only to insecticides. Pesticides include many different types of products with different functions or target (Table 1). The pesticide designation is formed by combining the name of the pest (e.g., insect or mite) with the suffix "-cide" (1).

Pesticides could be classified according to their toxicity, chemical group, environmental persistence, target organism, or other features. According to the Stockholm Convention on Persistent Organic Pollutants, 9 of the 12 persistent organic chemicals are pesticides. Classes of organic pesticides (consisting of organic molecules) include organochlorine, organophosphate, organometallic, pyrethroids, and carbamates among others (2, 3).

Most pesticides cause adverse effects when reaching organisms. The intensity of the toxic effect varies with time, dose, organism characteristics, environmental presence or pesticide characteristics. Their presence in environment determines the dose and time at which an organism is exposed and could represent a hazard for worldwide life due to their mobility. Hence, the persistence in the environment leads to a risk for life: the more persistent a pesticide is, the worse its environmental impact.

Pesticide persistence in environment is caused by either their physico-chemical properties or the lack of organisms able to degrade them. Light, heat or humidity could lead to loss of some pesticides by either volatilization or degradation (4). Contrastingly, degradation caused by organisms (biodegradation) could help decreasing considerably the pesticides persistence in environment. This information could be used to improve elimination of the

undesirable effects of pollutants by using organisms; such an approach has been called bioremedation.

The ability of organisms to bioremediate pesticides is mainly based on their biodegradation activity. Though bioremediation has been firstly achieved using microorganisms (bacteria or fungi), other organisms like plants or algae can be used. The aim of the present paper is to review the metabolic features which make organisms useful for bioremediation.

2. Overview

At this point, it is worth to mention that there is no convention on some words used in biodegradation. Here, we propose some words to improve communication and understanding bioremediation strategies. Albeit discussion of proper words is beyond aim of the present paper, we believe that before continuing is important to set up some concepts.

"Bioremediation" refers to any strategy used to eliminate undesirable effects of pollutants from environment. It would be desirable to eliminate pollutants but this is not always possible; though, some organisms could confine or immobilize them. For instance, organisms can accumulate contaminants, and reduce their presence and their environmental effect, but do not eliminate them from the environment. Such strategy, which is actually used (v.gr. phytoextraction (5)) should be included into the "bioremediation" concept. Those organisms able to bioremediate would be called bioremediators.

Pesticide	Target
Algicides	Algae
Avicides	Birds
Bactericides	Bacteria
Fungicides	Fungi
Insecticides	Insects
Miticides or Acaricides	Mites
Molluscicides	Snails
Nematicides	Nematodes
Rodenticides	Rodents
Virucides	Viruses

Table 1. Classification of pesticides according to their target.

Traditionally, bioremediation has been achieved by using microorganisms. Nevertheless, The fact that in past decades, several reports on bioremediation using plants, fungi, algae or enzymes (obtained from organisms) has broadened the scope of bioremediation. Words like phytoremediation or rhizoremediation have been used (5, 6), and perhaps it would be necessary to name properly each bioremediation strategy regarding the organism used (Table 2).

Bioremediator organism	Strategy
Microorganism	Microbioremediation or Bioremediation
Bacteria	Bacterial bioremediation
Fungi	Mycoremediation
Plants	Phytoremediation
Rhizosphere	Rhizoremediation
Algae	Phycoremediation
Biomolecules derived from organisms	Derivative bioremediation

Table 2. Classification of bioremediation strategies according to the organism involved.

The concepts of biodegradation and biotransformation overlap extensively, so that, they are synonymous in appearance. Biodegradation involves the biological reactions that modify the chemical structure of the compound, so, this implies a decrease in toxicity. In contrast, biotransformation reduces the pollutant concentration by either modification or translocation. Thus, biotransformation could end decreasing or increasing the undesirable effects. Their difference is clear in the case of pollutants translocation when biodegradation is not occurring but biotransformation does. Biotransformation concept has been developed for biological detoxification systems (7) and is a key concept in bioremediation strategies because they both are intended to eliminate undesirable effects of pollutants to organisms. Along the text, the word "Biodegrader" will be used for the organism able to biodegrade a certain compound. "Mineralization" refers to biodegradation leading to compounds like CO_2 or NH_3 which could be biologically assimilated (8).

In the earliest works on bioremediation, the practical purpose was to find or to isolate biodegrader microorganisms or consortia. In an admirable work, Alexander (8) reviewed several biodegrader consortia found in polluted environmental matrixes (soil, sediment or water). Among those tolerant or adapted microorganisms, there might be some proper bioremediators. A plausible explanation for this phenomenon might be that pesticides have exerted evolutionary pressure, so that, only organisms able to tolerate those doses of pesticides will survive. Even though not every tolerant organism is a biodegrader, every biodegrader should be tolerant. Thus, the evolutionary pressure exerted by the pollutant would have selected some tolerant bioremediators. In keeping with this, traditionally, bioremediation studies measured only final concentration of pollutants, but little or no attention to biochemical mechanisms responsible for biodegradation was given. Further research on factors affecting biodegradation process is required to improve selection of bioremediators and application of bioremediation technologies.

2.1. Factors affecting biodegradation process

Some metabolic features related to biodegradation efficiency have been investigated for microorganisms (8). Any factor which can alter growth or metabolism, would also affect biodegradation. Hence, physicochemical characteristics of the environmental matrix, such as temperature, pH, water potential, oxygen and substrate availability, would influence the

biodegradation efficiency (Figure 1). Two more factors are worth to mention: co-metabolism and consortia condition. Some biodegraders need other substrates to degrade pollutants (8). This phenomenon is called co-metabolism and is especially required for organochlorine compounds. In contrast, it has been shown that the presence of other carbon sources decreases organophosphate biodegradation (9).

When pesticide degradation occurs, it usually involves more than one microorganism, i.e. each microorganism contributes to biodegradation reactions on pesticides, but no example of mineralization by a single strain has been described. It seems that the presence of different microorganisms is essential for an adequate biodegradation. Reported microbiodegraders belong to basidiomycetes or to bacterial classes: gamma-proteobacteria (v.gr.: *Pseudomonas*, *Aerobacter*, *Acinetobacter*, *Moraxella*, *Plesiomonas*), beta-proteobacteria (v.gr.: *Burkholderia*, *Neisseria*), alpha-proteobacteria (v.gr.: *Sphingomonas*), actinobacteria (Micrococcus) and flavobacteria (*Flavobacterium*).

Pollutants might undergo biodegradation reactions like de-chlorination, cleavage, oxidation, reduction by different enzymes. Since biodegradation ability is based on enzymes which are promiscuous and have evolved to detoxifying enzymes, the shorter the duplication time of organism, the more adequate the organism is and the easier to obtain biodegraders. Thus, bacteria with duplication time around minutes are likeable to respond to natural or artificial pollutant-induced evolutionary pressure; this response consists in selecting biotransformation enzymes able to degrade them. These promiscuous enzymes are present in organisms even before the exertion of the evolutionary pressure, which could have induced genetic recombination or mutation leading to enzymes with better biodegradation ability. Copley (10) has excellently reviewed the evolution of metabolic pathways and those factors affecting the efficiency of pollutant biodegradation.

Though bacteria have been proved to be good biodegraders and bioremediators, some fungi, plants and algae could biodegrade pesticides too. Knowing the metabolism of those biodegrader species or strain improves the selection of bioremediation strategy for each site either by biostimulating the indigenous biodegraders (biostimulation) or adding exogenous to the site (bioaugmentation). Moreover, thanks to molecular biology, the metabolic biodegradation ability could be transferred from a biodegrader to another organism, thus improving its degrading capabilities. For instance, using genetic engineering, a whole mineralization pathway for paraoxon –the oxon metabolite of the organophosphate pesticide parathion- was built in a single strain of *Pseudomona putida* (11). Taking all this into account, it is clear that biodegradation enzymes play a key role in bioremediation processes and their knowledge could help in designing or choosing the most adequate strategy.

Biotransformation enzymes have been traditionally classified according to the phase they participate. There are three phases of biotransformation. Phase I consists of those enzymes catalyzing reactions which modify pollutant functional groups. In phase II, those enzymes catalyzing transfer reaction of whole groups or biomolecules to pollutants are classified. Phase III includes translocation processes rendering pollutants or their metabolites non bioavailable. For bioremediation purposes, biotransformation enzymes mainly belong to

four biochemical types: oxidoreductases, hydrolases, transferases and translocases (or pumps). Among oxidoreductases, the most frequent are monooxygenases (like cytochrome P450), dioxygenases, peroxidases and oxidases. Hydrolases like A-esterases are involved in biodegradation pathways. There are many types of transferases, and they are classified according to the group they conjugate to the xenobiotic: methyl-transferases, acetyl-transferases, glutathione S-transferases among others. For bioremediation purposes, only a couple of translocases have been identified and characterized: boths are pumps that translocate herbicides or glutathione-conjugates to vacuoles.

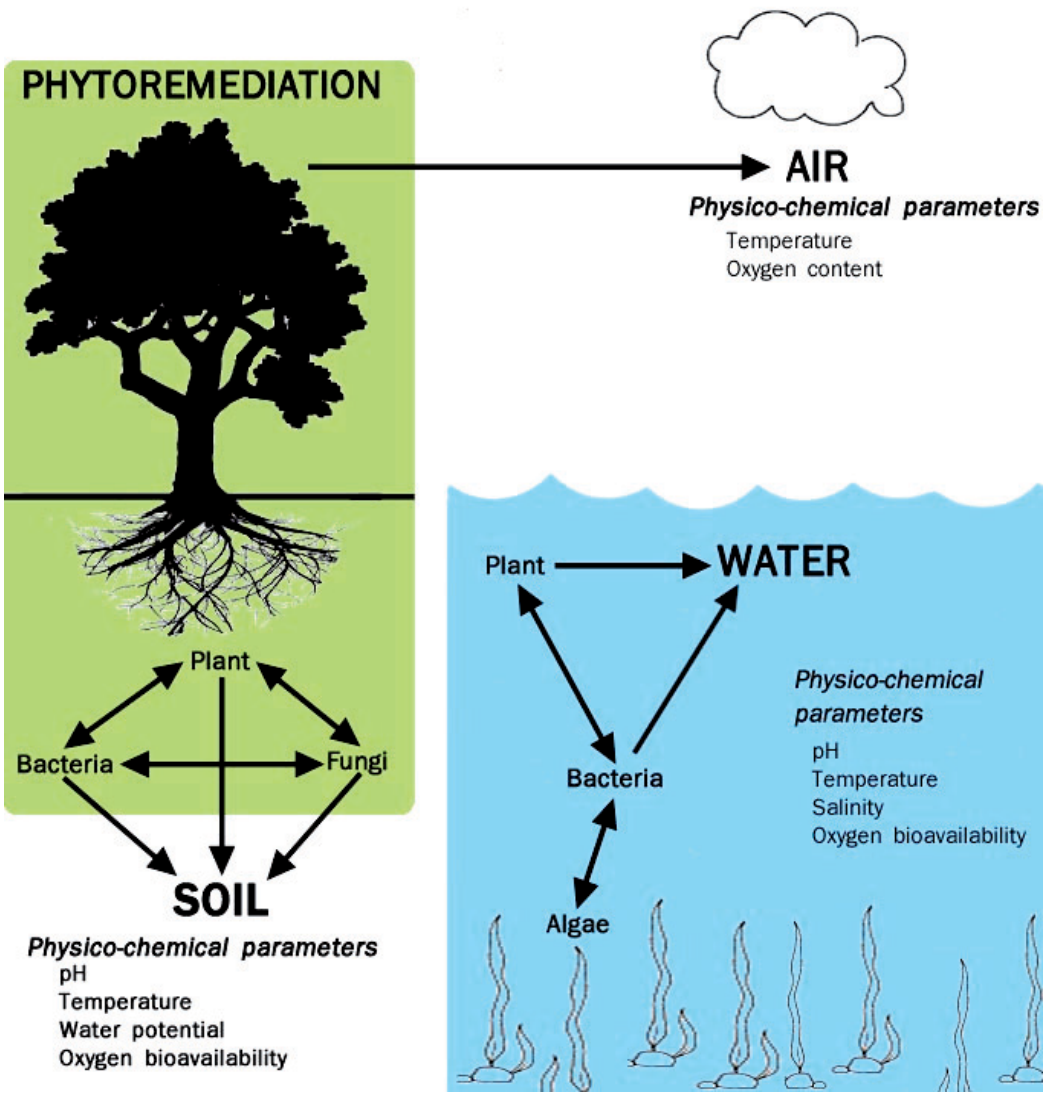

Figure 1. Factors affecting biodegradation and bioremediation in soil, water or air.

The biotransformation of every pollutant could be catalyzed by different enzymes depending on organism. There is no a sequence of reaction pre-determined and is independent of the classification described above. Detoxifying enzymes are promiscous and have different affinities and velocities. Their protein nature makes them susceptible to different factors like heat, pH or substrate availability. In general, biotransformation enzymes for bioremediation are present in bacteria, fungi, plants and animals. In the next section, main enzymes from bacteria, fungi and plants involved in organic pesticide degradation are briefly described. Afterwards, some examples of bacterial, plant, fungi or algae bioremediators are reviewed.

Cytochrome P450 (CYP): This consists of a superfamily of heme monooxygenases. They can catalyze reactions of oxidation, reduction or oxidative breakdown of xenobiotics (Figure 2). It seems that they are evolutively conserved since genomes from virus, bacteria, algae, plant, fungi and animals have isoforms of CYP codified (12-21). In eukaryotic organisms, CYP is found in smooth endoplasmic reticulum, and can biotransform a wide range of pollutants. A review about the biology of CYP can be found elsewhere (22). CYP catalyzes biodegradation of aromatic or alyciclic compounds and can activate toxics, i.e., CYP action on biomolecules might make them toxic or increase their toxicity.

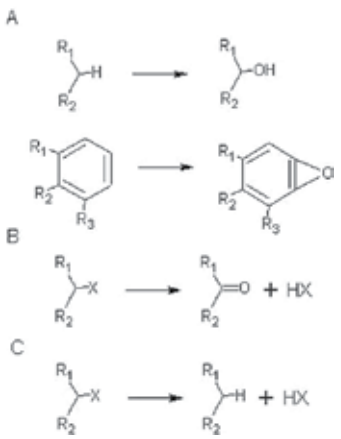

Figure 2. Scheme of reactions catalyzed by CYP: A) oxidation (monooxygenation), B) oxidative and C) reductive dehalogenation.

A-esterases: Esterases can be classified according to their interaction with organophosphates. A-esterases can catalyze the hydrolysis of organophosphate or carbamate pesticides (Figure 3), B-esterases are inhibited by organophosphates and C-esterases show no interaction with organophosphates. A-esterases include several enzymes like monophosphatases, phosphodiesterases or phosphotriesterases. They frequently use calcium and have been found in bacteria, fungi and animals (23). Human paraoxonase is an A-esterase and is involved in susceptibility to organophosphate pesticides; a review on human PON1 could be found elsewhere (24).

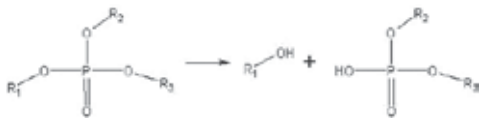

Figure 3. Scheme of reactions catalyzed by A-esterases.

Peroxidases and oxidases: They include some families of enzymes catalyzing redox reactions (Figure 4). Although they are produced by bacteria, fungi, plants and animals, reports on pesticide biodegradation exist for fungi. Peroxidases participate in cell response to oxidative damage and most of them are metalloproteins. They are extemely sensitive to the presence of azide, and inhibitor of metalloenzymes, with the exception of lignine peroxidases from fungi (25). It is known that ligninolytic fungi secrete peroxidases and oxidases to degrade lignine (25, 26). These enzymes are highly promiscuous.

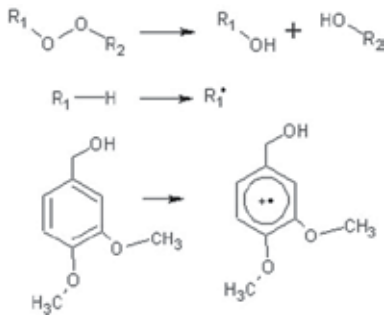

Figure 4. Scheme of reactions catalyzed by peroxidases.

Transferases: Among all known transferases, Glutathione S-transferase (GST) is the mainly involved in biodegradation for bioremediation purposes. GST includes a superfamily of enzymes that have been found in bacteria, fungi, algae, plants and animals (27-29). Even though they catalyze transference of glutathione to electrophillic pesticides, they can also show hydrolytic and peroxidase activities (29). Interestingly, GST can also catalyze the dehalogenation of rings (Figure 5, (30)).

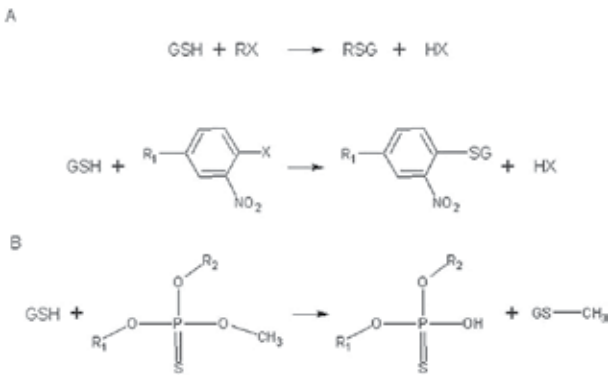

Figure 5. Scheme of reactions catalyzed by GST: A) dehalogenation, B) O-dealkylation.

Translocases: Translocation of molecules from a cell compartment to another is catalyzed by pumps named translocases. Some translocases are involved in the bacterial resistance to drugs, but this activity seems to lack relevance for bioremediation. Although it does not constitute a biodegradation itself, translocation is perhaps the only step of phase III biotransformation. In plants, translocation is part of secondary metabolism and herbicide-tolerance; interestingly, it has been suggested that a previous glutathionation is required for translocation to vacuoles (31, 32).

3. Bacterial bioremediators

Bacteria have been used extensively for bioremediation purposes. These studies have focused on the employment of bacteria, consortia or on the search for biotransformation enzymes. The fast growth, easy handling and low cost make them suitable for bioremediation. Unfortunately, there are some disadvantages such as the disposal of bacterial biomass, pathogenicity, bioactivation, among others. Bacteria can be found in soil, water or even in particles dispersed in air. Unfortunately, only a small fraction of bacteria (<10% from soil) can be cultured in laboratory conditions (33). Because of this, the number of studies about pesticide biodegradation mechanisms is less than those about biodegraders isolation, and then, little information on biochemical mechanisms or enzymes is available. For organochlorine pesticides, only few biodegration enzymes and genes have been described.

Bacterial biodegradation could take place in anaerobic or aerobic conditions. Although different enzymes participate in each condition, it seems that both, aerobic and anaerobic degradation should happen if a mineralization is expected to occur (34). It seems that anaerobic metabolism is more adequate for dechlorination (35, 36) and aerobic metabolism produces a cleavage in aromatic or aliphatic cyclic metabolites. The higher persistence of organochlorine in aerobic conditions (37) compared to anaerobic might be caused by the absence of enzymes or more likely by the oxidative damage following organochlorine metabolism. The removal of heteroatoms (like halogens) or heteroatom-containing groups are frequently among the first steps in biodegradation. These steps are catalyzed by monooxygenases, dioxygenases or peroxidases (37, 38), which in aerobic conditions could generate large quantities of free radicals. Thus, anaerobic conditions are more adequate for biodegradation of organochlorine pesticides, while aerobic are better for biodegrading hydrocarbon metabolites from pesticides (5). In spite of such requirements, some examples of organochlorine pesticides bioremediation could be accomplished *in situ* (34, 39).

Baczynski and co-workers(36) demonstrated that anaerobic biodegradation of dichlorodiphenyltrichloroetano (DDT), metoxychlor and gamma-hexachlorociclohexane (gamma-HCH), is affected by temperature and the ratio of desorbed pesticide. Moreover, only on chlorine atom could be cleaved from DDT in those conditions. This is in agreement with that reported by Alexander (8) who pointed out that biodegradation could produce molecules with at least one chlorine atom. Bacteria related to *Pseudomona*s, *Neisseria*, *Moraxella* and *Acinetobacter* able to degrade almost completely DDT were isolated from Yaqui valley in Sonora, Mexico (40). However, no information on biodegradation mechanism was compiled out.

Anabaena (a cyanobacterium), *Pseudomonas spinosa*, *Pseudomonas aeruginosa* and *Burkholderia* were shown to be good biodegraders of endosulfan (41, 42). The biodegrader KS-2P strain of *Pseudomona* was isolated from endosulfan polluted soil by repetitive enrichment in cultures. This strain could reduce the endosulfan concentration in days in a dose-dependent manner. As far as we know, no mineralization of endosulfan has been observed. Microorganisms from the *Pseudomonas*, *Bacillus*, *Trichoderma*, *Aerobacter*, *Muchor*, *Micrococcus* and *Burkholderia* genera have been shown to biodegrade dieldrin and endrin (43).

Even when HCH is considered as a persistent organic pollutant, it has been demonstrated that it could be bioremediated in situ (34). Murthy and Manonmani (44) identified a HCH-biodegrader consortium which contained species from *Pseudomonas*, *Burkholderia*, *Flavobacterium* and *Vibrio* genera. The biodegradation was achieved within hours. An excellent review by Phillips and co-workers (45) describes and enlists several HCH biodegraders. Interestingly, they could be grouped in two bacteria (*Sphingomonas* and *Pseudomonas*) and one white rot fungi (*Phanerochaete chrysosporium*). HCH mineralization seems to need aerobic and anaerobic conditions like those provided by particles, i.e. in one hand, oxygen could be bioavailable in soil, on the other, soil particles may present niches for anaerobic metabolism. This could explain also why bacteria grown on coffee beans exhibit better biodegradation than those in medium alone (35). Genes encoding enzymes able to degrade gamma-HCH have been named *lin* (37, 46), but further research on biochemical characterization is needed. Comparing biodegradation times for HCH, DDT and endosulfan, differences are observed. Listed in an increasing order of needed time for biodegradation: HCH<DDT<endosulfan. Evidently, this time varies according to the consortium or strains used.

It has been shown that some bacteria could degrade parathion (47) and fenitothrion by using A-esterases (48). From soil, Singh et al. have isolated a strain related to *Enterobacter* which can mineralize chlorpyrifos, parathion, diazinon, coumaphos and isazofos (49). Similarly, it has been found that a bacterial biodegrader related to *Serratia* can degrade diazinon (50). The A-esterase, can be encoded on genome or plasmid. A gene from the genome of a strain related to *Plesiomonas* which can hydrolize methylparathion was cloned to *Escherichia coli* (51). In contrast, the ability to degrade fenitothrion by a *Burkholderia* strain was found to be encoded on plasmids (9). Unfortunately, the presence of other carbon or phosphorous sources reduces the efficiency of organophosphate biodegradation. This limits severely the application of these biodegraders on bioremediation. Further research about parameters influencing biodegradation efficiency is needed to improve their usefullness for bioremediation.

4. Phytoremediators

Phytoremediation –the use of plants for bioremediation- has been less studied than those strategies using bacteria. Nevertheless, it has been proved to be more effective at large scale for soil, water and even for air pollution than bacteria. The mechanisms involved in the phytoremediation success include several bioremediation strategies like phytoextraction,

rhizodegradation, rhizofiltration, phytodegradation, phytostabilization (5, 52) (Figure 6). Several factors affect phytoremediation efficiency (Table 3). The enzymes involved in plant biotransformation are mainly CYP, carboxylesterases, GST and translocases (52). When using a plant, some cautions have to be considered; for instance, introduction of new species should be avoided and plant should tolerate transplantation and pesticide exposition. (5). Ramírez-Sandoval et al., (53) have showed that transplantation itself could induce oxidative stress in plant itself.

Phytodegradation and phytoextraction are the key mechanisms of plant defense (54). Maize (*Zea mays*) and giant foxtail (*Setaria faberi*) can biotransform some herbicides (55). Crop plants like brinjal (*Solanum melongena*), spinach (*Spinacea oleracea*), radish (*Raphanus sativus*) and rice (*Oryza sativa*) can bioaccumulate pesticides like DDT and benzene hexachloride (56). Basil (*Ocimum basilicum*) can bioremediate endosulfan from soil (53). Barley (*Hordeum vulgare*) can translocate herbicide metolachlor into vacuoles (31). Horseweed (*Conyza canadensis*) sequesters glyphosate in vacuoles (57). Also, it has been suggested that genetic engineering could be used to improve phytoremediation abilities of poplars (58) and plants in general (59).

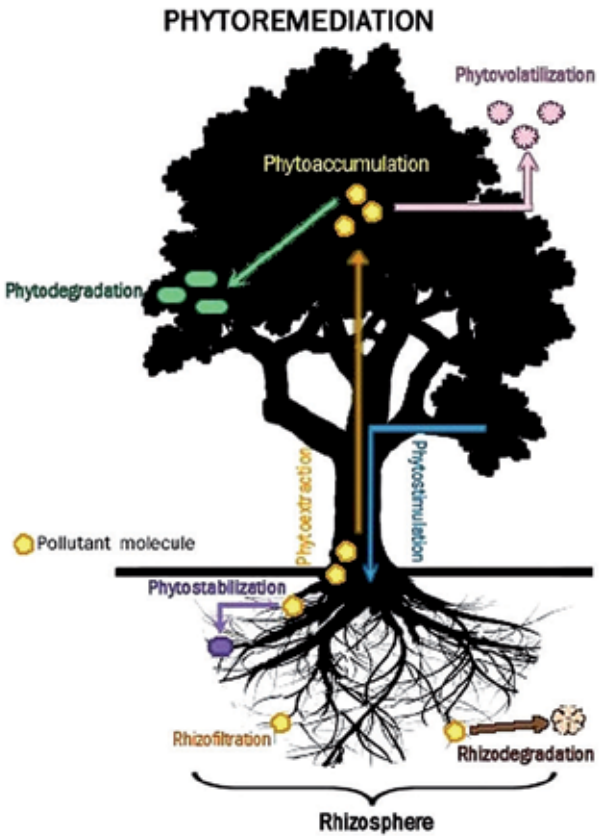

Figure 6. Mechanisms concerning in phytoremediation.

	Absorption	Elimination	
		Physical	Chemical
Site of the plant	Roots, leaves	Leaves, vacuoles	Rhizosphere, plant
Mechanism	Phytoextraction, Rhizofiltration	Phytovolatilization, Phytoaccumulation	Rhizodegradation, phytostimulation, phytodegradation
Limiting factors	Temperature, pH, molecular weight, hydrophobicity	Pollutant concentration, plant defense mechanisms	Class of pollutant, enzymes presence, bioavailability

Table 3. Factors involved in phytoremediation.

Since biodegrader microorganisms can be found in rhizosphere, pairs of plant-rhizosphere are unequivocally better bioremediators than taken separately. Plants exudate carbohydrates and mucilages that stabilize and nurture microorganisms around roots, providing better conditions for microbial growth than soil alone. As a matter of fact, the amount of microorganims around the plants roots are 10- to 100-fold those found in soil alone (60). In addition, some plants can provide co-substrates and oxygen to rhizosphere microorganisms, stimulating them to biodegrade pesticides. Phytostimulation has proved to be one of the most helpful strategies since it brings together the bioremediation capabilities of plant and its biorizhosphere -bacteria and mycorhiza (61).

The efficiency of the phytoremediation depends on several parameters like species, substrate, plant tolerance to pollution, among others. Nevertheless, phytoremediation has several advantages such as the control on bacterial biomass, the slow growth leading to few amounts of plant biomass, the large amounts of soil that could be treated. There are disadvantages or limitations such as the decrease in soil content needed for agriculture, times for accomplish bioremediation longer than microbioremediation, absence of native plants in the ecosystem, among others. Enzymes from microorganisms largely contribute to bioremediation when phytostimulation is performed. Because of this, some successful cases of phytoremediation could be explained by a combination of phyto- and rhizodegradation (53). Rhizoremediation have been used for remediation of the insecticide parathion and the herbicide 2,4-dichlorophenoxyacetic or 2,4-D (6). Pea (*Pisum sativum*) can stimulate endophytic bacteria to also degrade 2,4-D (62).

5. Myco- and phycoremediators

Although less studied, there is some cases worth to mention of biodegraders fungi or algae. Ligninolytic fungi have proven to be good bioremediators. Unfortunately, the nutritional, humidity and pH requirements for some species of fungi and algae represent a big obstacle for its use. Fungi secrete peroxidases, dioxygenases and oxidases able to biodegrade pesticides more efficiently than cytochrome P450 (25). Lignine peroxidase, laccase, and dichlorohydroquinone dioxygenase are some examples of biotransformation enzymes

produced by fungi like *Phanerochaete chrysosporium*, *Pleurotus ostreatus*, *Ganoderma australe* and *Fusarium ventricosum*; the three former are ligninolytic, and the latter is a saprobe. *P. chrysosporium* and *F. ventricosum* are members of soil microbial community.

It has been shown that *P. chrysosporium* can biodegrade endosulfan (17); a CYP and an intracellular peroxidase are likely involved. *F. ventricosum* has been also proved to degrade endosulfan (63). It has been shown that fungal peroxidases and dioxygenases are involved in biodegradation of pentachlorophenol (64, 65). The ligninolytic fungus *Ganoderma australe*, isolated from the stone pine (*Pinus pinea*), is a good biodegrader of lindane (66). This elegant work describes several parameters which has to considered to improve biodegradation like lag time, propagation velocity, biomass growth rate, biodegradation rate, biodegradation/biomass, biomass/propagation and biomass content.

Although in less extent, there are studies on algae ability to bioremediate pesticides in water. Bioremediation appears to occur thanks to bioaccumulation and biodegradation. As in aquatic plants, the biomass overproduction could be a serious disadvantage when using algae for bioremediation waterbodies. In some species, the physicochemical water parameters and other growing conditions might be a matter of caution on choosing these organisms. The unicellular green alga *Chlorella fusca* var vacuolata is able to biotransform the herbicide Metfluorazon by a CYP (14). Recently, it has been described that the alga *Chlamydomonas reinhardtii* can bioaccumulate and biodegrade herbicide prometryne (67).

Two cases of derivative bioremediation have been reported. 1) Using minced shepherd's purse roots, herbicide 2,4-D could be successfully degraded in the presence of hydrogen or calcium peroxide. Temperature did not influence degradation and moisture increased biotransformation (68). 2) An organophosphate hydrolase was immobilized in glass. Even when the activity was decreased in 50% respect to soluble enzymes, its half-life was 280 days and its activity was independent on pH or temperature (69). It was not clear if these characteristics were derived from immobilization or was inherent to enzyme. Regardless, it is clear that immobilized enzymes could be a bioremediation alternative with some advantages, such as the avoidance of biomass production or issues with other growth requirements which have to be dealt with when working with whole organisms.

6. Advantages and disadvantages of bioremediators

Bioremediation strategies show different advantages compared to physico-chemical or thermal treatments aimed to eliminate organic pollutants from environment (Table 4). We refer to maintainable to that strategy capable of being kept from more than a year in spite of the energy, economic and human resources spent to implement it. For instance, after a pesticide release, physicochemical remediation, micro-bioremediation or phytoremediation could be used in one occasion. Nevertheless, if a continuous or an intermittent pesticide release occurs along the year, some strategies should be applied again. Microbioremediation or phytoremediation would be self-maintained through all the year, while physicochemical and some microbioremediation strategies should be implemented each time a pesticide environmental release happens.

	Physico-chemical or thermal remediation	Microbio-remediation	Phyto-remediation
Advantages/Disadvantages			
Cost	High	Low	Low
Benefit/cost ratio	Low	High	High
Environmental friendly	No	Yes	Yes
Aesthetical	No	Some cases	Yes
Self-maintainable	No	Yes	Yes
Energy expenditure	Yes	Low	Low
Uses the metabolism of several organisms	No	Some cases	Yes
Could be used to bioremediate			
Soil	Yes	Yes	Yes
Water	Yes	Yes	Yes
Air	NK	NK	Yes
Requirements			
Specific infrastructure	Yes	Some cases	No
Posterior treatments of residues	Yes	No	No
Biological control or disposal of microorganisms	NA	Yes	No
Time	Short	Medium	Large
Research on bioremediator candidates	NA	Yes	Yes
Key points			
Bioaugmentation should be avoided	NA	Yes	Yes
Posterior pollutant production should be avoided	Yes	No	No
Substrate addition needed	NA	Some cases	No
Oxygenation	Some cases	Some cases	No

Table 4. Advantages and features of some remediation strategies.
NK=Not known, NA=not apply

Due to the exchange of gases, water and metabolites between plants and their surroundings, plants could be used for soil, water or air bioremediation. Water bioremediation could be achieved off-site by filtration throughout soil with bioremediators or *in situ* by aquatic plants able to bioaccumulate metals. Having the plant-rhizosphere ecology, phytoremediation encompasses the microorganism and the plant biodegradation. Moreover, plants exert biological control on rhizosphere biodiversity and quantity; in the same way, fungi and bacteria control them as a result of allelopathy and all the competitive interactions

between rhizosphere microorganisms. In understanding of this, it is reasonable that phytoremediation takes more time than microbioremediation, but the former requires no substrate input and generates fewer sub-products. This suggests that phytoremediation could be a more environmental friendly technology than microbioremediation.

Few bioremediators have been found for each pesticide. Certainly, a bioremediators would not biodegrade all kind of pesticides, or even the same kind of pesticides to which they were proved to bioremediate. To illustrate, it cannot be assumed that a parathion bioremediator will also efficiently biodegrade other organophosphates, let alone other kind of pesticides like organochlorine. Therefore, for each pesticide, adequate bioremediators have to be found. Furthermore, to avoid bioagumentation, it is essential to find the most satisfactory bioremediators.

7. Conclusions

The choice of the bioremediation strategy should be made on the basis of type of pesticide, environmental matrix and the organisms present in the ecosystem. Since, the organism is the only eligible factor, the knowledge about features, advantages or disadvantages of organisms could be a decisive factor on bioremediation proficiency. Some parameters have to be addressed to assure bioremediation. In bacteria and fungi, pH, temperature, cell count, biomass growth rate, substrate bioavailability, and moisture are some of them. Plants require less supervision, but finding the best phytoremediator could be a hard and time-consuming task. Derivative bioremediation is a promising strategy. To get all the benefits from this strategy is necessary to carefully select the most adequate enzyme, and to have it well-characterized. Regardless, further research on biodegradation or biotransformation mechanisms in plants, bacteria, fungi or algae is imperative if bioremediation strategies are to be implemented or improved.

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Growing population in the world demands increase in the food production and intense health care systems. Use of chemical pesticides is imperative for the management insects in agricultural and disease transmission, weeds and harmful microbes. Monitoring and estimating pesticide residue in crop plants, food, soil, water and other ecosystem has become significant in the recent concern on environment and ecosystem. The book comprises of new innovative trends to detect pesticide residue in crop plants, animal origin food and fishes. Different advanced extraction techniques of sample preparation for residue analysis are elaborately described. Apart from residue assays, metabolism and degradation of pesticide compounds fenamophos, chlorpyrifos, pirimiphos, heptachlor and organic pesticides are also documented. This book volume is of twelve chapters contributed by eminent scientists from eleven countries.

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