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Management of Hazardous Wastes

Edited by Hosam El-Din Mostafa Saleh and Rehab O. Abdel Rahman

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and **Rehab O. Abdel Rahman**

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Notice

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Meet the editors

Hosam El-Din Mostafa Saleh is a Professor of radioactive waste management in the Radioisotope Department, Atomic Energy Authority, Egypt. He was awarded MSc and PhD degrees in physical chemistry from the Cairo University. He is interested in studying innovative economic and environment-friendly techniques for management of hazardous and radioactive wastes. He has authored many peer-reviewed scientific papers and monographs and he is book editor of different books related to valuable international publishers.

Rehab O. Abdel Rahman is Associate Professor of chemical nuclear engineering at Atomic Energy Authority of Egypt. She has been awarded Ph.D. degree in nuclear engineering. She has authored more than 30 peer-reviewed scientific papers, 5 book chapters, and 1 monograph. She is an honored scientist of the ASRT and serves as verified reviewer in several journals and managing editor for IJEWM and IJEE.

Contents

Preface

The management of hazardous wastes is an attractive science for study not only for specialists but also for the public because of the complex nature of the problem. People have to recognize and deal with the environment and human health problems associated with water pollution, air pollution, solid waste, and groundwater contamination aspect.

Hazardous waste management deals with minimizing harmful effects on humans and environment through several processes of handling, treatment, transportation, storage, and disposal.

However, it is now realized that wastes have to be managed properly to preserve the environment for the coming generations. Moreover, huge quantities of hazardous waste deposited in landfills, ponds, fields, and other locations require removal or in situ treatment.

Novel researches are published annually on numerous issues facing waste management according to the increasing human activities and consequently environmental contamination facing the present and coming generations. The emphasis has been on publishing articles that address the management of environmental contaminants generally and hazardous waste including the radioactive one particularly.

Through an introductory chapter, the book editor provides a background material to facilitate understanding of the characterization and management of hazardous wastes. The content of the book ***Management of Hazardous Wastes*** was written by multiple authors and edited by experts in the field of *hazardous wastes managemen*t presenting a collection of chapters targeting an audience of practicing researchers, academics, PhD students, and other scientists. Such chapters could facilitate the task of accessing and interpreting scientific data within the closely related research fields.

To achieve the objective, the book reports the hazards associated with both toxic and radioactive waste and processes applied for their management. It established different types for biological remediation of hazardous wastes as one of the promising technologies.

The eight chapters within this book can be divided into two major parts covering some aspects of hazardous waste management conducting biological treatment.

The scope of the first part is quite broad and it discusses generally the management of hazardous and radioactive wastes in developing countries, especially in Malaysia. Moreover, the characterization and valorization of NORM wastes for construction materials and prediction of uranium transport in an aquifer are presented.

The second part deals with the novel microbial bioremediation system for radioactive waste treatment for environmental sustenance.

I would like to thank all the qualified authors from around the globe for their valuable contribution that gave value to this book with a wide variety of studies for the scientific media.

For 10 years of progress and continuous success, the role of *InTech* has been to publish detailed scientific theoretical and applied scientific papers, which can be presented as original research papers and review articles on all aspects of sciences. Particularly, I would like to acknowledge the Publishing Process Managers, Ms. Ana Simcic and Ms. Romina Rovan, for their prosperous cooperation, exceptional efforts, and prompt response to my requests.

Finally, we hope all success for this book to be a useful guide for readers and the scientific community.

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Section 1

Prospective on Hazardous Waste Management

Chapter 1

Introductory Chapter: Introduction to Hazardous Waste Management

Hosam El-Din M. Saleh

Additional information is available at the end of the chapter

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1. Introduction

Waste was associated with human society from prehistory to today and no doubt will continue for the future. People have to manage the produced waste. Disposal of waste into the surrounding locality has to date been the usual practice with little concern for the environment. Waste has to be managed properly to preserve the planet for the coming generations.

Waste generally generated accordingly with life continuity and related proportionally with the human activities such as agricultural, industrial, residential, institutional, municipal, commercial, mining, recreational, and others. This issue is strongly increasing and becomes a potential trouble in the community. The main focus of this study is on hazardous and radioactive wastes accompanying with their Different technologies developed for management.

Rapid trend of industry and high-technological progress are the main sources of the accumulation of hazardous materials. Nuclear applications have been rapidly developed recently, and several nuclear power plants have been started to work throughout the world. The potential impact of released radioactive contaminants into the environment has received growing attention due to nuclear accidents, which pose serious problems to biological systems.

Hazardous wastes are those that may contain toxic substances generated from industrial, hospital, some types of household wastes. These wastes could be corrosive, inflammable, explosive, or react when exposed to other materials. Some hazardous wastes are highly toxic to environment including humans, animals, and plants.

Radioactive waste was generated from use of radioactivity, in many but not all cases. Scientific society has approached the management of radioactive waste differently from the management of other waste types. Radioactive waste is defined as the material that contains or is contaminated with radionuclides at concentrations or activities greater than clearance levels

as established by regulatory authorities. The higher the concentration of radionuclides above the established levels, the greater the hazard the waste possesses. The hazard of radioactive waste also depends on the nature of the radionuclides, and, at the same concentration, different radionuclides have different levels of hazard.

The management of extremely increasing volumes of these wastes became a very important accordingly. Inadequate management of waste led to contamination of environment: water, soil, and atmosphere and to a serious impact on public health. Direct health impacts of mismanagement of waste are well known and can be observed obviously in developing countries.

Saving of the environment and human health from the detrimental effects of hazardous and radioactive wastes is achieved by the effective improvement of waste management programs.

In the scope of this study, the development in waste management planning and implementation of hazardous and radioactive wastes was presented.

2. Hazardous wastes

With increasing manufacturing processes, solid, liquid, and/or gaseous emissions generate as by-products. Some of these wastes are potentially harmful to human health and environment and thus need special techniques of management.

Wastes are classified as hazardous if they exhibit one or more of ignitability, corrosivity, reactivity, or toxicity. According to Resource Conservation and Recovery Act (RCRA), hazardous wastes are defined as any waste or combination of wastes which pose a substantial present or potential hazard to human health or living organisms because such wastes are non-degradable or persistent in nature or because they can be biologically magnified, or because they can be lethal, or because they may otherwise cause or tend to cause detrimental cumulative effects [1].

The management of hazardous wastes has become a specialized discipline because of the complex nature of the problem and the solutions available to humanity. The mismanagement examples of hazardous wastes causing disastrous human and environmental consequences are numerous. The management process is based on the definition and classification of the different wastes, and their toxic effects on human and taking in consideration the application of risk management to control human health and environmental impacts of hazardous waste. Hazardous waste management, therefore, deals with minimizing harmful effects on humans and environment by applying special techniques of handling, storage, transportation, treatment, and disposal of hazardous wastes.

3. Hazardous characteristics

A useful listing of hazardous characteristics is that provided by the United Nations [2] as part of recommendations relating to the transport of dangerous goods as illustrated in **Table 1**.

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UN class number	Hazardous characteristic
1	Explosive
3, 4	Flammable
5	Oxidizing
6	Poisonous/infectious
7	Radioactive
8	Corrosive
9	Toxic (delayed or chronic)/ecotoxic

Table 1. Hazardous characteristics: extracted from UN listing [2].

3.1. Industrial wastes

Waste generated from industrial sources can have non-hazardous and hazardous components, with non-hazardous waste usually representing the greater part of the volume. The hazardous component of this waste is relatively small in volume [3].

This type of waste was identified as hazardous waste when proceeds toxicity test, corrosively test, ignitability test, and some special character test. As a hazardous pollutant, it may impose serious impacts on surrounding environment and such impacts should be quantitatively examined to assess the influence on human health [4].

3.2. Household waste

Households generate small quantities of hazardous wastes such as oil-based paints, paint thinners, wood preservatives, pesticides, insecticides, household cleaners, used motor oil, antifreeze, and batteries. It has been estimated that household hazardous waste in industrialized countries such as the United States accounts for a total of about 0.5% (by weight) of all waste generated at home, while in most developing countries, the percentage probably is even lower [3].

3.3. Biomedical waste [5]

There are some of hazardous medical and dental wastes that, when disposed improperly, could cause harm to the environment. It also presents an occupational health hazards to the healthcare personnel who handle these wastes at the point of generation and those involved with their management, that is, segregation, storage, transport, treatment, and disposal.

Healthcare waste that is capable of producing injury or disease including many sorts of hazardous wastes such as:

- Infectious waste: Which contain pathogens namely bacteria, viruses, fungi, or parasites in concentrations sufficient to cause disease in susceptible hosts. Cultures and stocks of infectious agents from laboratory work; tissues and dressing generated from autopsies,

surgeries, and treatment of infected patients and animals; materials or equipment in contact with blood and infected body fluids.

- Pathological waste: Including tissue, organs, body parts, human fetuses, and animal carcasses, blood and body fluids.
- Sharps: It comprise syringes, needles, scalpels, saws, infusion sets, knives, blades, broken glass, or other items that can cause cut or puncture wounds.
- Pharmaceutical waste: It covers expired, unused, spilt, and contaminated pharmaceutical products, drugs, vaccines, and sera that are no longer required and need to be disposed of in appropriate manner.
- Genotoxic waste: This type combines cytostatic drugs, vomit, urine, or feces from the patients treated with cytotoxic drugs, chemicals, and radioactive materials. Genotoxic waste has mutagenic, teratogenic, and carcinogenic properties.
- Chemical waste: Discarded solid, liquid, or gaseous chemicals should be considered as hazardous if it is toxic, corrosive, inflammable, or reactive.
- Waste with high content of heavy metals: Mercury (thermometers, blood pressure gauges, amalgam), cadmium (discarded batteries), and lead (reinforced wood panels for radiation proofing in radiology department) generated from hospitals could be represented as a subcategory of hazardous chemical waste.
- Radioactive waste: The use of radioisotopes in vitro analysis of body tissues and fluids, in vivo organ imaging, tumor localization, and treatment and various clinical studies involving certain radionuclides need to be specially managed in a centralized treatment facility for radioactive wastes.

3.4. Radioactive waste

Nuclear applications have been rapidly developed recently, and several nuclear power plants started to work throughout the world. The potential impact of released radioactive contaminants into the environment has received growing attention due to nuclear accidents. Contamination of soil and water by radionuclides due to natural processes, global fallout from nuclear weapon testing, discharges from nuclear installations, disposal of nuclear waste, and occasional nuclear accidents (i.e., Chernobyl in 1986 and Fukushima in 2011) poses serious problems to biological systems. Radioactive waste includes a variety of radionuclides and occurs in a variety of physical and chemical forms. It can be generally classified as low-/intermediate-level radioactive waste and high-level radioactive waste [6].

Radioactive waste, arising from civilian nuclear activities as well as from weapon activities, poses a potential problem for handling and saving the environment for coming generations.

Radioactive waste includes a variety of radionuclides and occurs in a variety of physical and chemical forms. It can be generally classified as low-/intermediate-level radioactive waste and high-level radioactive waste. Nuclear research establishments include, for example, waste containing different organic components, toxic or chemically aggressive constitu-

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ents, radionuclides with specific properties (high mobility, high chemical activity, volatile elements, etc.), waste difficult for treatment and not appropriate for direct immobilization (e.g., spent organic ion exchange resins and spent liquid scintillation cocktails). For such waste, application of conventional treatment and conditioning options may not be efficient and appropriate in terms of economy, safety, and performance characteristics. In many cases, such wastes are stored awaiting an appropriate treatment and conditioning solution [7].

The primary sources of radioactive wastes in a country without nuclear fuel cycle activities are nuclear research, production of radioisotopes, application of radioisotopes, and decontamination and decommission of nuclear installations.

4. Hazardous and radioactive wastes management

Waste management is an important component of environmental policy all over the world. Priority of hazardous solid waste for environmental protection is formulated on source reduction and reuse, recycling, treatment, and landfilling [8].

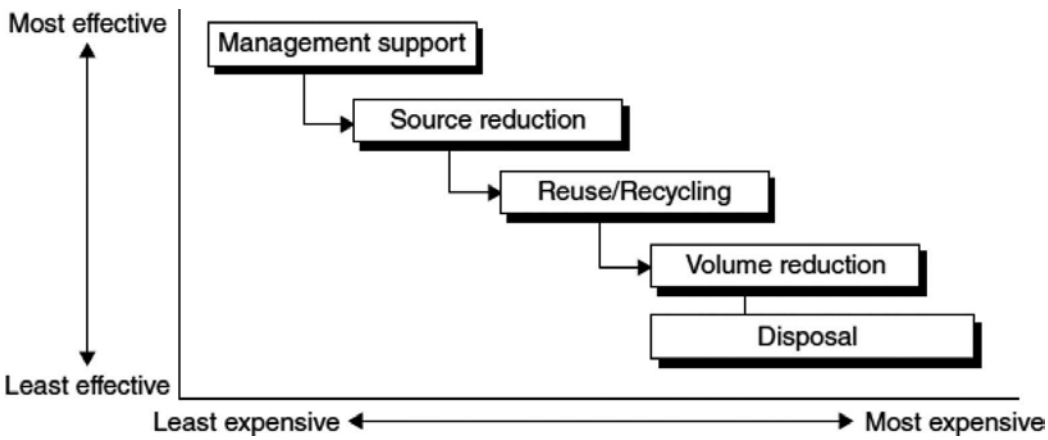

Figure 1. Minimization program for low- and intermediate-level radioactive wastes [11].

The objective of radioactive waste management is to deal with those wastes that in a manner that protects human health and the environment now and in the future without imposing undue burden on future generations [9]. Radioactive waste management involves many steps keeping the position of radioactivity clean. As nuclear power and arsenal grow, continuous monitoring and immobilization of waste over several decades and centuries and deposition in safe repositories assume great relevance and importance. The overall waste management generally includes the following steps: segregation and sorting of the radioactive wastes, treatment, conditioning, storage, transport, and final disposal. To achieve a satisfactory overall waste management strategy, component steps must be complementary and compatible with each other [10]. Consequently, for every nuclear activity, there should be a waste minimization

program that aims to reduce the amount of generated wastes. Such programs should cover the organizational, technological, and economic aspects of the performed waste minimization processes. The key considerations of the minimization program for low- and intermediate-level radioactive wastes are illustrated in **Figure 1**, based on the effectiveness and cost of processing [11].

4.1. Waste minimization

Waste minimization is a process aimed to reduce the amount and activity of waste to a level as low as reasonably achievable. This process is now applied at all stages of nuclear processing from power plant design through operation to decommissioning. It consists of reducing waste generation as well as recycling, reuse, and treatment, with due consideration for both primary wastes from the original nuclear cycle and secondary wastes generated by reprocessing and clean-up operations [12].

Some wastes may require treatment for safety, handling, or stability for interim storage reasons. Treatment methods can be generally classified as chemical, physical, and/or biological. For new wastes, there is an opportunity to influence the process design so that wastes generated will require little or no treatment. If treatment is required, it is usually easier to obtain most of the characterization, while the waste is in raw form and characterization requirements may be directed toward treatment process control. For historic wastes, many situations are possible. Wastes may have already undergone some degree of treatment with little or no precharacterization. In such a case, further characterization will be required both before and during treatment to obtain a sufficient degree of detailed information. Waste streams may have been inadvertently combined, leading to a much larger volume of material that must be checked for certain properties. Previously treated wastes need to be examined to determine the compatibility of the prior treatment process with the waste acceptance criteria for the conditioning and disposal phases [13].

However, the seeking of inexpensive methods has led to develop new technologies based on the utilization of plant in biosorption of hazardous elements such as radioisotopes. Phytoremediation is the using of plants to remove hazardous contaminants from the environment. This trend is a growing application in remediation studies due to its numerous advantages, such as environmental friendliness, cost-effectiveness, and high abundance [6, 14]. Phytoremediation like other traditional treatment processes has to follow the subsequent step called immobilization process, and it could be done to solidify and stabilize the resulting secondary solid waste in an inert matrix [15]. The efficiency of contamination removal by phytoremediation can be greatly enhanced by a proper selection of the species suitable for the nature of pollutant and according to its geographic location, the microclimate, hydrological conditions, soil properties, and accumulation capacity of the plant species.

4.2. Processing and immobilization

Processing of radioactive waste includes any operation that changes its characteristics such as pretreatment, treatment, and conditioning. Solidification/stabilization (s/s) of hazardous and

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radioactive wastes is an attractive technology to reduce their risks and facilitate their handling prior to disposal. The long-term safe landing of the solidified hazardous waste is an important request for keeping the surrounding environment more secure for the coming generations [16, 17]. Immobilization reduces the potential for migration or dispersion of contaminants including radionuclides. The International Atomic Energy Agency (IAEA) defines immobilization as the conversion of a waste into a waste form by solidification, embedding, or encapsulation. It facilitates handling, transportation, storage, and disposal of radioactive wastes. Conditioning means those operations that produce a waste package suitable for handling, transportation, storage, and disposal. Conditioning may include the conversion of waste to a solid waste form and enclosure of waste in containers [12].

Solidification/stabilization is typically a process that involves the mixing of a waste with a binder to reduce the contaminant leachability by both physical and chemical means and to convert the hazardous waste into an environmentally acceptable waste form for land disposal. Moreover, it provides the waste form acceptable mechanical performance to withstand transport and handling. Inorganic binders such as cement are effective in immobilizing heavy metals through chemical and physical containment mechanisms, but are not as effective in immobilizing most organic contaminants. Many substances in the wastes significantly affect the setting and hardening characteristics of binders, especially cement-based cementing systems [18].

The requirements for the waste form are to provide physical, chemical, and thermal stabilities of the solidified radioactive materials. Moreover, the immobilized final waste forms resist leaching, powdering, cracking, and other modes of degradation. Portland cement is the most widely inorganic-based system used for solidification/stabilization of hazardous, low- and intermediate-level radioactive wastes [19–21].

4.3. Disposal of hazardous and radioactive wastes

Waste disposal is the final step of waste management and ideally comprises placing hazardous waste in a dedicated disposal facility, although discharging of effluents into the environment within permitted limits is also a disposal option. Concepts for radioactive waste disposal have, however, developed considerably since that time and great consideration is now given to the necessary retention times and retention capacities for different types of waste, resulting in much-improved repositories and planned disposal facilities [12].

According to IAEA, the disposal of radioactive waste is defined as the emplacement of waste in an approved specific facility that is intended to isolate the waste from human and environment and to prevent or limit a release of potentially harmful substances such that human health and the environment are protected. However, the safe disposal of radioactive wastes is one of the main concerns for those who oppose the nuclear technology. Therefore, disposal plays an important role in public acceptance of civilian applications of nuclear technology in different nations [10].

The effect of climatic conditions, for example, flooding and freezing/thaw accidents on the solidified wastes, is one of the most issues taken into consideration to evaluate the performance

of this immobilized hazardous and radioactive wastes under the worst climatic conditions during the disposal process. Flooding accidents, one of the main dangerous problems that could face the solidified waste at the disposal site, should deserve special attention. Water is the primary agent of both creation and destruction of many natural materials, happens to be central of most durability problems in solidified waste materials [22, 23]. The rate of chemical deterioration is dependent on whether the chemical attack is confined on the surface of solidified waste material or also happening inside the material. The rate of deterioration is affected by the type and the concentration of ions present in water and by the chemical composition of the solid matrix.

5. Multi-barrier concept

Safety strategy for radioactive waste containment and isolation for the proposed storage and transportation focuses on two objectives: (1) to provide stabilization of the radioactive waste within the including package and (2) to limit the radiation exposure dose of the public during the transportation or other handling processes [24].

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Chapter 2

Characterization and Valorization of Norm Wastes for Construction Materials

Irene García-Díaz, Manuel Jesus Gázquez,
Juan Pedro Bolivar and Felix Antonio López

Additional information is available at the end of the chapter

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Abstract

The recycling of waste generated by industrial production processes is a topic of considerable environmental and economic interest. The minimization of waste disposal, avoiding its direct release into the environment, generates environmental benefits for industries in addition to the manufacture of the main product. Some of these wastes, measured by their radioactive element content, may be considered as naturally occurring radioactive material (NORM). Two of these NORMs are phosphogypsum (PG) and ilmenite mud (IM) come from the fertilizer industry and TiO_2 pigment industry, respectively. This chapter discusses the viability of valorization and/or recycling of PG and IM in the manufacture of sulfur polymer cement/concrete, Portland cement, and ceramic materials.

Keywords: Phosphogypsum, llmenite mud, sulfur polymer concrete/cement, portland cement, ceramic

1. Introduction

Some minerals or raw materials used in industrial process can contain radionuclides at concentrations which cannot be disregarded. The main natural radionuclides derive from the ^{238}U and ^{232}Th decay series. In some cases, industrial processing can lead to further enhancement of the concentrations in the product, by-product or in the waste materials.

These materials are known as naturally occurring radioactive material (NORM) used to describe materials that contain radioactive elements, radionuclides, found in the natural

environment. Long-lived radioactive elements of interest include uranium, thorium, and potassium, and any of their radioactive decay products such as radium and radon. However, the term is used more specifically for all naturally occurring radioactive materials where human activities have increased the potential for exposure compared with the unaltered situation. Sometimes artificially concentrated NORM is called technologically enhanced naturally occurring radioactive material (TENORM).

Environmental management of NORMs has taken on a new urgency in the last few decades. Increasingly, the governments are adopting more stringent regulations on radiation and environmental protection in general. Nowadays, the recycling and/or valorization of wastes generated by different industries are increasing exponentially [1–6]. Reduction in the disposal of these wastes has environmental and economic benefits for the industries and for the population.

This chapter is focused mainly in the valorization for construction materials of two NORMs coming from the fertilizer industry—phosphogypsum (PG) and the TiO_2 pigment industry—ilmenite mud (IM).

1.1. Fertilizer industry: phosphgypsum

The continuous rise in world population increases the demand for food production which involves a constant increase in the use of phosphate fertilizer [7] and the development of chemical industries related to their production. Phosphate rocks are used for manufacturing phosphoric acid and chemical fertilizers. These are mainly composed of carbonate fluorapatite ($Ca_{10}F_2(PO_4)_6CaCO_3$), quartz, goethite; minor mounts of Al-phosphates, anatase, monazite,

Country	^{238}U	^{232}Th	^{226}Ra	^{228}Ra
USA	259–3700	3.7–22	1540	–
USA:Florida	1500–1900	16–59	1800	–
Brazil	114–880	204–753	330–700	350–1550
Chile	40	30	40	–
Algeria	1295	56	1150	–
Morocco	1500–1700	10–200	1500–1700	–
Senegal	1332	67	1370	–
Tunisia	590	92	520	–
Egypt	1520	26	1370	–
Jordan	1300–1850	–	–	–
Australia	15–90	5–47	28–90	–

Source: IAEA Tech Report 419, p. 90.

Table 1. Activity concentration (Bq/kg) of radionuclides present in different phosphate rock samples.

barite, magnetite; and heavy metals and trace elements (Cd, Ni, As, and Sr) [8, 9]. The phosphate rock also contains certain levels of natural radioactivity being a major NORM due to its uranium (^{238}U) and thorium (^{232}Th) content [10], **Table 1**. So the fertilizer industry has an impact in conventional and radioactive environmental contamination [11, 12].

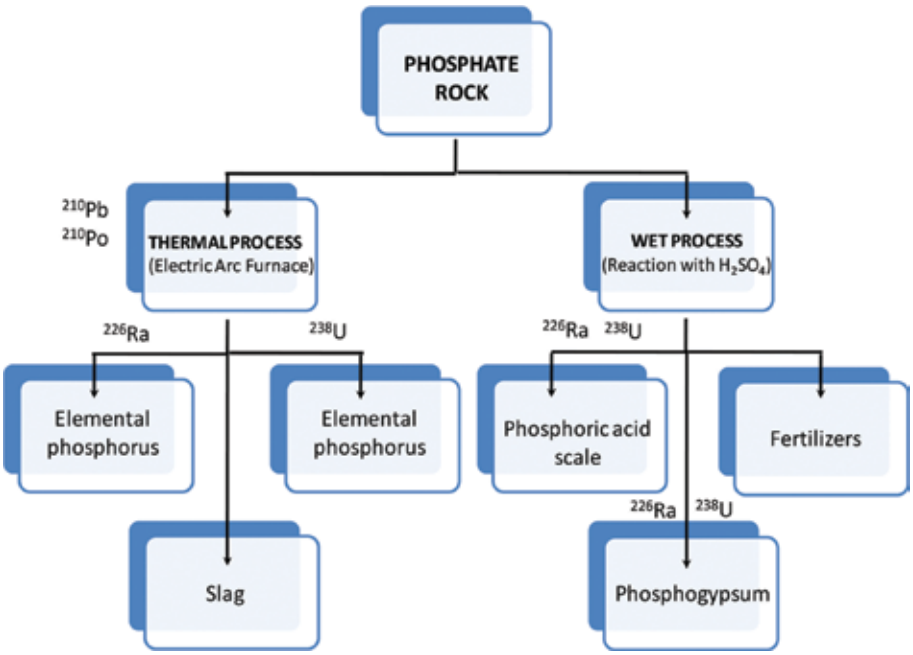

Figure 1. Schematic of phosphate processing [13].

Phosphate ore is processed to obtain phosphoric acid by the dry thermal or the wet acid methods, **Figure 1** [13].

Currently, over 90% of phosphoric acid production is by the wet acid method, involving the chemical attack of phosphate rock ore with sulfuric acid, generating a waste called phosphogypsum ($CaSO_4 \cdot 2H_2O$), Eq. (1). This process is economic, however, large amounts of PG are generated—around 5 tons per ton of phosphate rock.

$$Ca_5(PO_4)_3F + 5H_2SO_4 + 10H_2O \rightarrow 3H_3PO_4 + 5\,CaSO_4 \cdot H_2O + HF \quad (1)$$

$CaSO_4 \cdot H_2O$ is the main component of phosphogypsum, making up over 90% of it. Other impurities such as residual acids, fluorides (NaF, Na_3AlF_6, Na_3FeF_6 Na_2SiF_6, CaF_2), fluoride sulfate ions, H_3PO_4, $Ca_3(PO_4)_2$, $CaHPO_4 \cdot 2H_2O$, $Ca(H_2PO_4)2H_2O$, residual acids, some trace metals (e.g., Cr, Cu, Zn and Cd), and organic matter can be attached to the surface of the

gypsum crystals [13–16]. Due to the residual acid compounds, PG has a pH 3 and is thus classed as an acidic by-product.

The wet process causes the selective separation and concentration of naturally occurring radionuclides present in phosphate rock. Depending on the source rock, PG can contain as much as 60 times the level of radionuclides normally found prior to processing, with around 80% of ^{226}Ra, 86% of U, and 70% of Th ending up in the phosphoric acid, **Table 2** [13]. In particular, a great variation of ^{226}Ra activity is observed depending on the nature of the phosphate rock. For example, this ranges between 15 Bq/kg in Sweden and 114 Bq/kg in Florida. It is important to be aware of this radionuclide content due to the fact that ^{226}Ra produces radon gas (^{222}Rn) which has a short half-life of 3.8 days and is therefore highly active, that is, has a high radiation output and can cause significant damage to internal organs. High levels of ^{238}U and ^{210}Po activity have also been reported by various authors.

Country	Refs.	PG rock origin	^{238}U	^{236}Ra	^{210}Pb	^{210}Po	^{230}Th
Indonesia	[17]	Pt PetrokimiaGresik	43	473	480	450	–
China	[17]	Keiyan	15	85	82	82	
India	[17]	Vadorado	60	510	490	420	
Egypt	[17]	Nile Valley Rock	–	100	–	445	–
Florida	[14]	Central Florida	130	1140	1370	1030	113
Australia	[14]	Numerous	10	500	–	–	–
Sweden	[14]	Kola (USSR)	390	15	–	–	–
Spain	[18]	Morocco	140	620	–	82	280

Table 2. Radiochemical analyses of different PG types (Bq/kg) [13].

PG is stored without any prior treatment, requires a large land area, and can cause serious environmental contamination of soils, water, and the atmosphere. So PG management is one of the most serious problems currently faced by the phosphate industry.

1.2. TiO_2 industry: ilmenite mud

The titanium product most widely used is not in the form of titanium metal or alloy. It is a white powder—titanium dioxide (TiO_2) pigment. Because it provides whiteness and opacity, this is widely used in a huge range of products and industries from coatings and plastics, printing inks, as flux in glass manufacture, filler material for paper industries and rubber. It is also an important substance, although not used in such high volumes, in important sectors such as pharmaceuticals, foodstuffs, and cosmetics [19, 20]. The annual TiO_2 worldwide production is around 4.5 million t [21].

Two different commercial processes are used in TiO_2 pigment production—the chloride process using chloride gas and the sulfate process using concentrated sulfuric acid. Around

60% of total TiO_2 production is by the chloride process and 40% by the sulfate process [20–22].

The main feedstocks for the TiO_2 industry are rutile, synthetic rutile, and chloride slag, all used in the chloride route, and ilmenite and sulfate slag, used for the sulfate process. These ores can contain levels of naturally occurring radioactive material so this is a NORM industry, **Table 3**.

In the sulfate route, the main raw material used to produce TiO_2 is ilmenite ore ($FeTiO_3$). This contains approximately 43–65% TiO_2. A titanium slag (a co-product of the ilmenite ore coming from smelting process) which contains 70–80% titanium dioxide [23–25] is also used. The ilmenite contains around 300 Bq/kg of the radionuclides from the thorium series and around 100 Bq/kg for the radionuclides of the uranium series. This requires it to be classed as a NORM industry [26].

TiO_2 production begins with a highly exothermic reaction involving the digestion of a mix of ilmenite and titanium slag by highly concentrated (98%) sulfuric acid and water. The general steps for the digestion reaction are dissolution of the raw material, given in Eq. (2), followed by TiO_2 precipitation, Eq. (3).

$$FeTO_{3(S)} + 2H_2SO_{4(aq)} \rightarrow TiOSO_{4(aq)} + 2H_2O_{(aq)} + FeSO_{4(aq)} + MUD(s) \quad (2)$$

$$TiOSO_{4(aq)} + H_2O(aq) \rightarrow TiO_{2n} \cdot H_2O(s) + H_2SO_{4(aq)} \quad (3)$$

Raw materials	TiO_2 (%)	^{238}U	^{232}Th (Bq/g)
Rutile	93–96	100–740	80–360
Synthetic Rutile	88–95	40–80	140–1900
Chloride slag	85–86	2–80	1–120
Sulfate slag	79–86	2–80	10–120
Ilmenite	45–65	100–10,000	80–200

Table 3. Radionuclide activity of raw materials used in TiO_2 production (Bq/kg) [21].

After the sulfuric acid dissolution, the liquid contains titanylsulfate ($TiOSO_4$) and iron sulfate ($FeSO_4$).The liquor is then passed to a clarification tank, where the un-dissolved ore (solid ilmenite mud) is separated by flocculation and filtration. It is then neutralized and stored in a safe area (**Figure 2**).

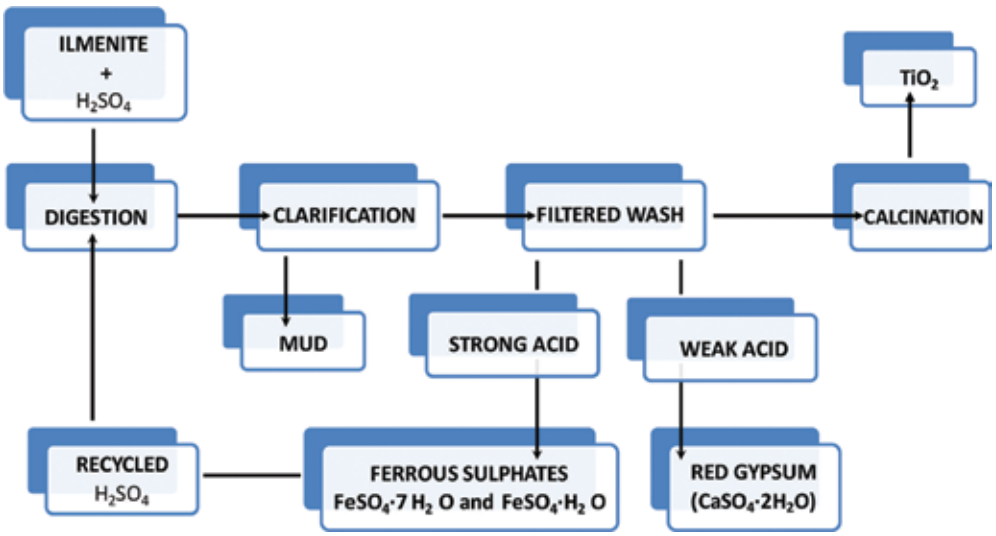

Figure 2. Sulfate route to TiO_2 production.

After ilmenite mud separation, the TiO_2 is precipitated from the clarified liquor by boiling it for several hours followed by cooling. Then, the $TiO_{2(S)}$ is separated by vacuum filters from the liquor, which is a "strong" acid, with a content 20–25% H_2SO_4. After filtration, the hydrated titanium dioxide slurry is placed in rotatory kilns where the TiO_2 crystals grow to their final size and the water and sulfur traces are removed [25]. The production of half a ton of TiO_2 pigment requires one ton of raw materials.

During the feedstock digestion with sulfuric acid, the radionuclides are distributed; U and Th believe will pass forward in liquor; Ra has low solubility and suggests 75–95% is not digested; and Pb has low solubility and expects majority to remain in the solid phase. Considering that after the digestion, the solid volume is reduced by a factor of 5–6 and the overall impact of the radionuclides activity in the residue, ilmenite mud, is increased. The activity concentration for the ilmenite mud is typically given as, ^{238}U series = 600–1000 Bq/kg (in a feedstock 100–200 Bq/kg) and ^{232}Th series = 1200–2500 Bq/kg (in a feedstock 400–500 Bq/kg) [24, 26].

The ilmenite mud remaining at the end is enriched in radium isotopes, ^{226}Ra (860 Bq/kg) and ^{228}Ra (2590 Bq/kg) as well as ^{40}K (280 Bq/kg). The increased radium concentration is due to the insolubility of radium as a sulfate [21]. The relatively high concentration of ^{40}K in ilmenite mud is because the main form of ^{40}K (the raw material had 18 Bq/kg) in the feedstock, and this remains unaltered by the process [24].

So the ilmenite mud is a highly acid waste that contains a large amount of natural radionuclides and also certain metals. All of these can adversely affect the environment and must be managed adequately.

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2. Norm wastes application for construction materials

2.1. Physico-chemical and radiological characterization of raw material

Table 4 shows the chemical composition for the major elements in PG and IM and in the raw materials [gravel, sand, red stoneware (RSM), and clinker] used in the various valorization processes. The PG and IM were collected from a PG piles and TiO_2 factory located at the Southwest Spain in the city of Huelva, respectively.

	SiO_2	Al_2O_3	CaO	Fe_2O_3	MgO	TiO_2	SO_3	P_2O_5	K_2O	Na_2O
PG	2.43	0.40	40.3	0.23	0.04	0.04	52.4	0.95	0.03	0.13
IM	11.9	1.44	0.73	12.5	0.94	52.9	7.79	0.02	0.16	0.16
Gravel	29.1	1.27	57.8	0.8	0.63	0.13	0.27	–	0.25	–
Sand	79.9	10.8	0.42	0.74	0.26	0.11	–	–	5.3	1.78
RSM	62.0	19.9	2.10	7.39	1.73	1.67	0.06	–	4.20	0.58
Clinker	22.0	5.3	66	2.8	1.4	–	0.53	–	1.0	0.04

Table 4. Concentration (%) of major elements.

PG is composed of 40% CaO and 54% SO_3. The main mineralogical phases by X-ray diffraction (XRD) are gypsum ($CaSO_4{\cdot}2H_2O$) and basanite ($CaSO_4{\cdot}1/2H_2O$). Also, a 0.95% of P_2O_5 is founded in PG sample, which remains later the washing in the industrial process [27].

The IM sample had a high concentration of titanium oxides (53%) and appreciable amounts of iron and silicon oxides (12.5 and 11.9%, respectively). Similar compositions have been observed elsewhere [24]. In the ilmenite mud, ilmenite (22%) and rutile (34%) were the main mineral phases observed by XRD. Other species such as Fe and Ti oxides ($Fe_3Ti_3O_{10}$), zircon ($ZrSiO_4$), and quartz (SiO_2) at 18.2, 13, and 12.1%, respectively, were also present.

In relation to the valorization studies for these industrial wastes, it is important take into account the particle sizes. The d (0.5) value of the particles of phosphogypsum and mud measured was ≤53 and 30 μm, respectively.

To evaluate the potential radiological impacts of these wastes when used in the production of building materials, the EU has proposed reference values for the natural radionuclide concentrations [28], defining an external risk index (I), also called the activity concentration index, according to the following equation:

$$I=\frac{C_{226_{Ra}}}{300}+\frac{C_{332_{Th}}}{200}+\frac{C_{40_K}}{300} \tag{4}$$

where C_{226Ra}, C_{232Th}, and C_{40K} are the activity concentrations for ^{226}Ra, ^{232}Th, and ^{40}K, respectively, expressed in Bq/kg.

This index should not exceed one ($I \leq 1$) for any material used in bulk quantities, for example, cement or concrete. It should not exceed six ($I \leq 6$) for superficial materials, for example, tiles or boards. This is to ensure that the external dose received by occupants does not exceed the reference value of 1 mSv/year [28–29]. Other countries use the equivalent radium concentration parameter, Ra(eq), given in Eq. (5):

$$\mathrm{Ra(eq)} = \mathrm{C}\left(^{226}\mathrm{Ra}\right) + 1.43\mathrm{C}\left(^{228}\mathrm{Ra}\right) + 0.077\mathrm{C}\left(^{40}\mathrm{K}\right), \tag{5}$$

where C(^{226}Ra), C(^{228}Ra), and C(^{40}K) are the activity concentrations for ^{226}Ra, ^{228}Ra, and ^{40}K in Bq/kg, respectively. Three hundred and seventy Bq/kg is taken as the reference.

The concentration of natural radionuclides from the ^{238}U decay series in phosphate rock (PR) used in Huelva was around 1650 Bq/kg (**Table 5**). This is over 50 times higher than the concentrations in unperturbed soils which are around 25 Bq/kg of ^{238}U and ^{232}Th [30]. Ilmenite ore (ILM) is also a NORM mineral since it is high in natural radionuclides from both the Th and U series with a total concentration of over 400 Bq/kg for ^{238}U and ^{232}Th nuclides [24].

Code	^{210}Pb	^{238}U	^{232}Th	^{226}Ra	^{228}Ra	^{40}K	I	Ra(eq)
PR	1600 ± 90	1650 ± 70	25 ± 2	1580 ± 80	22 ± 2	<18	5.4	1612
PG	624 ± 37	97 ± 6	8.2 ± 1.0	589 ± 34	8 ± 1	<18	3.9	621
ILM	82 ± 4	105 ± 10	315 ± 20	110 ± 10	300 ± 20	30 ± 5	1.9	541
IM	247 ± 11	184 ± 12	250 ± 15	521 ± 30	1919 ± 112	334 ± 21	11.4	3291

Table 5. Natural radionuclide concentrations (Bq/kg) and radium equivalent (Bq/kg) and external risk index (I) calculated in raw materials and NORM wastes.

The activity concentration of ^{238}U and ^{226}Ra in the pure PG used in this work is around 100 and 600 Bq/kg, respectively, which are significantly higher than the average world-wide values from unperturbed soils. The ilmenite mud (IM) also has a total radionuclide concentration higher than 1000 Bq/kg. ^{226}Ra and ^{228}Ra are the radionuclides with the highest activity concentrations in IM at around 500 and 2000 Bq/kg, respectively. This is a considerable fraction of the radioactivity content originally present in the raw material.

2.2. Use of phosphogypsum and ilmenite mud in sulfur polymer cement/concrete manufacturing

The manufacture of Portland cement is associated with a high energy consumption (850 kcal/kg of clinker) due to the high temperature required to produce Portland cement clinker (around 4500°C). This has negative environmental effects as around 850 kg of $CO_2$is emitted into the atmosphere per ton of clinker produced, as well as other greenhouse gases and $NO_x$and SOx. It also involves massive quarrying for raw materials (1.7 ton to produce 1 ton of clinker) [3, 4].

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In contrast, the manufacture of traditional binders such as sulfur polymer concretes (SPCs) does not require a heavy energy input. SPCs are based basically in the sulfur properties. To manufacture, a thermoplastic material is melted and mixed with an aggregate after which the mixture poured, molded and allowed to harden. These materials have been studied extensively over the past few decades.

Various trials have been carried out in which sulfur was used as the basis of construction materials [31–34]. In these studies, SPCs were prepared by heating of a mix of elemental sulfur (un-modified sulfur) and mineral aggregates. However, the SPCs manufactured using elemental sulfur exhibited durability problems. They failed under repetitive cycles of freezing and thawing, humid conditions, or immersion in water [31, 35].

Extensive research has established the reason for the failure of these SPCs and determined ways of preventing it. When the hot mixture of elemental sulfur and aggregate is cooled to cast SPCs the liquid sulfur binder crystallizes at 114°C as monoclinic sulfur (Sβ) with a decrease in volume of around a 7%. When the temperature goes below 96°C, the Sβ begins to transform to orthorhombic sulfur Sα which is the stable form of sulfur at ambient temperature [35]. This transformation is rapid (<24 hours) and causes the material failure. The Sα form is denser than Sβ. In the material, high strength is induced by the change in the material. Thus, the sulfur binder can become highly stressed and cause durability problems, mainly when it is exposed to water conditions.

It is necessary modify the sulfur-based concrete to reduce the expansion and contraction during thermal cycling and achieved a product with good durability. Several chemical substances to inhibit the transformation of monoclinic to orthorhombic sulfur and to improve SPC products have been studied. Such modifiers are now available. Among the modifiers, commonly used are dicyclopentadiene (DCPD) or a combination of dicyclopentadiene, cyclopentadiene, and dipentene [31–36].

Sulfur concrete has properties, which for certain applications are superior to Portland cement concrete (PCC). Compared to traditional PCC, the main advantage of sulfur concrete is its high durability. SPC is also resistant to aggressive chemical environments and has good mechanical properties, high frost resistance, low absorbability, antiseptic qualities, water resistance, and good thermal insulating and dielectric properties [37]. SPCs have a fast setting time reaching nearly full mechanical strength in <24 hours. These features make SPCs an ideal material for a wide range of applications in different fields, such as in road and railway infrastructure, hydrotechnical constructions, waste water treatment plants, and landfills [37]. Due to their generally good properties and higher adhesive strength, SPCs are can also be used for stabilization/solidification in aggregate recycling and radioactive waste storage [35, 38–40].

Valorization of PG and IM by incorporating them into sulfur polymer concretes was studied by the authors of this chapter.

Different mixtures were prepared with percentages of PG and IM between 10 and 30 wt%. Each sample was denominated as SPC-XPG-Y and SPC-XIM-Y, where “X” is the percentage wt% of elemental sulfur and “Y” is the percentage wt% of phosphogypsum or ilmenite mud of the mixture. The reference sample was SPC.

The SPCs were prepared by first heating the aggregates (gravel/sand and NORM waste) at 130–135°C for 4 h. Then, the sulfur, gravel, sand, and NORM waste are heated together in a preheated mixing bowl, where the temperature is controlled at 135–140°C for 10 minutes. After this time, the modified sulfur STX™ is added with continuous mixing. Finally, the mixture is stirred at 140–145°C for 4–5 minutes. The molds (4 × 40 × 160 mm) are preheated to approximately 120°C before adding the sulfur polymer concrete. The material is compacted using a vibration table at 3000 rpm for 30 seconds. The storage of the moulds is done at a room temperature. The specimens are de-molded 24 hours after placement in the steel moulds.

The compressive Cs and flexural strength Fs in SPC cements are shown in **Table 6**. The compressive strength of the SPCs has values between 55–62 MPa for PG and 58–64 MPa for IM. These values are in line with those reported by other authors. For example, López et al. [38] present values of 54 and 58 MPa for metacinnabar SPC with sulfur/HgS ratios of 0.4 and 2.6, respectively. Abdel-Mohsen et al. [31] obtained a compressive strength of 54 MPa for SPC with fly ash, where the sulfur/fly ash proportion was 0.9. SPCs with lead wastes, have been studied by Lin et al. [39], and the final compressive strength of the SPC was 48.5 MPa. Between 65 and 73 MPa was the SPC strength obtained using a recycled aggregate coming from the ceramic industry [40].

	Fs (Mpa)	Cs (Mpa)	Ratios sulfur/waste
SPC-21	7.07 ± 0.66	57.70 ± 1.98	–
SPC-17PG-10	9.36 ± 0.24	55.41 ± 1.37	1.70
SPC-19PG20	11.20 ± 0.14	62.11 ± 0.86	0.95
SPC-21PG30	10.72 ± 0.60	56.76 ± 5.20	0.70
SPC-17IM10	9.83 ± 1.82	58.45 ± 1.62	1.70
SPC-21IM20	13.25 ± 0.77	64.38 ± 1.62	1.05
SPC-21IM30	9.40 ± 0.52	36.77 ± 2.58	0.70

Table 6. Values of compressive strength (Cs) and flexural strength (Fs) in the SPCs at 1 day old the ratios of sulfur to waste.

These results can be compared with Portland cement, which has a Cs > 52.5 MPa. At 28 days of curing Portland cement has a flexural strength of 10.1 ± 1.2 MPa and compressive strength of 61.3 ± 1.0 MPa [41]. These values of strength are similar or slightly lower than the values obtained for the SPC with a percentage of between 20 and 30% of PG or between 10 and 20% of IM at 1 day old.

The strength obtained tends to increase as the sulfur/waste ratio increases up to between 0.95 and 1.05 (**Table 6**). A thin layer of sulfur-coated PG particles is a good binder for aggregates increasing the mechanical strength. However, a decrease in the compressive strength is observed with a larger proportions of sulfur. This is due to the fact that this level of sulfur increases the thickness of the sulfur layer around the particles and leads to brittleness.

Table 7 shows the water activity coefficient (WAC) results recorded for SPC-21PG-30 and SPC-21IM-20. Water absorption by capillarity was studied gravimetrically in prismatic concrete specimens (50 × 29 × 9 mm) in water at room temperature. The water level was sufficient to wet only the lower surface of the test specimens. After 28 days, the WAC coefficient values of the samples are very low and certainly less than that of the SPC. However, all WAC values for SPC-PG and SPC-IM samples are smaller than those for concrete made from Portland cement as reported by several authors [42, 43]. WAC coefficients of 5.0 and 6.2 kg/m^2 after 28 days of immersion in water are measured for an ordinary Portland cement and for a concrete made of Brazilian Portland cement (CPII E-02) (slag-modified Portland cement), respectively. The use of a sulfur/modified sulfur mix reduces the WAC coefficient producing a very impermeable concrete.

Mixture	Water absorption (g cm^{-2})			
	3 hours	72 hours	7 days	28 days
SPC 21	0.07	0.20	0.30	0.90
SPC-21PG-30	0.10	0.16	0.20	1.20
SPC-21IM-20	0.01	0.04	0.05	0.06

Table 7. Water absorption by capillarity as a function of time for cement with and without mud.

	SPC-17PG-10	SPC-19PG-20	SPC-21PG-30	SPC17-IM-10	SPC21-IM-20	SPC21-IM-30
%Waste	10	20	30	10	20	30
^{210}Pb	70 ± 5.0	143 ± 9.0	219 ± 13.0	28 ± 4	40 ± 4	76 ± 7
^{238}U(^{234}Th)	21 ± 2.0	12 ± 2.0	38 ± 3.0	25 ± 4	61 ± 5	84 ± 7
^{232}Th(^{212}Pb)	9.4 ± 0.7	8.6 ± 0.6	8.1 ± 0.5	54 ± 3	115 ± 7	182 ± 11
^{226}Ra	63 ± 4.0	115 ± 7.0	179 ± 11.0	51 ± 3	123 ± 7	194 ± 11
^{228}Th	8.7 ± 0.7	<6	6.8 ± 0.7	50 ± 2	100 ± 6	168 ± 11
^{228}Ra(^{228}Ac)	8.6 ± 0.8	8.8 ± 0.8	6.9 ± 0.7	212 ± 13	426 ± 26	674 ± 39
^{40}K	528 ± 32.0	394 ± 24.0	347 ± 21	512 ± 31	493 ± 32	413 ± 24
INDEX "I"	0.43	0.56	0.75	1.40	2.70	4.20
Ra(eq) (Bq/kg)	117	253	158	394	770	1189

The radium equivalent activity and the activity concentration index "I" are also given.

Table 8. Natural radionuclide concentration (Bq/kg) in SPC samples.

Taking into account that the wastes come from a NORM industry, it is necessary to carry out a radioactivity characterization of the SPCs obtained (**Table 8**). The radionuclides with the highest activity concentration in the PG-SPCs are ^{226}Ra (and its daughters of short half life) and ^{40}K. As expected, the activity concentration of ^{226}Ra shows a linear dependence (y = 5.49x + 8.95;

$R^2 = 0.9988$) on the percentage (x) of PG added. However, ^{40}K concentration decreases linearly ($y = -5.82x + 529$; $R^2 = 0.9041$). This is due to the fact that the potassium content in PG is practically negligible (<18 Bq/kg for ^{40}K, or <0.06% in natural potassium).

In SPC-IM samples, the radionuclide with the highest activity concentration is ^{228}Ra. The activity concentration of ^{228}Ra also shows a linear dependence ($y = 18.7x + 59.6$; $R^2 = 0.9976$) on the percentage (x) of IM added to the SPC-IM samples. A similar behavior was showed for ^{210}Pb, ^{234Th}, and ^{232}Th. In relation to the ^{40}K, the activity concentration decreases when we increase the IM content in the sample.

The activity concentration index (I) in the SPC-PG samples is below the EU reference values for bulk building materials (**Table 8**). The Ra(eq) is also below the threshold of 370 Bq/kg considered in USA and some other countries. However, in the SPC-IM samples, the index I is higher than the EU reference values for materials used in bulk amounts. Nevertheless, these could have other civil construction applications, for example, in marine platforms or bridges. The values of Ra(eq) are also higher than the reference level of 370 Bq/kg.

Due to the high radionuclide content to assess the risk of the use is important study the environmental mobility of these. In relation to the toxicity characteristic leaching procedure leaching test (TCLP, USEPA) [44], it can be confirmed that none of the radionuclides ^{234}Th, ^{226}Ra, ^{228}Ra ^{40}K, and ^{210}Pb were detected in the leachates as their concentrations were below 1 Bq/L (leaching coefficients LC < 0.1%).

Sample	pH	Ac (wt%)	^{238}U(Bq/L)	^{210}Pb(Bq/L)	^{210}Pb Lc (%)	^{238}U Lc (%)
SPC-21	2	1.63	0.078 ± 0.003	<0.01	<0.01	0.27
	4	1.01	0.143 ± 0.012	0.039 ± 0.019	0.19	0.48
	6	1.25	0.078 ± 0.005	0.017 ± 0.011	0.09	0.26
	8	0.95	0.0106 ± 0.0003	<0.01	<0.01	0.03
	10	0.75	<0.01	<0.01	<0.01	<0.01
SPC-21PG-30	2	1.50	1.09 ± 0.05	0.97 ± 0.07	1.17	0.18
	4	1.89	0.73 ± 0.05	0.15 ± 0.03	0.78	0.03
	6	1.99	1.14 ± 0.04	0.067 ± 0.017	1.22	0.01
	8	1.46	0.09 ± 0.03	<0.01	0.09	<0.01
	10	0.87	0.05 ± 0.01	<0.01	0.05	<0.01
SPC-21IM-20	2	0.37	0.463 ± 0.025	0.871 ± 0.020	0.86	0.26
	4	0.36	0.363 ± 0.078	0.891 ± 0.027	0.88	0.30
	6	0.34	0.340 ± 0.020	0.862 ± 0.020	0.84	0.20
	8	0.38	0.111 ± 0.008	0.906 ± 0.023	0.90	0.18
	10	0.46	0.009 ± 0.004	0.807 ± 0.026	0.77	0.05

Table 9. ^{238}U and ^{210}Po concentration and leaching coefficients with respect to pH.

In acidic media, pH 2 and 4, the concentration of ^{238}U in SPC-21 was between 0.078 and 0.143 Bq/L and for pH 8 0.011 Bq/L. These values are in agreement with those recorded for inland

water bodies which are between 0.005 and 0.5 Bq/L [45]. The SPC-21PG-30 leachate has a mean ^{238}U concentration of around 1 Bq/L in acidic media higher than SPC-21, falling to 0.07 Bq/L for basic media (close to the upper limits allowed for groundwater affected by mining residues [1 Bq/L]) [46]. This, plus the results outlined in **Table 9**, shows that the contamination of water by uranium isotopes by SPCs containing 30% waste would be negligible.

The activity concentration of ^{210}Po in SPC-21 is between 0.039 and 0.017 Bq/L for acid media, below the mean 0.01 Bq/L recorded for basic media. In acid media, the ^{210}Po concentration is higher in SPC–21PG–30 with values around the levels for inland water bodies but lower at pH of 8 and 10, that is, 0.01 Bq/L. The ^{238}U concentration is around twice that of ^{210}Po in the leaching so Po tends to be more strongly fixed to a particular material than U.

The activity concentration of ^{238}U in the acidic media in SPC-21IM-30 is around 0.38 Bq/L, this value falling between 0.1 and 0.009 Bq/L for pH = 8 and 10, respectively. The contamination of water by uranium isotopes due to the SPC-IM would be negligible. At all pH values, the ^{210}Po concentration is very similar, around 0.8 Bq/L, with the leaching coefficient for ^{210}Po being in general higher than for ^{238}U by a factor of 3. For pH = 10, it can be higher by a factor of 15.

This result confirms that phosphogypsum and ilmenite mud can be successfully immobilized and valorized in the manufacture of SPC construction materials, which also allow for the safe disposal of waste sulfur.

2.3. Use of ilmenite mud in commercial cement manufacturing

Industrial wastes are widely used within the cement industry [3, 4, 47–49] either as partial or total substitution of raw material in clinker production or as an addition to clinker for cement production. In addition, it is important to note that the use in cement production of waste generated in several industrial activities should be undertaken with caution. Certain requirements should be met: (a) the physical and chemical properties of the cement generated should be at least comparable to the physical and chemical properties of the "normal" cement; and (b) the "new" cement should not cause any additional environmental problems (in other words, one environmental problem should not be replaced by another).

It is known that the moderate addition (5% or less) of pure TiO_2 in the manufacture of Portland clinker increases the hydraulic activity (the capacity to react with water in order to gain high resistance) of the cement, acts as a mineralizer, reduces the porosity and increases the resistance [50, 51].

Taking into account that IM contains around 50% TiO_2, we decided to explore the effects of adding the ilmenite mud (IM) directly to cement. The mixtures were IMA (97.5% Cem–2.5% IM), IMB (95% Cem–5% IM) and IMC (90% Cem–10% IM). Below we analyze and evaluate the main mechanical, elastic and thermodynamic properties of these cements. Their properties were compared with those of ordinary Portland cement (OPC)—Type I (clinker 97% and natural gypsum 3%) with compressive strength around 52.5 N/mm^2).

The first step after the formation of the three dried mixtures of clinker with ilmenite mud was to determine the appropriate water/cement (W/C) relation in weight terms for obtaining a

"normal" consistency of the paste formed [52]. The results of experimentation show that the optimum water–cement ratio for each of the samples was 0.27 for OPC, IMA, and IMB and 0.28 for IMC. The setting times of the three cements formed using different proportions of IM as an additive were obtained with a Vicat apparatus [52]. The setting time for OPC is 139 min. This time is extended to 158, 171, and 203 min for IMA, IMB, and IMC, respectively. On the other hand, the final setting time was 224 for OPC, and 201, 206, and 263 min for IMA, IMB, and IMC. The setting times for the three IM cements are comparable to those obtained for commercial cement and fulfill the requirements of national legislation. Note that the initial and final setting times are prolonged by increasing the proportion of IM used.

Mechanical resistance tests (compressive and flexural strength) were carried out for the new cements with ilmenite mud added and for the commercial cement (CEM). The results obtained are given in **Table 10**

Sample	Flexural strength		Compressive strength	
	2 days	28 days	2 days	28 days
Commercial cement (CEM)	6.8 ± 0.3	10.1 ± 1.2	34.4 ± 0.4	61.3 ± 1.1
IMA (2.5% IM)	6.8 ± 0.1	9.8 ± 0.6	38.1 ± 1.4	55.3 ± 1.9
IMB (5% IM)	6.7 ± 0.3	9.0 ± 0.3	34.8 ± 0.9	55.9 ± 1.3
IMC (10% IM)	4.8 ± 0.3	8.0 ± 0.2	24.2 ± 0.8	45.5 ± 1.0

Table 10. Compressive and flexural strength values (MPa) of new cement produced by adding IM to OPC (ordinary Portland cement).

These results show that the addition of a little ilmenite mud to the cement (2.5 and 5%) improves the mechanical strength. In particular, after to 2 days, the bending strength of the new cement that had 2.5% IM added is not affected but its compressive strength increased by approximately a 10%. After 28 days, the values decrease slightly, but is still within acceptable limits (>52.5 MPa) [42].

In order to analyze the possible risks of short-time expansion, which can affect the ilmenite mud cements formed, the Le Chatelier test was performed [52]. The results obtained for the new cements show that the presence of IM in the cements formed does not cause any significant modification in their expansion behavior in comparison with the commercial cement. This applies irrespective of the proportion of IM used as an additive, within the range tested.

Calorimetric analyses of the three IM cements were performed (**Table 11**) in order to determine the total heat liberated in their hydration process. These values clearly indicate that the addition of IM diminishes the rate of heat release in the acceleration–deceleration peak, down to 12.4 kJ/kg h in the IMC sample. This is 28% lower than that obtained for the commercial cement. The addition of IM also retards the appearance of this peak until 7.23 h in the IMB sample or 8.61 h in the IMC sample. The total heat liberated in the cements formed with IM is slightly lower in all the samples, at around 245 kJ/kg.

Sample	Acceleration–deceleration peak			Total heat after 60 hours (kJ/kg)
	V(kJ/kg h)	t_V (h)	Q_V (kJ/kg)	
Cement	17.60	6.40	86.4	254.3
Clinker	12.50	7.20	71.4	258.3
IMA	16.06	6.85	73.0	240.0
IMB	14.90	7.23	71.8	247.0
IMC	12.40	8.61	67.5	245.0

Table 11. Calorimetric analysis of the new cement obtained with IM, clinker and cement taken as reference.

Finally, a thorough analysis from the environmental point of view was carried out. The external risk index (I) of samples was measured. For IMA, the value is 0.68, that is, below unity. Therefore, this sample can be used without restrictions. However, sample IMB presents an index slightly above unity at 1.09. IMC has a value close to 2 which is a problem from a radiological point of view. In relation with radium-equivalent Ra(eq), the values obtained for IMA, IMB, and IMC were 190, 308, and 544 Bq/kg, respectively, showing that IMA and IMB are below the 370 Bq/kg level, but IMC is above it.

To assess the risk of the use of IM to the environment, a TCLP leaching test was carried out [29]. The pollutant concentrations (metals and radionuclides) in the leaching solutions obtained from the mobility tests were measured. The leaching test shows that the concentrations of radioisotopes are of the same order of magnitude as typical ones in continental waters [53], and it can be ensured that their potential radiological impact is negligible.

	IMC	CEM	US EPA
Cr	70	89	5000
As	2.7	1.1	5000
Se	22	16	1000
Cd	<1	<1	1000
Ba	330	317	100,000
Pb	8.8	8.8	5000
Ti	2.7	<1	–
Fe	<1	<1	–

Limit values given in the US EPA standard.

Table 12. Leachability concentrations of metals (μg/L) obtained by TCLP test from the CEM and IMC samples.

The content of Cr(VI) in the samples must also be analyzed, because European directive [54] prohibits the marketing and use of cement which, when hydrated, contains more than 2 mg/kg of water-soluble hexavalent chromium, determined as percentage by mass of dry

cement. In **Table 12**, we can see that the concentration of Cr (total) in the IMC sample is 70 μg/L and taking into account that the dilution factor used was 20, the final concentration of Cr by mass of dry cement is 1.4 mg/kg, lower than 2 mg/kg. Also, values for other heavy metals are similar or even lesser than the cement taken as reference.

In order to gain a better understanding of how the ilmenite mud impurities are incorporated in the final cement product, a detailed study by SEM was made of the IMC cement sample. This sample is the one that contains the biggest proportion of ilmenite mud (10%). In **Figure 3**, one can see a main dark matrix (point 1) corresponding to the cement formed, with a composition typical of a calcium silicate hydrate (C–S–H) gel and a Ca–Si ratio = 2.2 ± 0.1. The bright particles embedded in the matrix are formed by iron plus titanium (point 2). This can be deduced from the corresponding X-ray spectra obtained by XRMA at these points, which are also shown in **Figure 3**.

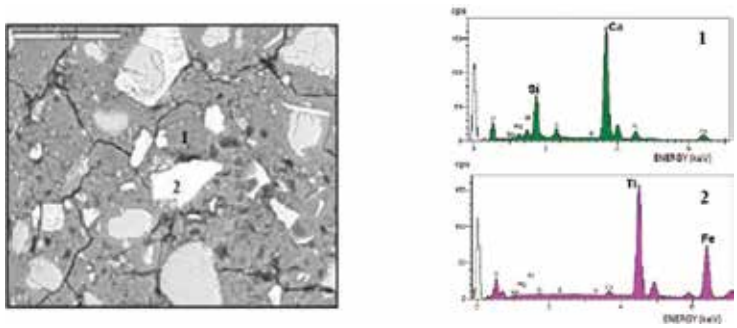

Figure 3. SEM image obtained in the BSE mode of the cement formed by mixing conventional clinker with 10% IM (IMC sample). The X-ray spectra corresponding to the numbered points in the image are also shown.

All the SEM-XRMA results indicate that the particles trapped in the matrix are high in iron and titanium. This is important because it reduces the potential leaching of the iron and titanium contaminants in the cement formed and, consequently, limits their potential harm to the environment.

2.4. Ilmenite mud as additive in the manufacture of ceramic

In line with the research to date, and noting the composition of the IM [24], another possible valorization method for IM is its use in the production of ceramic tiles. Three fundamental factors need to be borne in mind when considering the possibility of using ceramic tiles for the recycling of IM. Firstly, it is essential to study whether the presence of this residue modifies the mechanical properties of the tile compared with commercial tile. Secondly, these studies require prior physico-chemical analysis (elemental, mineralogical, and morphology) of the waste and the components used in the generation of tile tested. Finally, it is essential to check its environmental impact mainly associated to the potential problem of leaching of metals and radionuclides included in the tile matrix [55–58].

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Ilmenite mud exhibits a high content of iron oxide ($Fe_2O_3 \approx 10\%$). This is a similar value to that of red stoneware (RSM) which is made from natural clays with high iron oxide content ($Fe_2O_3 > 7\%$), (**Table 4**). For this reason, its valorization in ceramic tiles has been trialed [59]. Mixtures of a commercial red stoneware (RSM) with different concentrations of ilmenite mud (3, 5, 7, 10, 30, and 50%) were shaped by uniaxial pressing (Nannetti S hydraulic press) at 40 MPa in a steel die to produce tiles measuring 50 × 50 × 5 mm, which were fired in an electric furnace at 1150°C following a fast-firing process for 8 minutes.

The sintering behavior of the ceramic was evaluated on the basis of water absorption, apparent porosity, and bulk density. The water absorption E (wt%), was measured according to EN ISO 10545-3 [60] for ten representative specimens. The apparent porosity and the bulk density were measured according to ASTMC373-88 [61]. Also, the linear shrinkage, LS (%), and the bending strength, BS (MPa), were measured according to EN 843-1 [62].

The results shown in **Table 13** indicate that the linear shrinkage increases with the concentration of ilmenite mud showing values up to 7%. Lower values are advantageous because they reduce cracking and volume changes during firing. Also, apparent porosity generally increases with the concentration of ilmenite mud. This physical property is very important, and it is directly related with the water absorption and open porosity [63]. Moreover, the water absorption decreases with the addition of 3 and 5% of sludge (2.82 and 2.48%, respectively). In accordance with the European Standard EN 14411 [64], tiles are categorized according to the water absorption coefficient (E). So tiles with low water absorption belong to group I, which in turn, is divided in two sub-groups (a) $E \leq 0.5\%$ (called BIa group) and (b) $0.5\% < E \leq 3\%$ (called BIb group). Tiles with $3\% < E \leq 6\%$ and $6\% < E \leq 10\%$ belong to the BIIa and BIIb groups, respectively. This fact is important because of low values of both E and P provides a high resistance to freeze-thaw cycles, requiring less drying time.

RSM/IM (%)	Linear shrinkage LS (%)	Apparent porosity P (%)	Water absorption E (wt%)	Bulk density B ($g \cdot cm^{-3}$)	Bending strength BS (MPa)
100/0	3.8 ± 0.1	12.5 ± 0.7	5.30 ± 0.06	2.36 ± 0.02	35.1 ± 0.9
97/3	5.6 ± 0.1	6.71 ± 0.3	2.82 ± 0.05	2.38 ± 0.04	41.1 ± 0.8
95/5	6.0 ± 0.1	5.98 ± 0.2	2.48 ± 0.03	2.42 ± 0.05	40.2 ± 0.8
93/7	6.0 ± 0.1	10.0 ± 0.5	4.18 ± 0.04	2.40 ± 0.03	37.5 ± 0.7
90/10	6.4 ± 0.2	12.0 ± 0.7	4.72 ± 0.06	2.55 ± 0.03	36.5 ± 0.7
70/30	6.3 ± 0.1	19.8 ± 0.8	9.02 ± 0.10	2.22 ± 0.02	33.2 ± 0.8
50/50	6.6 ± 0.1	20.9 ± 0.9	9.18 ± 0.12	2.27 ± 0.05	30.8 ± 0.9

Table 13. Linear shrinkage and technological properties of fired tiles (results show the average values of 10 measurements).

The bulk density increases with the concentration of sludge up to 10% due to the high density of sludge (3.7 g cm^{-3}). The bending strength increases with the addition of IM up to 10% due

to the beneficial effect of ilmenite on sintering during firing as denoted by the decrease in porosity (**Table 13**).

The 97/3 and 95/5 samples show the highest values of bending strength, that is, 41 and 40 MPa, respectively. These are above 30 MPa, the minimum value required for group BIb [64]. On the other hand, values corresponding to 100/0, 93/7, and 90/10 samples are greater than for the BIIa group (22 MPa). Finally, in the 70/30 and 50/50 samples, bending strength decreases due to the increase of interconnecting open pores.

In order to analyze how the ilmenite mud is incorporated to the final product, a detailed study by FESEM was made. The images (**Figure 4**) show good sintering and a homogeneous microstructure free of internal defects such as cracks.

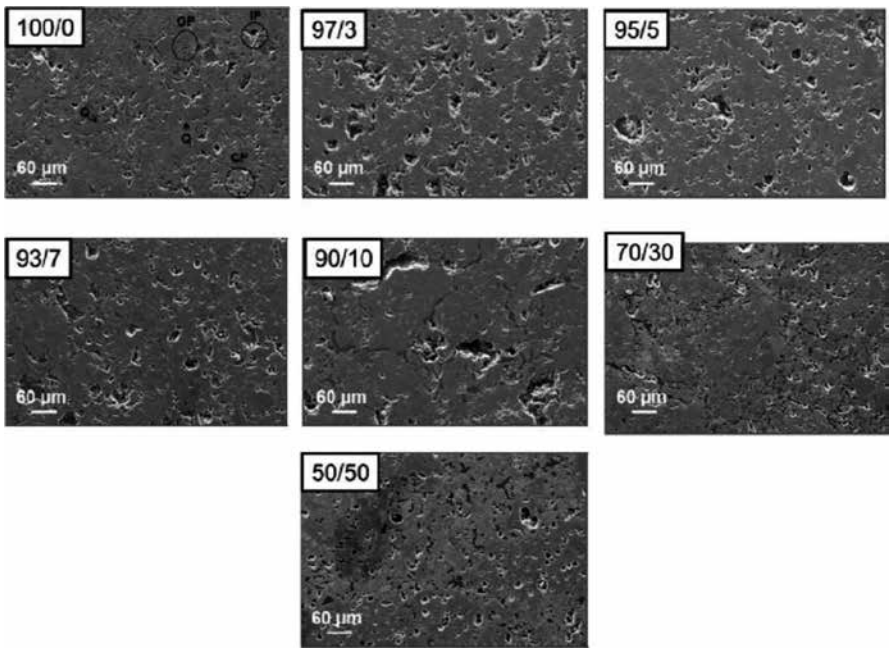

Figure 4. Secondary electron images (low magnification) on polished surfaces of fired tiles. Q: quartz; OP: open porosity; CP: closed porosity; and IP: interparticle porosity.

It is important to note the existence of both open (<5 μm) and closed (<10 μm) pores in the samples. Once the allotropic transformation is carried out at 573°C, quartz particles produce shrinkage giving rise to cracks when the piece cools [65]. The addition of the moderate percentages of ilmenite mud has a benefit, decreasing the open porosity as we can see in 97/3 and 95/5 samples.

Recall that the original mud had a total radionuclide concentration of 2000–3000 Bq/kg, with the highest activity concentrations for ^{226}Ra and ^{228}Ra being around 500 and 2000 Bq/kg, respectively, (**Table 5**). In the tiles, the activity concentration of these radionuclides increased with increasing ilmenite mud content, as did those of ^{228}Ra, ^{228}Th, and ^{238}U. The activity

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concentration of ^{40}K was constant in all samples. The index (I) for ceramic samples is lower than six for all of the analyzed materials. This means that ilmenite mud suitable material for use in the ceramic industry in comparison with other additives [66].

A TCLP leaching test was also applied to evaluate the potential environmental impacts generated by hazardous metals and radionuclides contained in tiles. The results indicate that increasing the proportion of ilmenite mud in mixture decreases the leached metals. Obviously, the firing process makes metal less leachable. These values are lower than the limits imposed by the US EPA and will not have a significant impact when this material is released into the environment. The only exception is ^{210}Po (**Table 14**).

	^{238}U	TF	^{234}U	TF	^{232}Th	TF	^{230}Th	TF	^{210}Po	TF
RSM	29 ± 3	1.5	36 ± 4	1.8	50 ± 17	1.7	129 ± 28	7.6	7.3 ± 1.9	0.4
97/3	10.1 ± 2.1	0.6	10.1 ± 2.1	0.5	18 ± 6	0.6	54 ± 10	2.5	12 ± 2	0.7
95/5	4.8 ± 1.4	0.2	6.3 ± 1.7	0.3	33 ± 11	1.0	75 ± 17	3.0	9.6 ± 1.9	0.6
93/7	6.3 ± 1.4	0.3	5.1 ± 1.2	0.3	10.5 ± 3.6	0.4	24 ± 5	1.3	10.8 ± 2.9	0.3
90/10	6.4 ± 1.4	0.4	6.4 ± 1.4	0.4	5.1 ± 1.6	0.2	8.0 ± 2.0	0.4	5.5 ± 0.8	0.3
IM	2.1 ± 0.7	0.1	5.3 ± 1.2	0.3	37 ± 9	1.0	99 ± 18	4.3	9.7 ± 2.8	0.1

Transfer factor (%) in TCLP.

Table 14. Average concentration (mBq/L) of each sample analyzed by alpha spectrometry.

The motilities of U- and Th-isotopes are of the same order of magnitude for the various samples studied, that is, 10–100 mBq/L, apart from Po which is one order of magnitude lower (<10 mBq/L). This fact is explained by the tendency of Po to bind onto the particulate material [15].

Therefore, that the impact of the use of ilmenite mud as an additive in ceramics is negligible from the environmental point of view.

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Chapter 3

The Management of Hazardous Waste in Developing Countries

Daniel Mmereki, Adrew Baldwin, Liu Hong and Baizhan Li

Additional information is available at the end of the chapter

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Abstract

This book chapter discusses the management of hazardous waste in developing countries, with particular emphasis on industrial hazardous waste, medical waste, and household hazardous waste. It seeks to identify the current situation and also aims to provide a review of the existing strategies that are particularly related to hazardous waste management. In developing countries, hazardous waste management systems lack a systematic approach to administer waste management programmes; inability to effectively collect and manage wastes as well as to reduce the negative impacts of those activities. The current regulatory frameworks and regulations do not adequately address hazardous waste treatment and final disposal. There are inadequacies in the implementation of regulations associated with hazardous waste management due to fragmented responsibilities among government departments and local authorities. The chapter provides practical best processes for the management of hazardous waste aimed at improving the current situation.

Keywords: hazardous waste, developing countries, recovery, recycling, disposal

1. Introduction

Wastes are classified as being hazardous when they display one or more radioactive or hazardous properties, including explosive, oxidizing, flammable, irritant, harmful, toxic, carcinogenic, as well as harmful effects on the environment and human health [1]. The relevant property or properties are determined by property testing or where applicable, concentration based criteria.

Hazardous wastes are materials which are discarded after use from e-products, vehicles, clinical and medical products, fuel products (e.g. oil), gas exploration and extraction. Scientific research indicates that these include materials such as industrial solvents, waste oils, industrial sludges and chemical wastes. Households, small businesses, farms, and the healthcare and construction sectors also generate quantities of hazardous waste including batteries, electrical equipment, healthcare risk waste, solvent based paint and varnish waste, sheep dip, and fluorescent lamps [2]. Hazardous waste not only poses risks to the surrounding air, water, and soil, but also do harm to the ecological environment and human health through diversified channels [3]. Developed countries (such as United States and some European Union members states) are the main producers of hazardous waste in the world [4]. The management of hazardous wastes is of great importance due to environmental health, social, and economic impacts. During the past two decades the world experienced a dramatic increase in the amount of hazardous waste generated [5]. In developing countries, the management of hazardous is exacerbated by lack of comprehensive legislation, unauthorized scrap yards dealing with e-waste, and end of life vehicles. Poor conduct and inappropriate disposal methods exercised during the handling and disposal of hazardous wastes are increasing significant health hazards and environmental pollution due to the harmful nature of the waste [6].

Past research studies [5, 6] have established that the key driver to hazardous waste management is the involvement of all the stakeholders including waste generators, regulators, decision makers, waste processors, and informal and formal sectors. These stakeholders have a crucial role in improving the system by ensuring the development and delivery of an effective and efficient hazardous waste management program. The aim of this book chapter is to describe the management of hazardous waste in developing countries, in terms of treatment, prevention of hazardous waste, and the best practices of hazardous waste management.

2. Definition and sources of hazardous waste

In recent decades, researchers have defined and classified hazardous waste as waste with inherent chemical and physical characteristics, such as toxicity, ignitability, corrosivity, carcinogenicity, or other properties [6]. Nevertheless, in developing countries there is insufficient definition and classification, which leads to difficulties in identifying the needs of treatment and disposal of hazardous waste. For instance, in China, hazardous waste has been classified into three types, household hazardous waste, industrial hazardous waste, and medical waste [7]. It has been established that in many developing countries, only industrial hazardous and medical waste are disposed separately [8]. Other hazardous waste type, such as household hazardous waste (HHW) are somewhat neglected. Thus, in the developing countries, there is limited classification carried out in terms of the composition, type, and purpose.

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3. The current situation

In developing countries, the quantities of hazardous wastes has not been documented because these waste streams are incorrectly managed, thereby posing greater environmental impacts than reported. Based on the 2013 environmental statistics annual report released by the Ministry of Environmental Protection of China, it has been observed that the Chinese industries produced 31.57 million tons of hazardous wastes belonging to 49 types in 2013. Furthermore, 53.9% of these hazardous wastes is recycled and reused, while 22.2 % is treated and disposed, and 25.7% is stored [11]. Therefore, determining the accurate estimates of hazardous is not an easy task [6]. As a result, this waste stream may end up mixed with general domestic or commercial, or disposed of in an uncontrolled manner through burning, burying, or discharged to sewer, water, or ground surface. Currently, in developing countries there are limited options for generators of hazardous waste to manage it appropriately [8]. In some cases, there are no commercial hazardous waste collection services, retail take back systems and periodic drop-off services provided by the municipalities. In terms of responsibilities, developing countries have not designed and implemented producer responsibility for hazardous waste materials including human and farm animal medicines, waste oil, oil filters, paint and paint containers, pesticides and herbicides (household), ink and ink containers from publishing organizations [9]. In particular, there are no national take back schemes for unused or expired human medicines and e-products considering the widespread consumption of medicines and e-products. In addition, no pilot schemes have been carried out focusing on collection of hazardous waste such as plant protection product containers, unused or out-of-date animal health medicines and pesticides, waste oils, oil, filters, empty cartridges, aerosols, e-waste, paints, batteries, and other hazardous waste [10].

4. Legislation framework

The Basel Convention on the Control of Transboundary Movements of Hazardous Wastes and their Disposal is the most important regulatory framework for the management of hazardous waste. However, other developing countries such as China has adopted several regulations and standards based on the international safety standards in combination with the Chinese situation. These are developed in order for waste producers to:

- Minimize waste in fuel production and fuel cycles, materials classification and purification.
- Guarantee a high volume reduction [7].

Based on the previous scientific research, it has been established that in developing countries, the Basel Convention is not adequately implemented [8]. Consequently, hazardous waste from developed is still received by the developing countries. As noted by some scholars, some of the problems related to legislation in developing countries include: unambiguousness in waste classification due to the subsuming unspecific nature of the waste codes, and the liberty given to waste generators to choose their own names for the wastes they generate; potential over-

lapping of source-based and constituent-based waste codes [12]. Furthermore, there are no identification standards of ignitable and reactive characteristics for hazardous wastes [10]. Therefore, the present methods and standards continue to hamper and impede the development of sustainable management systems in the developing countries.

5. Treatment and disposal of hazardous waste

The treatment of hazardous waste should take place under regulated and controlled conditions [13]. Hazardous waste management includes the possession, transportation, handling, storage, and ultimate disposal of waste. However, in developing countries the treatment of hazardous waste takes place in unregulated or uncontrolled conditions, and in some cases hazardous waste are exported to developing countries by the developed countries [14]. It has been established that for most parts, hazardous wastes are treated in unlicensed facilities using conventional methods such as landfilling. The remainder of the waste stream is treated at authorized facilities with low technologies and low environmental standards [15]. These include incineration plants, landfills and oil recovery. An important fraction of hazardous wastes is still mixed with non-hazardous wastes, being mainly landfilled and producing serious environmental impacts regarding heavy metals and persistent organic pollutants content in landfill leachate, thus creating a great health risk to municipal workers, the public, and the environment. The main problems affecting the management of hazardous waste in developing countries include the following:

- Lack of necessary rules, plans, regulations, and instructions on different aspect of collections and disposal of waste.
- Lack of policy directions or incentives for existing local authority or private sector landfill operators.
- Inadequate Institutional capacities.
- Lack of hazardous waste prevention activities.
- Shipment of hazardous to developing countries from developed countries.
- Mixing of hazardous waste with domestic waste or commercial waste.
- Lack of collection facilities for hazardous waste.
- Failure to quantify the hazardous waste generated in reliable records.
- Existing environmental permits mostly fail to contribute to Best Available Technology (BAT).
- Not all regional environmental authorities register the generated waste types in the permit using waste classification codes.
- Information about waste production rates corresponding to the recorded waste generation is usually missing. The developing countries waste information system is not detailed

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enough to gather information about concentrations of hazardous substances, only total mass.

- Lack of enforcement of and compliance with the existing regulations.
- Lack of priority sectors for prevention of hazardous waste.
- Lack of conformance with the best international practices on hazardous waste management.
- Inadequate infrastructure and self-sufficiency in hazardous waste management.
- Absence of dedicated waste managers and committees, as well as plans responsible for monitoring hazardous waste practices.
- Absence of dedicated national facilities.
- Lack of guidance, awareness, capacity building training on the management of sectoral hazardous waste management.
- Absence of specific policies dedicated to the management of hazardous household waste.

This suggests that integrated strategies for hazardous wastes recovery are needed in the developing country industry that may reduce the disposal rate of these wastes in communal landfills and impulse resource recovery and recycling of valuable materials of these wastes. Therefore, it is necessary to develop high technologies; promoting recovery and recycling centers and hazardous waste management strategies, which are environmentally, socially, economically, and technically feasible.

Doing so recognizes the proximity of best international practices to manage hazardous waste: maximizing the reuse, recycling, and recovery of potential materials, precious metals and where practical through the provision of a range of local treatment options; ensuring the availability of recovery and disposal outlets and stimulating green economy opportunities within nations.

Currently, many developing countries have no dedicated hazardous landfill disposal facilities. A few countries such as South Africa have hazardous landfill disposal facilities [16]. However, in many developing countries there are considerable challenges to develop hazardous waste landfills, including social acceptance, regulatory, technically skilled manpower, financial resources, limited technology or provision of such infrastructure. It is demonstrated that while landfill is the least favoured option on the waste hierarchy, it is recognized that for some non-recoverable or non-combustible hazardous wastes it will need to be considered [15].

Satisfactory infrastructure and monitoring of movement of hazardous waste are non-existent [17]. Indiscriminate disposal of solid waste materials such as e-waste, used oil, poses major environmental problems including the soil contamination and threat to animals. Hazardous wastes such as wastewater from healthcare facilities are often discharged into storm channels, which are not periodically cleaned. Monitoring of the health and environmental risks associated with these practices is not done due to the lack of technically skilled manpower, health and safety personnel.

Also, scientific, engineering, and organizational challenges, which need to be taken care, are indicated in **Table 1**. What is so worrying here is that the recycling base in the developing countries is very weak.

Engineering challenges	Scientific challenges	Organizational challenges
• Scientific collection, transportation, segregation, and disposal of hazardous waste • Practical techno-economical solutions ◦ Recycling ◦ Reuse ◦ Recovery	• Value addition of recyclables for reuse • Disposal of process waste and residues • Eco-friendly recovery solution	• Organization and structuring hazardous waste management system • Training and awareness

Table 1. Scientific, engineering and organizational challenges in developing countries.

5.1. Industrial hazardous waste

Industrial hazardous waste is defined as waste generated from industrial sectors and pose immediate danger to the environment and the public [17]. Industrial hazardous wastes are characterized in terms of toxicity (acute, chronic, and extrinsic), inflammability, reactivity, and corrosiveness. The main important sources of industrial hazardous wastes are mining, chemical, mechanical, pulp and paper industries, cement production facilities, wood remanufacturing facilities, etc. Important industrial hazardous wastes include used oil and oil contaminated materials, spent solvent [14]. Industries in developing countries such as Lebanon are estimated to generate 3000 to 15,000 tons/year of hazardous wastes due to deficient physical infrastructure and absence of environmental management plans, which will inevitably amplify environment impacts associated with industrial activities [18]. Nevertheless, there is limited data on the quantities of industrial hazardous waste, where is going, where it is generated and disposed of. However, due to the accelerated development of economies, mass manufacturing and processing industries and less strict standards on environmental quality assessment, the quantities of industrial hazardous waste (IHW) will continuously increase. Meanwhile, in developing countries it has been confirmed that an important fraction of hazardous waste is still mixed with non-hazardous wastes, mainly through landfilling, and producing serious environmental impacts regarding heavy metals and persistent organic pollutants content in landfill leachate [16]. Scientific research has observed that wastes are not separated in origin into hazardous and non-hazardous materials. Furthermore, non-hazardous are also not separated into recyclables, non-recyclables, and domiciliary. In fact, scientific research has found that developing countries lack regulations that specifically deal with industrial hazardous wastes. In addition, in the developing countries there are no hazardous waste management

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plans and authorized facilities to manage, treat, and eliminate hazardous wastes. The separation of all the waste streams for possible waste reuse is not implemented in the developing countries. This suggest that there is an urgent need to establish policies geared towards stimulating industries, commerce and societies to manage hazardous waste in a sustainable manner; encouraging the shift from traditional waste management practices to "cradle-to-cradle", and a reduction of the problem of management of hazardous wastes.

5.2. Medical waste

Medical waste is waste generated from health-related facilities such as hospitals, clinics, health centre, research institute, etc. [19, 20]. It contains toxic chemicals, heavy metals, and may contain substances that are genotoxic or radioactive, pathological waste, sharps (e.g. needles, syringes, scalpels, knives, broken glass, etc.), infectious waste, bulk human blood and blood products. Presently, in developing countries, there is no statistical data on medical waste. However, in most cases, the estimated quantities of medical waste are extrapolated using bed occupancy and number of beds in health institutions [16]. For instance, in Bangladesh, Dhaka City, it is reported that 37±5 tonnes of medical waste is generated from hospitals, clinics, and other healthcare facilities [21]. However, scientific studies have shown that secure disposal of medical waste remains quite lower in several developing countries [22]. Meanwhile, significant fraction of medical waste is disposed as municipal solid waste (MSW) or discharged without monitoring and control; incineration is the only predominant formal way to treat medical waste. In addition, in developing countries, majority of the incineration facilities are not fully maintained and operational; use primitive technologies and simple equipment, which may consequently cause pollution [23].

5.3. Household hazardous waste

In the home, it has been pointed out that there are several jobs which generate hazardous waste because of the product used may contain hazardous substances. It has been confirmed that such products include paints, cleaners, stains and varnishes, car batteries, motor oil, and pesticides [13, 24]. As a result, the used leftover contents from such consumer products are known as household hazardous waste. Other HHW include e-waste. It is estimated that developing countries will discard 400-700 million obsolete computers by 2030. Most of the waste is sent to Africa or Asia under false pretences as donations from developed countries, especially Europe and the United States. Currently, in developing countries, it has been observed that a few national and local regulations have been developed and/or drafted for HHW management [25]. Due to the lax enforcement and monitoring, valuable hazardous wastes such as lead batteries, waste mineral oils, photographic chemical wastes, waste mercury lamps, and certain electronic waste containing heavy metals and printed circuit boards (PCBs) are improperly disposed of, thereby causing significant negative impacts on public health and the environment. Furthermore, in developing countries some HHW is almost managed as municipal solid waste [26]. The informal recyclers are the dominant sector recovering and recycling valuables. **Table 2** presents hazardous waste and country practice in developing countries. Most importantly, in developing countries, safe disposal methods of

hazardous wastes including incineration, landfilling, and special treatment (e.g. microwave, sterilization, etc.) are not well-founded. Thus, this inevitably accelerates pollution of HHW.

Waste	Practice						
	Equipment availability	**Legal framework**	**Public awareness**	**Technology**	**Inventory**	**Separation of hazardous waste**	**Incentives or penalties**
HHW	–	Nascent or non-existent	Lack of capacity and awareness	Low technology incineration	Lack of information on HHW	Unfriendly environmental collection, treatment and disposal system	Low incentives or penalties
Medical waste	–	No specific policy	Lack of capacity and awareness	Low technology incineration	Lack of information on medical waste	Inadequate collection, treatment and disposal system	Low incentives or penalties
Industrial hazardous waste	–	No specific policy	Lack of capacity and awareness	Low technology incineration	Lack of information on industrial waste	Comingled with MSW	Low incentives or penalties

Table 2. Hazardous wastes and country practice.

6. Prevention of hazardous waste

Scientific research studies have proven that in waste management, prevention is at the top of the hierarchy and represents the preferred policy approach to materials management and an alternative to reduce the wastage of materials or resources. As a result, it is substantiated that prevention of waste is preferable to its generation and to the monetary and environmental costs incurred as a result of its generation. It is proven that radioactive waste will continue to be a priority in order to achieve greater resource efficiency [27]. For this reason, an integrated approach should be adopted to design programs, and agencies to lead and coordinate a wide range of prevention initiatives to reduce the potential environmental and public health impacts [16]. These should include development of programs focusing on a number of strategic objectives in order to achieve the goal of prevention which will include the prevention of radioactive and hazardous wastes. This suggests that to reduce the public health and environmental impacts of radioactive and hazardous wastes, a range of regulations and innovative processes must be developed to control the content of these potentially hazardous and harmful substances.

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7. Innovative processes used for treatment of hazardous wastes in developing countries

The negative environmental and public health impacts caused by hazardous wastes as well as the use of complex equipment, infrastructure, sophisticated controls, and dangerous processes have encouraged developing countries to develop innovative processes to treat these wastes. These innovative processes play a critical role in the final treatment of hazardous wastes, including the protection of the surrounding soil where these wastes are disposed of, and such technologies are cost-effective, easy to operate in the face of limited infrastructure, technical knowledge and expertise, and suitable for developing countries. One of these innovative processes is phytoremediation. Phytoremediation involves the planting of trees to prevent and repair environment from hazardous wastes, including restoration of degraded soil, and conservation. It is used in China, India, Pakistan, etc. However, scientific research has found that phytoremediation has limitation of long lasting and of low efficiency [28, 29]. Therefore, there is an urgent need for long lasting and high efficiency processes for the treatment of hazardous wastes.

8. Best practices of hazardous waste management in the world

It is important to note that policy makers, regulators, product producers, generators, and holders of hazardous waste need to play a critical role in ensuring that such materials are prevented, minimized, collected, and treated properly in accordance with the waste hierarchy or "cradle-to-cradle" [12, 29]. The best practices in managing radioactive and hazardous waste include the following:

- Adopting alternative strategies (e.g., physico-chemical treatment).
- Treatment with or without heat recovery. For instance, in Chile, used oil and spend solvents are used as raw materials for alternative fuels production [16].
- Making new regulations where necessary and appropriate.
- Ensuring that other government departments and public bodies fulfill their roles and responsibilities identified in hazardous waste management.
- Facilitating a two-way communication with sectoral and stakeholder interests.
- Municipalities fulfill their important role in providing small-scale collection services and generally raising awareness in hazardous waste management.
- Prevention: engaging with priority sectors on hazardous waste prevention waste activities through cleaner technologies and better compliance with regulation.
- Devising future new policies on the management of hazardous waste by also taking into consideration the generation rates of these types of wastes and potential savings from recovery of these wastes [30].

9. Conclusion

This book chapter discusses hazardous waste management in developing countries, with emphasis on industrial hazardous waste, medical waste, and household hazardous waste. It identifies the current situation on hazardous waste management. In developing countries there is lack of information on the quantities of hazardous waste generated, lack of capacity and awareness; low incentives or penalties; lack of clear roles and responsibilities for stakeholders; limited infrastructure; and inadequate institutional framework. Other challenges include: lack of technically skilled manpower, financial resources, know-how, testing facilities, and equipment; and the absence of an integrated framework regarding the monitoring and management of hazardous wastes. Also, inadequate collection, treatment and disposal system; and the lackadaisical response by the government makes it difficult for the local authorities to identify targets to be achieved either annually or strategically on a long term basis for solid waste management. For the successful implementation of best practices of hazardous waste management, there is an urgent need to consider the experiences of developed countries and this should be combined with the socio-economic context of the developing countries. Waste minimization through source reduction, reuse, and recycling has to be effectively implemented to decrease the amount of hazardous waste generated and disposed of. For this achievement, there is the need for a drastic reform of the current regulations in the developing countries.

10. Recommendations for developing countries towards hazardous waste management

The environmentally sound management of hazardous wastes is becoming a major concern in developing countries due to the diversity of the waste stream and toxic material within it, as well as the negative environmental and public health impacts caused. Hence, several practical recommendations are suggested which include the following:

- Creation of public awareness of the potential of recycling hazardous wastes.
- Source reduction.
- Capacity building and human resources development for hazardous wastes recycling.
- Monitoring and evaluation of hazardous wastes management systems as well a reporting programmes.
- Development of appropriate infrastructure, technical knowledge, and expertise.
- Strengthening and reforming existing regulatory frameworks.
- Development of decision support for identifying appropriate technologies for treatment of hazardous wastes.
- Provision of funding for identification of emerging best and state-of-the art technologies.
- Development of regional hazardous waste management system.

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Conflict of interests

The authors declare that there is no conflict of interest regarding the publication of this book chapter.

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Chapter 4

Prediction of Uranium Transport in an Aquifer at a Proposed Uranium *In Situ* Recovery Site: Geochemical Modeling as a Decision-Making Tool

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Additional information is available at the end of the chapter

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Abstract

Roll fronts are some of the most important uranium deposits and are quite common in the United States. Generally, a roll front has an oxidized zone and a reduced zone, the latter being the zone of high mineralization and a target for *in situ* recovery (ISR) mining. The challenge remains the gathering of information to enable making informed decisions regarding post-mining groundwater quality. In this study, potential uncertainties in uranium sorption on iron oxyhydroxides or hydrous ferric oxides (HFO) following mining were assessed, as these oxidized zones create a greater risk for future uranium transport than fully reduced zones. Using two different geochemical databases, uncertainties in predicting uranium sorption on HFO based on a post-recovery restoration scenario were studied. The scenario was assessed using one-dimensional PHREEQC geochemical modeling simulations with respect to: uranium, oxygen, carbon dioxide, and iron hydroxide concentrations. The results of the simulations showed that uranium concentrations in solution are likely to be controlled by the amount of HFO available for sorption and the concentration of uranium-carbonate complexes formed in the solution. The presence of calcium, through the dissolution of calcite, was found to reduce the adsorption of uranium onto HFO as the resulting uranium-calcium-carbonate complexes are quite soluble. Overall, the simulations provide a procedure for predicting down-gradient uranium concentrations based on ultimate restoration levels at uranium ISR sites. This is important for risk assessment, regulatory enforcement, and decision making.

Keywords: hydrous ferric oxides, *in situ* recovery, reactive transport modeling, uranium, PHREEQC

1. Introduction

Uranium is a toxic element due to its radioactivity and chemical toxicity. In the environment, it exists as the soluble uranyl ion, UO_2^{2+} (U(VI)) and the reduced form U(IV). The oxidized form is capable of forming complexes with ligands such as chlorides, fluorides, phosphates, nitrates, sulfates, selenides, tellurides, carbonates, and organic moieties, and these influence its speciation and transport [1–4]. The toxicity and bioavailability of uranium is dependent on its speciation upon uptake as well as changes in speciation during transport in the human body [5]. Ingestion of uranium in drinking water has been shown to negatively affect the kidneys, causing their inflammation, a condition called nephritis [5, 6]. It can also affect the bone marrow by replacing calcium, thereby weakening the bones and causing osteoporosis [4].

In the United States, roll fronts are important deposits of uranium. These deposits develop as groundwater containing uranium species migrates through porous and permeable sandstone or conglomerate aquifers (**Figure 1**). Groundwater transports the leached uranium from the source rock, for example, a volcanic ash fall deposit and re-deposits it upon migrating into a reducing environment within the aquifer [7] (**Figure 1**). *In situ* leach mining reverses that process using a leach solution of oxygen and carbon dioxide, rendering uranium soluble (**Figure 2**). The pregnant solution from the extraction wells is pumped to the treatment plant where uranium is recovered in a resin ion exchange or liquid ion-exchange (solvent extraction) system. The uranium is then stripped from the ion-exchange resin, and precipitated chemically, usually with hydrogen peroxide [8]. Geochemical changes that may or may not occur outside of the recovery zone are important for local groundwater users, regulatory agencies, and other stakeholders to understand when evaluating the potential effects on surrounding groundwater quality [7].

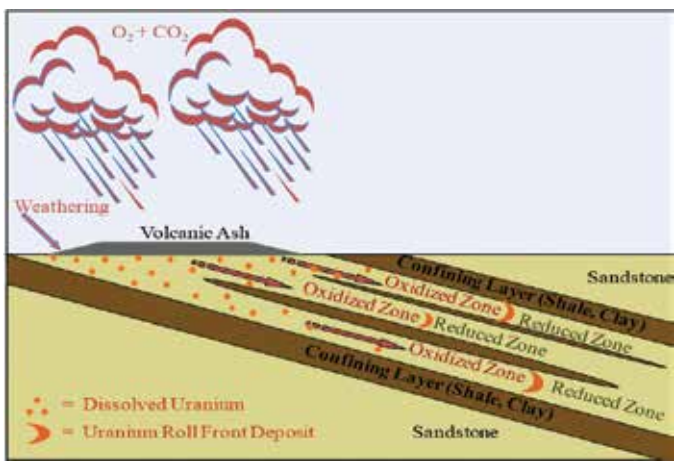

Figure 1. Formation of uranium roll front deposits involving weathering of uranium from a source, transport of soluble uranium within an oxidized zone and precipitation of uranium as it contacts a reducing zone.

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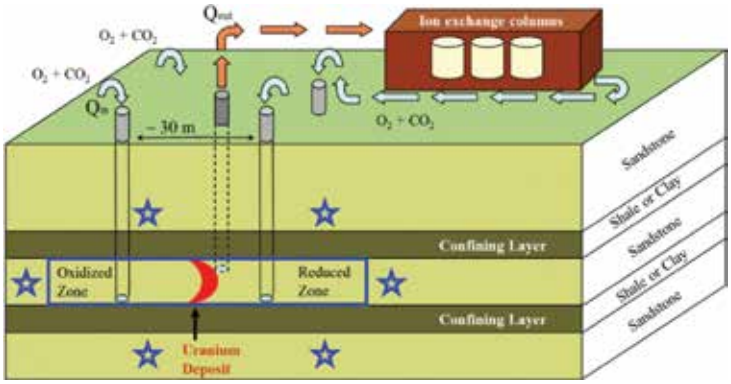

Figure 2. Generic uranium *in situ* recovery facility (not to scale). The box indicates the main recovery zone, while the stars indicate groundwater monitoring wells.

Figure 2 illustrates a generic uranium ISR facility where the stars represent monitoring wells that are continually tested for any changes in groundwater quality. In areas with reducing conditions down-gradient (containing pyrite and/or organic carbon), uranium is precipitated and/or adsorbed onto the organic carbon, as this is how the uranium ore was originally emplaced. However, if there is sandstone with HFO coatings (without organic carbon or pyrite) in the down-gradient solid-phase materials, adsorption of uranium onto HFO will most likely be the factor controlling future uranium concentrations [7].

In **Figure 2**, the groundwater flow during the formation of the uranium ore would have been from left to right (i.e., oxidized to reduced). Reversal of the current groundwater flow pattern would lead to post-recovery groundwater contacting the oxidized solid phase. Such changes in groundwater flow patterns through geologic time scales are well known at uranium ISR sites [9].

Domestic and agricultural groundwater users proximal to uranium ISR sites are concerned about potential influences of uranium ISR on local groundwater quality. Due to the reactions at a uranium deposit, the local groundwater can be high in uranium, radium, and radon concentrations. However, the surrounding groundwater outside of the local ore body is generally much lower in radionuclide concentrations and can meet drinking (30 μg L^{-1} according to the United States Environmental Protection Agency, US EPA) and/or agricultural water quality standards.

In this study, different parameters were used to model the uncertainties in uranium sorption on HFO, as these oxidized zones could create a greater potential for future uranium transport than fully reduced zones.

2. Materials and methods

The study site is the proposed Dewey-Burdock uranium ISR site near Edgemont, South Dakota, USA [10]. However, the procedures can be generically applied to any uranium ISR site. During

the preliminary drilling work, some drill cores of the aquifer rock were collected and the content of HFO and calcite in them determined according to the method used by Breit and Goldhaber [11]. Local groundwater was sampled and analyzed according to accepted methods [12, 13].

Geochemical modeling has been used since the 1960s to study hydrochemistry and has become an increasingly popular tool in the study of water-rock interactions [14, 15]. Geochemical modeling has a wide range of applications, including use in the research of fundamental solution processes on small laboratory scales and extending to large-scale aquifer geochemistry modeling for use in regulatory practices. Applications of geochemical modeling include the following [14–16]:

- Determination of speciation and complexation of inorganic species within solution
- Calculation of saturation indices and the subsequent dissolution and precipitation of minerals
- Cation exchange and adsorption/desorption of species on surfaces
- Reactions involving gases, liquids (mixing), and solid phases, including organic material
- Change in solution chemistry during redox or temperature variation

Geochemical modeling uses predominantly thermodynamic equations due to a lack of data of environmental kinetic reactions. A common approach used by computer codes uses numerical methods to solve the nonlinear set of equations that are comprised from mass action (equilibrium constants) and mass balance equations [15]. This method assumes that there is a local chemical equilibrium established in the system. There are several computer codes available for geochemical modeling, including PHREEQC, WATEQ4F, MINTEQA2, and the Geochemist's Workbench.

In this study, the PHREEQC geochemical modeling code was used for simulating the reactions occurring in the aquifer. PHREEQC stands for PH REdox Equilibrium (in C language) and is widely used for simulating a variety of reactions and processes in natural waters or laboratory experiments. PHREEQC requires an input file in which the problem is specified via KEYWORDS and associated data blocks. Some of the keywords include SOLUTION_SPREAD, EQUILIBRIUM_PHASES, SURFACE, TRANSPORT, and END and are defined as follows. A full description of many alternatives for input and the mathematical backgrounds can be found in the manual of the program by Parkhurst and Appelo [17].

SOLUTION_SPREAD defines one or more aqueous solution compositions (it is also an alternative input for the keyword SOLUTION).

EQUILIBRIUM_PHASES defines assemblage of minerals and gases to react with an aqueous solution.

SURFACE defines the composition of an assemblage of surfaces.

TRANSPORT specifies parameters for advective-dispersive-reactive transport, optionally with porosity.

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END demarcates end of a simulation.

Forward geochemical models are models in which the final composition of a solution after a chemical reaction is calculated through the solving of mass balance and mass action equations using numerical methods [18]. Geochemical models can be combined with a groundwater flow model to produce a reactive transport model. The flow path distance is divided into a series of discrete cells, a process called discretization. In each cell, a reaction or equilibration occurs and the composition of the solution is calculated using the geochemical model. The solution is then transported using the flow model to an adjacent cell, and the composition following further reactions or equilibrations in each cell are calculated again using the geochemical model [18]. The process is repeated for a defined number of steps, usually referred to as"shifts".

The simulations, dimensions, and all the groundwater and solid-phase geochemistry were based on the proposed site.

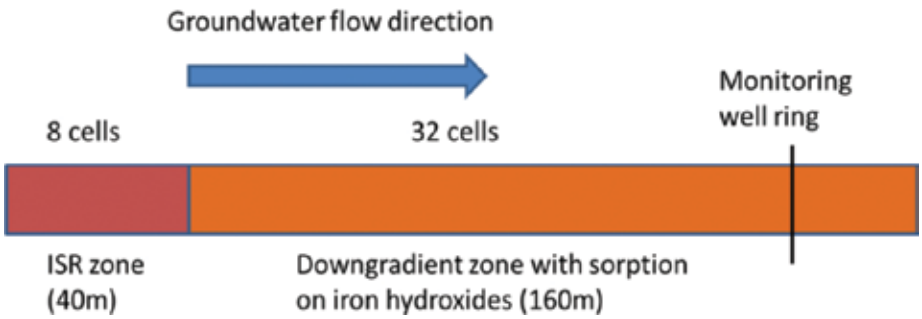

Figure 3. Schematic of one-dimensional model domain. ISR is *in situ* recovery.

One-dimensional reactive transport simulations were created with PHREEQC [17] to represent long-term groundwater flow away from a restored uranium ISR site (**Figures 3** and **4**). Each cell represents a length of 5 m with 8 cells for the ISR zone (40 m) and 32 cells for the down-gradient transport zone (160 m) (**Figure 3**). The groundwater flow rate is 5 m per year (which is represented by 1 cell), resulting in 40 years required for the water to traverse 200 m (i.e., 5 m is equivalent to 1 cell which in turn is equivalent to 1 year). Groundwater monitoring wells are often installed at a certain distance away from the ISR zone, and in this site, they are planned to be placed at 125 m, meaning it will take the water 25 years to get to them. No dispersion component was included in the models. Geochemical reactions in the ISR zone were not considered while down-gradient reactions include calcite ($CaCO_3$) equilibrium and sorption of uranium on HFO. Initial conditions assumed local background groundwater quality for the down-gradient zone, and groundwater quality in the ISR zone was the same as for background groundwater except for higher concentrations of oxygen, uranium, and carbon dioxide (**Table 1**; **Figure 4**). These are based on the likely conditions that could follow site restoration after mining. Restoration would usually involve cleanup of excess contaminants (after mining operations have ceased) using background groundwater and is a regulatory requirement by the US EPA that should be conducted before monitoring ceases.

```
TITLE Script for one dimensional reactive transport simulations

# Local background solution defined as solution 0 (the solution coming into cell 1 during the simulation.
SOLUTION_SPREAD
    -units    mg/l
 Description      Number    pH     pe     Temp    O(0)       Alkalinity          ... (solution input defined in Table 1)
                                                  mg/l    mg/l as CaCO3          ...
         684         0          6.78      1.2     12.8     1.2                   ...

# ISR Zone solutions defined for cells 1-8. Solutions input as shown in Table 1.
SOLUTION_SPREAD
    -units    mg/l
 Description      Number    pH     pe     Temp    O(0)       Alkalinity          ... (solution input defined in Table 1)
                                                  mg/l    mg/l as CaCO3          ...
     NoRemed         1-8  6.78    1.2     12.8       8              269          ...

# Local background solution defined for cells 9-40, equilibrated with calcite and HFO surface. Solutions input as shown in Table 1
SOLUTION_SPREAD
    -units    mg/l
 Description      Number    pH     pe     Temp    O(0)       Alkalinity          ... (solution input defined in Table 1)
                                                  mg/l    mg/l as CaCO3          ...
  Background        9-40  6.78    1.2     12.8     1.2              269          ...

EQUILIBRIUM_PHASES 9-40 Oxidized zone
    Calcite   0 0.0


SURFACE 9-40 Add HFO sorption in oxidized zone
    -equilibrate with solution 0
    Hfo_sOH    0.009      600       25.6
    Hfo_wOH    0.036

# Defining transport parameters. Groundwater flow is 5 m per year
TRANSPORT
    -cells                40
    -shifts               100
    -time_step            31536000 # seconds
    -diffusion_coefficient 0
    -thermal_diffusion    2   0
    -print_cells          1-40
    -punch_cells          1-40
    -multi_d              false
END
```

Figure 4. Example of PHREEQC script used for one-dimensional reactive transport simulations.

All incoming groundwater (left side in **Figure 3**) during a simulation was of background groundwater quality. Geochemical model testing included variations in: (i) the geochemical database, (ii) post-recovery ISR zone groundwater quality, (iii) amount of iron in the down-gradient solid phase, (iv) down-gradient calcium concentrations, and (v) post-recovery ISR zone carbon dioxide concentrations. Two different geochemical databases were used with the PHREEQC geochemical modeling program: (i) the Wateq4f database [19] that is available upon downloading the PHREEQC program, and (ii) a modified PHREEQC database (hereinafter called the "updated database") with more recent thermodynamic data on uranium carbonate complexes from Guillaumont et al. [20] and calcium-uranium-carbonate/magnesium-uranium-carbonate complexes from Dong and Brooks [21]. The same thermodynamic data for uranium sorption onto HFO were used for both databases and were based on the study by Dzombak and Morel [22]. Differences in predicting uranium sorption on HFO were simulated using post-recovery restored groundwater (uranium = 200 μg L^{-1}, oxygen = 8 mg L^{-1}, all other constituents were the same as those for incoming groundwater in **Table 1**). These post-recovery groundwater constituents are approximate values for simulation purposes only and were not measured values since the Dewey-Burdock site is only proposed at this point. Iron concentrations of 500 and 2500 mg kg^{-1} were evaluated based on preliminary iron extraction results from the site. For the simulations, these Fe amounts were converted to an equivalent HFO or hydrous ferric oxide (HFO, FeOOH) as used by PHREEQC according to the method in Appelo and Postma [23]. Because uranium concentrations were quite sensitive to the presence of calcium for the updated database, a simulation using lower calcium concentrations

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in the down-gradient groundwater was added. Initial simulations used a calcite saturation index of 0.0 and simulations with lower calcium concentrations used a calcite saturation index (SI) of −0.5. The background calcium concentration was 365 mg L^{-1}. The resulting calcium concentration in the down-gradient zone with a saturation index of 0.0 (fully saturated) was 387 mg L^{-1} and a saturation index of −0.5 (slightly undersaturated) produces a calcium concentration of 316 mg L^{-1}. A high carbon dioxide concentration with a log pCO_2 of 0.5 was used compared to the natural groundwater conditions of approximately −1.5. The excess CO_2 was assumed to be the left over amount from the lixiviant following the ISR process. The simulations were conducted at 25 years post-restoration, the time taken for the groundwater to flow from the mining zone to the monitoring well ring.

Parameter	Local background	ISR zone
pH	6.78	6.78
Redox potential (pe)	1.2	1.2
Temperature	12.8	12.8
O_2 (aq) (mg L^{-1})	1.2	8
CO_2 (as $logP_{CO2}$)	−1.5	0.5
Alkalinity (mg L^{-1} as $CaCO_3$)	269	269
As (μg L^{-1})	4.72	4.72
B (mg L^{-1})	0.143	0.143
Ba (mg L^{-1})	0.012	0.012
Ca (mg L^{-1})	365	Equilibrated with calcite
Cl (mg L^{-1})	9.67	9.67
F (mg L^{-1})	1.83	1.83
Fe (mg L^{-1})	18.5	18.5
K (mg L^{-1})	117	117
Mg (mg L^{-1})	0.552	0.552
Na (mg L^{-1})	118	118
Se (μg L^{-1})	0.883	0.883
Si (mg L^{-1})	4.9	4.9
SO_4^{2-}(mg L^{-1})	1460	1460
Sr (mg L^{-1})	7.37	7.37
V (μg L^{-1})	0.873	0.873
U (μg $^{-1}$)	0	200
Zn (mg L^{-1})	0.067	0.067

Table 1. Initial water quality conditions used for simulations.

3. Results and discussion

The key parameter changes were evaluated, and the resulting simulations at 25 years post-restoration are presented in **Figures 5–12**. **Figures 5** and **6** show the influence of iron concentrations on the adsorption of uranium. Without any sorption, concentrations of up to 200 mg L^{-1} that are in the original background groundwater are observed. For the Wateq4f and updated databases, the predictions show a slight decrease in uranium concentration in water when iron concentration is reduced from 2500 to 500 mg kg^{-1}. It should be noted here that the apparent dispersion in uranium concentrations (**Figure 5**) is not dispersion included in the simulations, but is rather created by the adsorption/desorption of uranium to HFO through time.

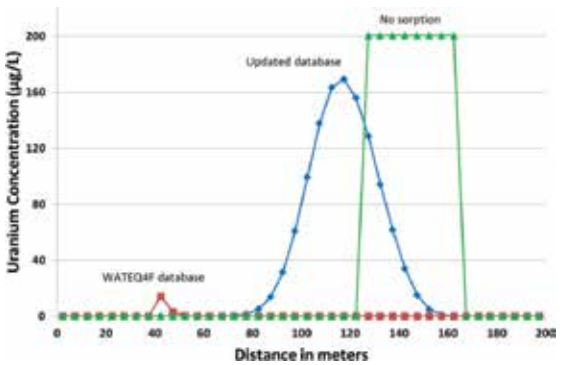

Figure 5. Uranium concentrations in groundwater at 25 years based on **2500 ppm Fe**. The triangles show no sorption, diamonds show the updated database and squares show the Wateq4f database.

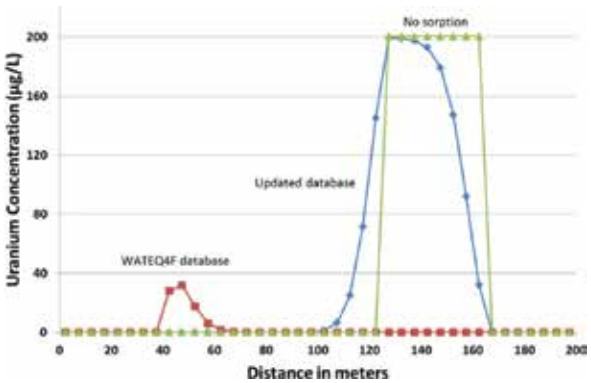

Figure 6. Uranium concentrations in groundwater at 25 years with **500 ppm Fe**. The triangles show no sorption, diamonds show the updated database, and squares show the Wateq4f database.

Differences in the databases are apparent with the updated database showing far less adsorption of uranium (**Figures 7** and **8**). This is attributed to the inclusion of the calcium-uranium-

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carbonate complexes, $Ca_2UO_2(CO_3)_3^0$ and $CaUO_2(CO_3)_3^{2-}$, based on Dong and Brooks [21]. These complexes render uranium much more soluble, decreasing the sorption potential. A $MgUO_2(CO_3)^{2-}$ complex from Dong and Brooks [21] was also added, but the influence of this complex in separate simulations (not presented here), given the Mg concentration that were evaluated, created a minimal influence on uranium concentrations remaining in solution. This may not be the case at sites with higher Mg concentrations and would have to be considered carefully when studying those sites. Simulations (not presented here) that used only the updated uranium carbonate complexes based on Guillaumont et al. [20] compared to the Wateq4f database, without the added calcium-uranium-carbonate complexes, showed no difference in uranium concentrations.

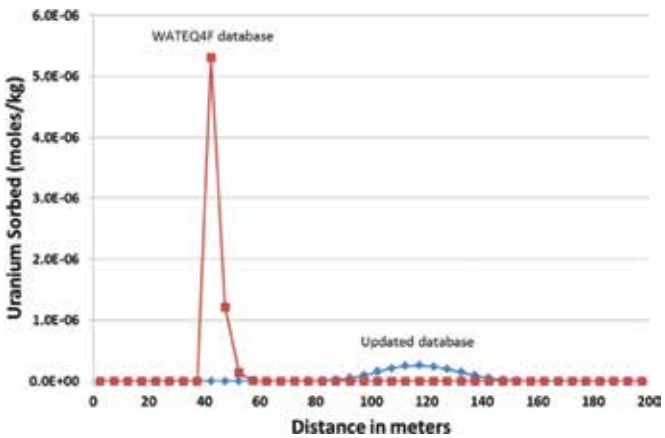

Figure 7. Sorbed uranium concentrations at 25 years with **2500 ppm Fe**. The diamonds show the updated database, and the squares show the Wateq4f database.

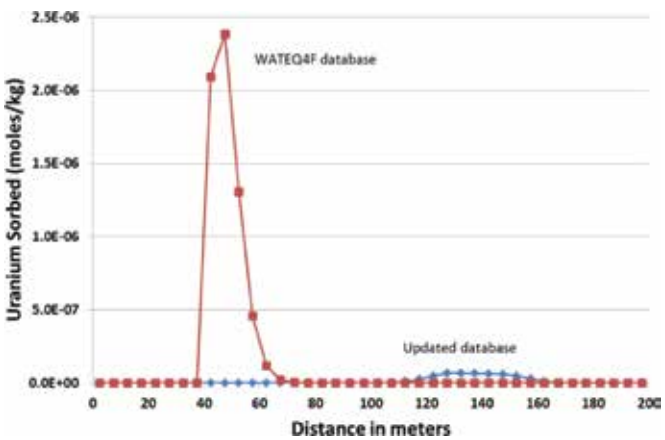

Figure 8. Sorbed uranium concentrations at 25 years with **500 ppm Fe**. The diamonds show the updated database, and the squares show the Wateq4f database.

Because the updated database relies heavily on calcium-uranium-carbonate complexes, a lower calcium concentration was tested as pointed out earlier. The results (**Figures 9** and **10**) show how sensitive the simulations are to slight changes in calcium concentrations. The lower calcium concentrations result in more sorption of uranium and thus slower movement and lower concentrations of uranium in down-gradient groundwater.

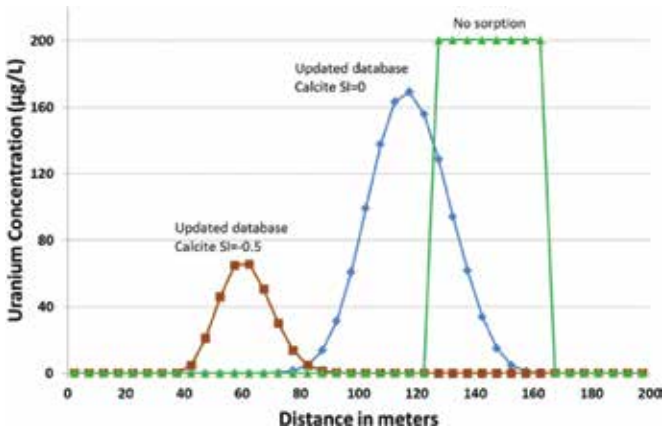

Figure 9. Uranium concentrations in groundwater at 25 years with **2500 ppm Fe**. The triangles show no sorption, the diamonds show the updated database, and calcite saturation index set to 0.0. The squares show the updated database, and calcite saturation index set to –0.5.

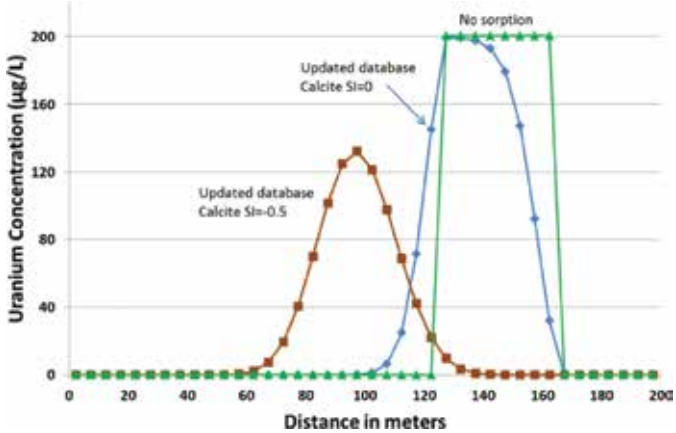

Figure 10. Uranium concentrations in groundwater at 25 years with **500 ppm Fe**. The triangles show no sorption, the diamonds show the updated database, and calcite saturation index set to 0.0. The squares show the updated database, and calcite saturation index set to –0.5.

Higher carbon dioxide concentrations in the recovery zone are quite likely as CO_2 is often used in the uranium ISR process as pointed out earlier. For Fe = 500 mg kg^{-1} in the updated database, the increased CO_2 (log pCO_2 = 0.5) dissolves down-gradient calcite (SI = 0), keeping Ca and

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alkalinity in solution, and making uranium mobile in the groundwater (**Figure 11**). The large decrease in uranium concentrations at approximately 120–140 m was quite unexpected (**Figure 11**). Because the calcite concentration in the aquifer rock was quite low (0.15 wt%), the lowered pH created by the higher CO_2 concentration consumed all of the calcite in the first down-gradient cell (cell 9). This is described chemically as follows:

$$CO_{2(aq)} + H_2O_{(l)} \rightarrow HCO_3^-{}_{(aq)} + H^+{}_{(aq)} \quad (1)$$

$$2H^+{}_{(aq)} + CaCO_{3(s)} \rightarrow Ca^{2+}{}_{(aq)} + CO_{2(g)} + H_2O_{(l)} \quad (2)$$

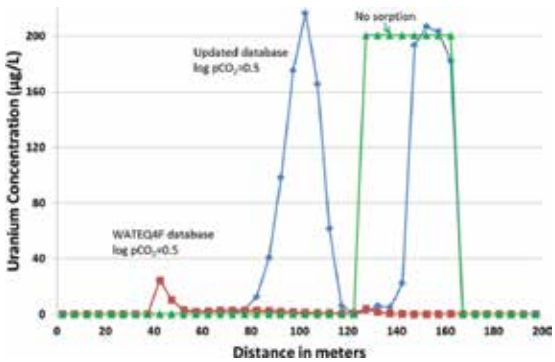

Figure 11. Uranium concentrations in groundwater at 25 years with **500 ppm Fe and CO_2 in recovery zone of log pCO_2 = 0.5 and down-gradient calcite = 0.15 wt%.** The triangles show no sorption, the diamonds show the updated database, and the squares show the Wateq4f database.

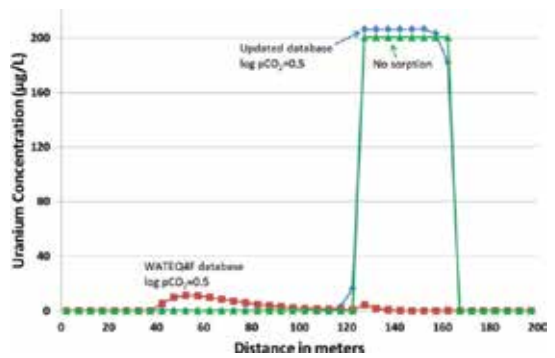

Figure 12. Uranium concentrations in groundwater at 25 years with **500 ppm Fe and CO_2in recovery zone of log pCO_2 = 0.5 and infinite calcite down-gradient**. The triangles show no sorption, the diamonds show the updated database, and the squares show the Wateq4f database.

Adding an infinite amount of calcite created conservative transport conditions for uranium for the higher CO_2 scenario (**Figure 12**). The output from the higher CO_2 and lower calcite scenario was further examined by evaluating the uranium in solution and the sorbed uranium in cell 9 through time (**Figures 13** and **14**). The abrupt decrease of uranium concentrations in cell 9 at 5 years corresponds to the time when all the calcite in that cell has been dissolved, due to the acidity produced by the additional CO_2 [in Eq. (2) above]. The resulting drop in pH increases the sorption of uranium to the HFO (**Figure 14**). Through time, as the background groundwater begins to enter cell 9 and the ISR zone groundwater moves down-gradient, the subsequent increase in pH decreases the uranium sorption and releases uranium back into the groundwater (**Figures 13** and **14**). It is noteworthy that uranium can reach concentrations in groundwater that are actually higher than the original post-restoration uranium concentration of 200 μg L^{-1} (**Figure 13**) as desorption occurs. The unexpected "split plume" shown in blue in **Figure 11** is a strong case, where the amount of solid-phase calcite along with the updated database created unusual results. However, these results can be explained based upon further evaluation of the geochemical processes (**Figures 13** and **14**).

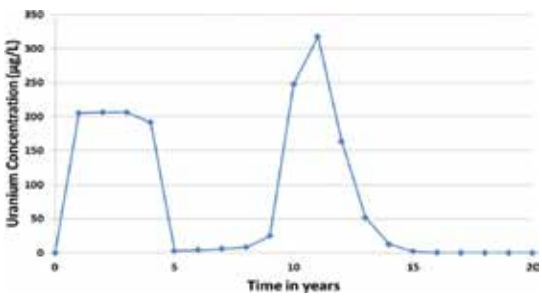

Figure 13. Uranium concentrations in groundwater at cell 9, the first cell down-gradient from the uranium recovery zone. The simulation used the updated database and the following parameters: **500 ppm Fe, a log pCO_2 in the recovery zone of 0.5, and down-gradient calcite = 0.15 wt%**.

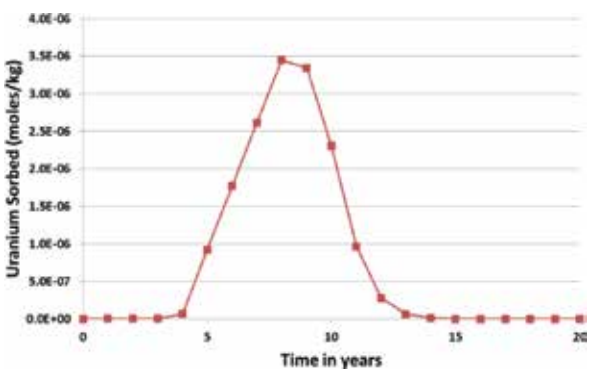

Figure 14. Sorbed uranium concentrations at cell 9, the first cell down-gradient from the uranium recovery zone. The same simulation database and parameters as in fig13 were used.

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4. Conclusions

The study provides a procedure for predicting down-gradient uranium concentrations based on ultimate restoration goals at uranium ISR sites. However, this tool relies on assumed amounts of HFO (based on preliminary site data) and their assumed sorption strengths (based on literature values and new information on calcium-uranium-carbonate complexes). Notwithstanding, the results provide a powerful tool for determining important controlling parameters that could assist scientists in conceptualizing and evaluating site conditions. At any uranium ISR site, more accurate site predictions could be made using (i) actual groundwater quality from the post-restoration ISR zone, (ii) actual down-gradient mineralogy (i.e., amount of Fe and calcite), and (iii) batch or column studies of true sorption potential in the down-gradient zone.

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Chapter 5

Overview of Hazardous Waste Management Status in Malaysia

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Additional information is available at the end of the chapter

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Abstract

This chapter reviews the status of hazardous waste management in Malaysia. It highlights the sources of the hazardous waste, government policies on waste generation and management, the involvement of the stakeholders, and the various management procedures adopted in Malaysia. Currently, the manufacturing sector is the major contributor in hazardous waste generated in Malaysia. Other sectors that contribute include household, agriculture, medical, and other industrial sectors. Malaysian government's resolve on human health protection and safeguarding the environment prompted various acts, regulations, and orders such as the popular Environmental Quality Act (EQA) 1974. The regulations made pursuant to the Environmental Quality Act have continuously improved to address the issues on the definition and classifications of hazardous waste and the management process in Malaysia. The management of hazardous waste in Malaysia is effectively growing as a result of continuous review of the regulations and enforcement of the acts. The stakeholders in the industries have also been active in keeping to the EQA regulations to keep the environment safe as much as possible.

Keywords: hazardous waste generation, health effect of hazardous waste, hazardous waste management, Malaysia Government policies, stakeholders involvement

1. Introduction

Malaysia is one of the fast growing nations in the global economy. Malaysian economy ranked 24th in the global competitive index of 2013 [1] and has grown to occupy the 18th position in the 2015 global competitive index ranking [2]. Economic growth comes with some burden on

the environment which includes waste generation, greenhouse gas emission from energy systems, deforestation, etc. The continued increase in waste generation in Malaysia has been associated with the growing population and the growing economy. These factors create high demand for goods and services by the growing classes of people with an aim to meeting their varying lifestyles, while the environment bears the consequence of the increasing waste generation [3]. The quantity of municipal solid waste generated in Malaysia was analyzed in 2010 by Agamuthu [4] with a projection of 30,000 tons/day of waste generation by 2020; in a review by Aja and Al-Kayiem [5], it was found that in 2013, the waste generation in Malaysia was 33,000 tons/day which exceeded the projection cited earlier. In a recent review by Fazeli et al. [6] on waste to energy, it was noted that the growing economy of Malaysia contributes to the environment burden levied by high energy consumption and high volume waste generation. Wastes generated in Malaysia are categorized based on level of potential hazard. According to the Department of Environment, waste is defined as "any substance prescribed to be scheduled waste or any matter whether in a solid, semi-solid, or liquid form, or in the form of a gas or vapor, which is emitted, discharged, or deposited in the environment in such volume, composition, or manner as to cause pollution" [7]. Scheduled wastes are the categories of waste listed in the First Schedule of the Environmental Quality (Scheduled Wastes) Regulations 2005 [8, 9]. Some categories of the scheduled waste are classified as environmental hazardous waste due to the toxic and hazardous nature of such wastes.

Environmentally hazardous substance (EHS), under the Malaysian Environmental Quality Act (EQA) 1974, is defined as "any natural or artificial substances including any raw material, whether in a solid, semi-solid, or liquid form, or in the form of gas or vapor, or in a mixture of at least two of these substances, or any living organism intended for any environmental protection, conservation, and control activity, which can cause pollution" [10–12]. There are currently 3839 items in the EHS reference list [13] and in a situation where a potentially hazardous material is not on the list, such substances are classified using the globally harmonized system (GHS) classification scheme and assigned a hazard category as implemented by the Department of Occupational Safety and Health, Malaysia [10].

There are currently 77 categories defined in the First Scheduled Waste of the Environmental Quality in Malaysia as EHS, which are classified into five groups as detailed in **Table 1**. The hazardous wastes in the five groups are from different sources such as industrial sector, agricultural sector, health sector, and households. Industrial waste poses potential serious hazard to the environment as most industrial processes employ chemical or chemically produced materials. In agriculture, hazardous wastes are generated through the use of pesticides [14], herbicides and even the use of inorganic fertilizer which has fluoride as by-product of phosphate fertilizer production [15, 16]. The use of organic manure also constitutes a hazard in agriculture by the dissolution of manure nitrate into ground water. This causes health hazards in most developing countries where there is no access to treated water and ground water is used as alternate source [17, 18]. Medical wastes include hospital disposables contaminated with blood and tissues, used pharmaceutical products, expired and used drugs, chemical wastes, radioactive isotopes used for diagnosis and treatment, etc. which require careful disposal [19–21]. In homes, several hazardous wastes are generated in meeting the

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desired lifestyle of the people. Such wastes include caustic cleaner, toxic paints, flammable solvents, pesticides, expired/unused drugs, mercury, etc. [22, 23].

SW1:	**Metal and metal-bearing wastes**
SW 101	Waste containing arsenic or its compound
SW 102	Waste of lead acid batteries in whole or crushed form
SW 103	Waste of batteries containing cadmium and nickel or mercury or lithium
SW 104	Dust, slag, dross, or ash containing arsenic, mercury, lead, cadmium, chromium, nickel, copper, vanadium, beryllium, antimony, tellurium, thallium or selenium excluding slag from iron and steel factory
SW 105	Galvanic sludge
SW 106	Residues from recovery of acid pickling liquor
SW 107	Slags from copper processing for further processing or refining containing arsenic, lead, or cadmium
SW 108	Leaching residues from zinc processing in dust and sludge form
SW 109	Waste containing mercury or its compound
SW 110	Waste from electrical and electronic assemblies containing components such as accumulators, mercury-switches, glass from cathode-ray tubes and other activated glass or polychlorinated biphenyl-capacitors, or contaminated with cadmium, mercury, lead, nickel, chromium, copper, lithium, silver, manganese, or polychlorinated biphenyl
SW 2:	**Wastes containing principally inorganic constituents which may contain metals and organic materials**
SW 201	Asbestos wastes in sludge dust or fiber forms
SW 202	Waste catalysts
SW 203	Immobilized scheduled wastes including chemically fixed, encapsulated, solidified, or stabilized sludge
SW 204	Sludge containing one or several metals including chromium, copper, nickel, zinc, lead, cadmium, aluminum, tin, vanadium, and beryllium
SW 205	Waste gypsum arising from chemical industry or power plant
SW 206	Spent inorganic acids
SW 207	Sludge containing fluoride
SW3:	**Wastes containing principally organic constituents which may contain metals and inorganic materials**
SW 301	Spent organic acids with pH less or equal to 2 which are corrosive or hazardous
SW 302	Flux waste containing mixture of organic acids, solvents, or compounds of ammonium chloride
SW 303	Adhesive or glue waste containing organic solvents excluding solid polymeric materials
SW 304	Press cake from pretreatment of glycerol soap lye
SW 305	Spent lubricating oil
SW 306	Spent hydraulic oil
SW 307	Spent mineral oil–water emulsion
SW 308	Oil tanker sludge

SW 309	Oil–water mixture such as ballast water
SW 310	Sludge from mineral oil storage tank
SW 311	Waste oil or oily sludge
SW 312	Oily residue from automotive workshop, service station, oil, or grease interceptor
SW 313	Oil contaminated earth from re-refining of used lubricating oil
SW 314	Oil or sludge from oil refinery plant maintenance operation
SW 315	Tar or tarry residues from oil refinery or petrochemical plant
SW 316	Acid sludge
SW 317	Spent organometallic compounds including tetraethyl lead, tetramethyl lead, and organotin compounds
SW 318	Waste, substances, and articles containing or contaminated with polychlorinated biphenyls (PCB) or polychlorinated triphenyls (PCT)
SW 319	Waste of phenols or phenol compounds including chlorophenol in the form of liquids or sludge
SW 320	Waste containing formaldehyde
SW 321	Rubber or latex wastes or sludge containing organic solvents or heavy metals
SW 322	Waste of non-halogenated organic solvents
SW 323	Waste of halogenated organic solvents
SW 324	Waste of halogenated or unhalogenated non-aqueous distillation residues arising from organic solvents recovery process
SW 325	Uncured resin waste containing organic solvents or heavy metals including epoxy resin and phenolic resin
SW 326	Waste of organic phosphorus compound
SW 327	Waste of thermal fluids (heat transfer) such as ethylene glycol
SW 4:	**Wastes which may contain either inorganic or organic constituents**
SW401	Spent alkalis containing heavy metals
SW402	Spent alkalis with pH more or equal to 11.5 which are corrosive or hazardous
SW403	Discarded drugs containing psychotropic substances or containing substances that are toxic, harmful, carcinogenic, mutagenic, or teratogenic
SW404	Pathogenic wastes, clinical wastes, or quarantined materials
SW405	Waste arising from the preparation and production of pharmaceutical product
SW406	Clinker, slag, and ashes from scheduled wastes incinerator
SW407	Waste containing dioxins or furans
SW408	Contaminated soil, debris, or matter resulting from cleaning-up of a spill of chemical, mineral oil, or scheduled wastes
SW409	Disposed containers, bags, or equipment contaminated with chemicals, pesticides, mineral oil, or scheduled wastes
SW410	Rags, plastics, papers, or filters contaminated with scheduled wastes

SW411	Spent activated carbon excluding carbon from the treatment of potable water and processes of the food industry and vitamin production
SW412	Sludge containing cyanide
SW413	Spent salt containing cyanide
SW414	Spent aqueous alkaline solution containing cyanide
SW415	Spent quenching oils containing cyanides
SW416	Sludge of inks, paints, pigments, lacquer, dye, or varnish
SW417	Waste of inks, paints, pigments, lacquer, dye, or varnish
SW418	Discarded or off-specification inks, paints, pigments, lacquer, dye, or varnish products containing organic solvent
SW419	Spent di-isocyanates and residues of isocyanate compounds excluding solid polymeric material from foam manufacturing process
SW420	Leachate from scheduled waste landfill
SW421	A mixture of scheduled wastes
SW422	A mixture of scheduled and non-scheduled wastes
SW423	Spent processing solution, discarded photographic chemicals, or discarded photographic wastes
SW424	Spent oxidizing agent
SW425	Wastes from the production, formulation, trade, or use of pesticides, herbicides, or biocides
SW426	Off-specification products from the production, formulation, trade, or use of pesticides, herbicides, or biocides
SW427	Mineral sludge including calcium hydroxide sludge, phosphate sludge, calcium sulfite sludge, and carbonates sludge
SW428	Wastes from wood preserving operation using inorganic salts containing copper, chromium, or arsenic of fluoride compounds or using compound containing chlorinated phenol or creosote
SW429	Chemicals that are discarded or off-specification
SW430	Obsolete laboratory chemicals
SW431	Waste from manufacturing or processing or use of explosives
SW432	Waste containing, consisting of or contaminated with, peroxides
SW 5:	**Other wastes**
SW 501	Any residues from treatment or recovery of scheduled wastes

Table 1. The 77 categories of first scheduled waste in the five grouping [7].

Malaysian government is set to ensure that industrial processes meet her environmental protection rules and regulations. It is a resolve demonstrated in a demand that chemicals must be produced and used such that risks and significant adverse effects are minimized on the environment and human health. The process involves cross-sector commitment of all stakeholders to coordinate approaches and common principles in the adoption and strength-

ening of good practices for a safe and ecologically sustainable chemicals management regime [8, 24].

2. Hazardous waste generation in Malaysia

The manufacturing sector in Malaysia was the first identified generators of toxic and hazardous waste. The hazardous waste problems were very much noticeable between the 1970s and 1980s; this is connected to the boom in the manufacturing sector between 1966 and 1988. The manufacturing sector in 1966 contributed 11% to the nation's gross domestic product (GDP), 24% in 1988 [25], and 24.6% in 2010 [26], while in 2012, the manufacturing sector contributed 24.2% to the Malaysian GDP [27]. The volume of hazardous waste generated from the Malaysia industrial sector in 1987 was about 400,000 tons, yet there was no institutional mechanism for managing the wastes [25]. In 2008, the hazardous waste generated was 1304902.74 metric tons [28, 29], while in 2011, it grew to 1622031.52 metric tons [29]. The hazardous waste generation for 2008 and 2011 is reported in **Tables 2** and **3**, respectively, showing the waste categories as presented in **Table 1**.

No	Waste category	Waste code	2008 waste generation	
			MT/Year	Percentage
1	Dross/slag/clinker/ash	SW 104, 107, 406	208319.53	15.96
2	Gypsum	SW 205	366771.99	28.11
3	Mineral sludge	SW 427	107122.05	8.21
4	Heavy metal sludge	SW 204, 105, 108	91730.67	7.03
5	E-waste	SW 110	102808.53	7.88
6	Oil and hydrocarbon	SW 305, 306, 307, 308, 309, 310, 311, 312, 314, 315, 415	129701.99	9.94
7	Clinical/pharmaceutica1	SW 404, 403, 405	26967.95	2.07
8	Batteries	SW 102, 103	34283.59	2.63
9	Acid and alkaline	SW 206, 401, 414	38179.66	2.93
10	Used container/oil filter	SW 409	38876.05	2.98
11	Spent solvent	SW 322, 323	38062.81	2.92
12	Contaminated paper and plastic	SW 41O	17270.40	1.32
13	Ink and paint sludge	SW 416, 417, 418	18695.75	1.43
14	Residue	SW 501	13544.07	1.04
15	Rubber sludge	SW 321	15512.02	1.19
16	Mixed wastes	SW 422, 421	33928.70	2.60
17	Pheno1/adhesive/resin	SW 325, 319, 303	6184.99	0.47
18	Catalyst	SW 202	5225.53	0.40
19	Others	NA	6627.73	0.51

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No	Waste category	Waste code	2008 waste generation	
			MT/Year	Percentage
20	Arsenic	SW 10l	–	–
21	Chemical waste	SW 430, 429	1169.75	0.09
22	Contaminated land/soil	SW 408	1324.77	0.10
23	Photographic waste	SW 423	418.77	0.03
24	Contaminated active Carbon	SW 411	934.42	0.07
25	Pesticide	SW 426	12.26	0.00
26	Mercury	SW 109	469.31	0.04
27	Asbestos	SW 201	668.94	0.05
28	Thermal fluids	SW 327	–	–
29	Sludge contain cyanide	SW 412	84.78	0.01
30	Peroxide agent	SW 432	5.73	0.00
Total			1304902.74	100.00

Table 2. Hazardous waste generation by category for year 2008 [28, 29].

No	Waste category	Waste code	2011 Waste generation	
			MT/Year	Percentage
1	Dross/slag/clinker/ash	SW 104, 107, 406	370789.09	22.86
2	Gypsum	SW 205	278139.00	17.15
3	Mineral sludge	SW 427	207445.01	12.79
4	Heavy metal sludge	SW 204, 105, 108	173837.06	10.72
5	E-waste	SW 110	152722.04	9.42
6	Oil and hydrocarbon	SW 305, 306, 307, 308, 309, 310, 311, 312, 314, 315, 415	133260.91	8.22
7	Clinical/pharmaceutica1	SW 404, 403, 405	44674.52	2.75
8	Batteries	SW 102, 103	41246.65	2.54
9	Acid and alkaline	SW 206, 401, 414	38152.48	2.35
10	Used container/oil filter	SW 409	36706.83	2.26
11	Spent solvent	SW 322, 323	30976.89	1.91
12	Contaminated paper and plastic	SW 41O	23332.03	1.44
13	Ink and paint sludge	SW 416, 417, 418	19224.56	1.19
14	Residue	SW 501	18118.39	1.12

No	Waste category	Waste code	2011 Waste generation	
			MT/Year	Percentage
15	Rubber sludge	SW 321	16130.66	0.99
16	Mixed wastes	SW 422, 421	10708.41	0.66
17	Pheno1/adhesive/resin	SW 325, 319, 303	7904.42	0.49
18	Catalyst	SW 202	6229.05	0.38
19	Others	NA	5505.33	0.34
20	Arsenic	SW 101	2131.57	0.13
21	Chemical waste	SW 430, 429	1327.61	0.08
22	Contaminated land/soil	SW 408	1072.87	0.07
23	Photographic waste	SW 423	587.63	0.04
24	Contaminated active carbon	SW 411	510.03	0.03
25	Pesticide	SW 426	487.10	0.03
26	Mercury	SW 109	434.18	0.03
27	Asbestos	SW 201	194.11	0.01
28	Thermal fluids	SW 327	178.00	0.01
29	Sludge contain cyanide	SW 412	5.09	0.00
30	Peroxide agent	SW 432	–	–
Total			1622031.52	100.00

Table 3. Hazardous waste generation by category for year 2011 [29].

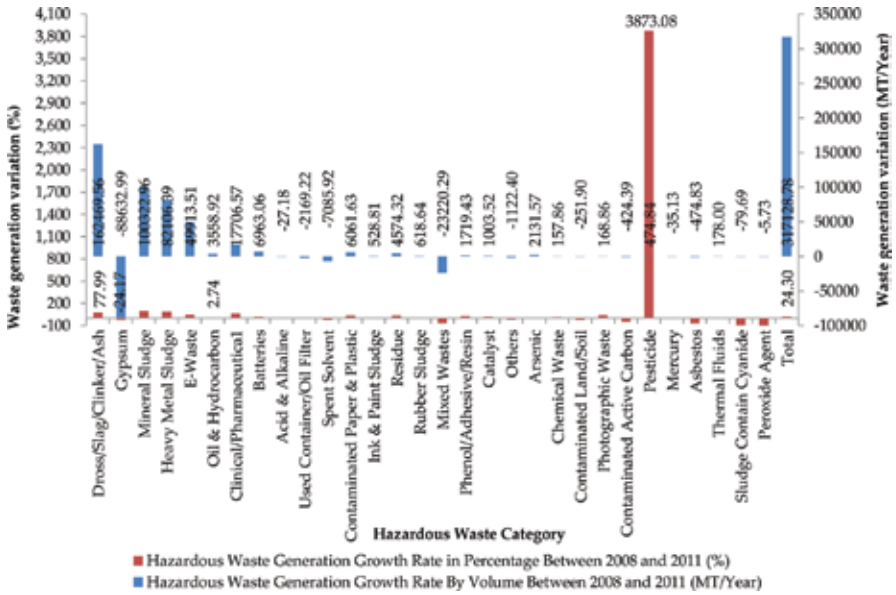

Figure 1. Analysis of the hazardous waste generation growth rate between 2008 and 2011.

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The highest volume of hazardous waste generated in 2008 was from gypsum with a total volume of 366771.99 metric tons, while in 2011, Dross/Slag/Clinker/Ash was the highest with 370789.09 metric tons of hazardous waste. Pesticide showed the highest percentage growth which is connected to the rapidly growing agricultural status of Malaysia, most especially the oil palm. In 2008, the hazardous waste generated from pesticides was 12.26 metric tons which drastically shot up to 487.1 metric tons in 2011, showing an increase of 474.84 metric tons in three years translating to 3873% increase waste generation compared to the waste generated in 2008. An analysis of the variation in the hazardous waste generation between 2008 and 2011 by categories is analyzed in **Figure 1**. It was found that even though oil and hydrocarbon was a strong industry in Malaysia, the change in the waste generation was (3558.92) lower than the change in clinical and pharmaceutical waste which increased by 17706.57 metric tons between 2008 and 2011. Similar observation can be made from the figure for other waste categories such as mineral sludge, batteries, and heavy metal sludge. There was no reference on the reason for the change, but it can be attributed to the sound safety and environmental regulations in the oil and gas industries championed by PETRONAS in Malaysia [30].

3. Malaysian hazardous waste management strategy

The core objective of pollution control and waste management was centered on human health protection and environmental cleanliness [31]. The Environmental Quality Act (EQA) of 1974 is the foundation of almost all the environmental protection and waste management policies in Malaysia. The EQA was enacted in 1974 and came into force in 1975 and has since been amended in 1976, 1985, and 1996 to meet the changing technologies and meet international standards. Hazardous waste management during the boom in the manufacturing sectors was unsuccessful because no regulation was in place till 1989; thus, there were no comprehensive hazardous waste management facilities in Malaysia. Industrial wastes were disposal freely into refuse disposal sites [25] which were unsuitable as destinations for hazardous waste without proper treatment. The improper disposals led to poor air quality near the disposal sites, contamination of ground water, and surface water bodies by chemical and biological agents from the waste dumps/disposal sites causing adverse effects on human health and the environment [32]. To protect the environment and the health of the citizens, the Sixth Malaysia Plan 1991–1995 clearly defined the policy statement for full integration of environmental concerns into all development processes of the nation with direct focus on sustainable development [33, 34]. As the adverse effects became known, industries began to treat, recycle, and reuse some of their waste materials [35]. The Malaysia Government also stepped up programs to render all scheduled waste harmless by enacting policies for scheduled wastes to be treated at the waste generation point or at specially designed treatment plants following the National Policy on the Environment. This policy, launched in 2002, is aimed at harmonizing economic development goals in line with environmental imperatives following the dictates of the Eight Malaysia Plan [36]. The enforcement of the regulations with some tax rebates to promote compliance and penalties for non-compliance made the industries become active players in waste reduction and recycling [37].

4. Malaysian hazardous waste management legislation and policy

In the Environment Quality Act, 1974 as amended, legislation on hazardous waste management has the main objective of controlling/regulating waste generation and improving waste management process and procedure in Malaysia. The legislation describes waste management process from generation, storage, handling, treatment, and final disposal. The EQA, 1974 is the primary legislation upon which other subsidiary environmental legislations and policies are built. Other environment regulations are also in place for the management of hazardous wastes as shown below:

- Environmental Quality (prescribed activities) (environmental impact assessment) Order 1987: This order is established following the dictates of EQA 1974, section 34A. The article 18a of the order is centered on waste treatment and disposal of toxic and hazardous waste outlining the developmental plan and procedure for sustainable management of hazardous waste. The plans and procedures are stipulated for the construction of incineration plants, off-site recovery plants, off-site waste water treatment plants, secure landfill sites, and off-site waste storage facilities [38].
- Environmental Quality (scheduled wastes) Regulations, 1989: This document contains regulations specifically for the management of scheduled waste from generation to final disposal. It classifies the most common hazardous scheduled wastes generated in Malaysia and defines a case of incompatible scheduled waste which is a condition, where a non-hazardous waste can be treated as hazardous waste. These regulations have been replaced by Environmental Quality (scheduled wastes) Regulations, 2005.
- Environmental Quality (prescribed premises) (scheduled wastes treatment and disposal facilities) Order, 1989: The order prescribed the premises occupation or use a holder of a license issued will cover. The premise occupation include off-site storage facilities, off-site treatment facilities, off-site recovery facilities, scheduled waste incinerators, land treatment facilities, and secure landfills.
- Environmental Quality (prescribed premises) (scheduled waste treatment and disposal facilities) Regulations, 1989: These regulations support the order 1989 and set procedure for licensing for prescribed premises (scheduled waste treatment and disposal facilities).

In compliance with the Basel Convention on control of transboundary hazardous waste, import and export orders were formulated under the Malaysian Customs Act, 1967 which prohibits importation or exportation of hazardous wastes unless with prior written approval from the Director General of the Department of Environment. The two orders are as follows:

- Custom (Prohibition of Export) Order (Amendment) (No. 2) 1993 now replaced with Custom (Prohibition of Export) Order 1998.
- Custom (Prohibition of Import) Order (Amendment) (No. 2) 1993 now replace with Custom (Prohibition of Import) Order 1998.

The Department of Environment of Malaysia does not encourage the import of hazardous waste into the country. Waste generators are allowed to export waste for recycling, recov-

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ery, or treatment with prior written approval from the importing state to discourage abuse of other nations' rights. On importation of used electrical and electronics equipment, Malaysia does allow such importations, provided the products are not older than three years from manufacturing date following the guideline policies for the classification of used electrical and electronic equipment in Malaysia 2008, revised 2010 [39].

5. Scheduled waste management facilities

The waste management facilities used by the various waste management operators in Malaysia depend on the waste that the operators handle. Below is the current hazardous waste management facilities used in Malaysia.

- Scheduled/hazardous waste transport facilities.
- Off-site waste storage and waste transfer stations/facilities.
- Secure landfill—for final disposal of stabilized wastes.
- Scheduled waste incineration plant which can be on-site or off-site activities depending on the type of waste and volume generation.
- Clinical waste incineration—specifically for the management of clinical and pharmaceutical wastes.
- Off-site physio-chemical waste treatment facilities for waste stabilization or solidification for final landfilling.
- Centralized waste treatment facility (e.g., electroplating park).
- Resource recovery—this involves the recovery of reusable materials from hazardous waste such as oily wastes, metal dross/metal hydroxide, and catalyst.
- Land treatment—treatment of contaminated land.
- Waste water/sewage treatment facilities.

To establish any of the facilities, the operator needs to apply for a license through the office of the Director General of the Department of Environment. The licensing process is well detailed in Part III of the EQA, 1974 as amended. The process involves the following four stages:

i. Environmental impact assessment (EIA)—proposes site inspection to access suitability for the operation against environmental pollution following the developmental plan of Malaysia.

ii. Processing of the written permission—Provision of all qualifying document for the operator to prove capability to run the operation in conformity with the EQA, 1974 and other environmental regulations.

iii. Pre-licensing inspection.

iv. Processing of the operating license.

5.1. Classification hazardous waste management facilities

A hazardous waste facility is any of the government-approved waste management facility that observes ethical practices and sustainable development. The facilities include contiguous land, waste storage facility, waste recovery facility, recycling facility, incinerator, and secure landfill [40]. A hazardous waste facility can function independently depending on the type of hazardous waste that it handles or may require a combination of technologies as in the case of commercial facility processing different types of wastes. The different facilities available in Malaysia for hazardous waste management include the following:

- **Waste recovery/recycling facilities:** This type of facility is used to recover material for reuse and is saleable for economic benefits. Examples of recoverable products are typically solvents, oils, acids, or metals etc.
- **Treatment facilities**: The use of treatment facilities is mainly for materials that require changes in the physical or chemical characteristic before disposal. This process uses thermal, physical, biological, or chemical methods to reduce the potential harm in the waste before disposal.
- **Land disposal facilities:** This is the final destination of stabilized waste that need no further usage before being permanently buried below soil surface.
- **Fully integrated facility:** This is one major commercial facilities operator for the management of Hazardous waste in Malaysia Kualiti Alam Sdn Bhd which operates fully integrated facility.

5.2. Exclusive right to Kualiti Alam Sdn. Bhd

In the mid-1960s through 1980, Malaysia experienced rapid economic growth in the manufacturing sector which triggered the generation of hazardous wastes in Malaysia and the associated negative effects on the environment [25]. The Malaysian government recognized the growing problem of hazardous waste generation in the country and worked out general waste management strategies to carter for her waste generations. The growing concern on hazardous waste generation led to a survey by a Danish consultancy corporation, which findings helped in drafting regulations on hazardous waste management in 1984. Further surveys on hazardous waste generation and the effect on the environment were conducted by the Department of Environmental (DOE) in 1985 [25]. After several surveys and review of reports of findings on the growing problems of hazardous waste, the Malaysian Government issued the first formal legislation on hazardous waste in 1989. The legislation was supported with the development of a national scheduled waste program aimed at developing an integrated scheduled waste management system which was given to two private companies to design. In 1995, one of the companies, Kualiti Alam Sdn. Bhd, a consortium of Malaysian and Danish companies was given approval to establish integrated scheduled waste plant and was granted the exclusive right operate the plant for 15 years. Kualiti Alam Sdn Bhd was given

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the responsibilities for waste collection, transportation, treatment, and final disposal of hazardous waste [41, 42].

5.3. Integrated scheduled waste management system

The integrated scheduled waste management system of Kualiti Alam Sdn Bhd is a centralized integrated waste management center (WMC) developed to use a combination of multiple technologies in the treatment and final disposal of different types of scheduled wastes. The waste management center initially has four integrated treatment facilities comprising incineration plant, physio-chemical treatment plant, solidification plant, and secure landfill but currently includes another facility for clinical waste treatment as shown in **Figure 2**. The facility treats all the categories of scheduled wastes except radioactive waste, pathological waste, and explosive waste.

Figure 2. Kualiti Alam end-to-end facilities of the waste management center.

5.3.1. Incineration

Kualiti Alam Sdn Bhd treats about 120,000 metric tons of Malaysia's industrial wastes per year [43]. Industrial wastes are categorized following the organic carbon content where wastes that contain organic carbon level above 10% are disposed only by incineration. Kualiti Alam Sdn

Bhd incineration plant design incorporates a rotary kiln, secondary combustion chamber, and flue gas-cleaning system. The incinerator, as shown in **Figure 3** operates at high temperature, thus volatilely destroys all the hazardous scheduled waste channeled to it including polychlorinated biphenyls PCBs contaminated wastes. The ash produced at Kualiti Alam incineration process is around 14,000 metric tons of bottom ash which are disposed to secured landfills lined with impermeable layers [43]. A full landfill will be covered to protect it from rainwater and to minimize seepage using low-density polyethylene liner, but this covering process is not 100% effective. A recent study investigated a sustainable method of managing the ash and found nearness of the ash composition to cement [43].

Figure 3. Kualiti Alam incineration facility.

5.3.2. Stabilization/solidification and physical and chemical treatment facilities

Hazardous waste solidification plant of Kualiti Alam stabilizes neutral inorganic waste and reduces hazardous substances mobility. The system traps contaminants within their host medium and bind them into solid matrix [44]. The facility has a capacity of 15,000 MT/year. Physical and chemical methods are often used in combination with solidification, to separate or transform hazardous substances to less harmful materials. Inorganic wastes are reduced to neutral pH values and other management method like stabilization will be employed.

5.3.3. Secured landfills

The secured landfill is the final destination of stabilized or reduced waste. Incineration by-products like slags, fly ash, and flue gas cleaning products with other residues do undergo solidification and finally deposited in the secure landfill. The landfill is constructed with some monitoring sensors as shown in **Figure 4**. The monitoring system is a specific requirement from the department of environment to prevent ground contaminations.

5.4. Inspection of hazardous waste management facilities

Inspections of waste management facilities are carried out by the Department of Environment, which is a part of the procedural standards for the licensing of waste management facilities or license renewal for operators. The inspections include inspection of schedule of compliance

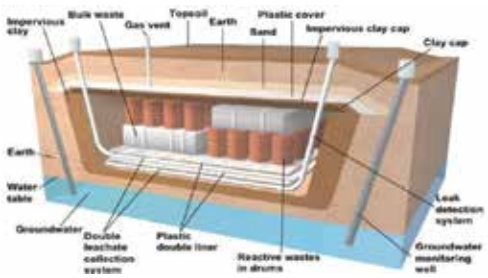

Figure 4. Secured landfill.

for written permission and pre-license inspection. A routine annual inspection for operating facility which requires license renewal is one of the major inspections. This routine inspection helps the Department of Environment to monitor and keep updated record of the various operating facilities. There is also an unscheduled inspection when there is sign or report of operator contravening the regulation of the EQA 1974 and other acts. Hazardous waste management facility inspections from pre-licensing period to operations depend on the type of facility the operator applied for.

Scheduled hazardous wastes	Recovery	PCT	Solidification	Incineration	Secured landfill
Inorganic waste					
Reactive agent, acidic substances and alkalis	√	√			
Metallic solids/heavy metals	√	√	√		
Metallic solution/sludge of heavy metals	√	√	√		
Inorganic sludge		√	√		
Inert inorganic waste					√
Organic waste					
Organic solvents	√			√	
Organic oil contaminates	√			√	
Agro-pesticides/herbicides				√	
Chlorinated hydrocarbons				√	
Resins, organic sludge, and paints	√			√	

Table 4. Waste treatment options for some hazardous waste categories.

5.5. Scheduled hazardous wastes treatment and disposal methods

There are several treatment methods available for hazardous waste management. The waste management methods considered by government are waste reduction (most sustainable procedure), recover/reuse, physio-chemical treatment (PCT), thermal treatment/incineration,

solidification, and biological methods. Some waste treatment options used in the management of scheduled wastes are analyzed in **Table 4**.

6. Conclusion

The focus in this chapter has been the status of hazardous waste management in Malaysia. As highlighted, industrial waste is the major source of hazardous waste in Malaysia, and the nations' approach to hazardous waste management is very well designed and in line with the nation's development plan. The management process is designed such that only licensed operators can handle and treat hazardous waste. Close monitoring of the industries is enforced and sanctions swiftly imposed on erring operators to help keep everyone in line. There is still much to do in the management of agro-hazardous waste as most of the wastes are associated with fertilizer and pesticides usage. The oil/hydrocarbon industry was found to be more conscious following the growth in the waste generated between 2008 and 2011. Clinical waste, which is high-potent hazardous substance, is growing so much and the regulators should pay more attention to that sector and devise more stringent rules on its management. Zero waste generation (reduction) concept is the most sustainable option for Malaysia and can only be achieved if rules are set for waste generators to pay higher fees per kilogram of waste generated.

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Section 2

Microbial Bioremediation of Hazardous and Radioactive Wastes

Chapter 6

Bioremediation for Tanning Industry: A Future Perspective for Zero Emission

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Additional information is available at the end of the chapter

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Abstract

Bioremediation is one of the recent technological advancements for treating heavy metals containing industrial wastes. Leather industry utilizes almost 90% of chromium-based tanning agent for converting raw skins/hides into leather. Apart from chromium, metals such as aluminum, titanium, iron, and zirconium are widely used for various end applications. Hence, effluent after tanning processes contains higher concentration of heavy metals. Though Cr(III) is less toxic than Cr(VI), there is higher possibility of oxidation during subsequent treatment processes. Therefore, several methodologies have been developed to remove the heavy metals from the effluent before processing it for common effluent treatment. Phytoremediation is one of the eco-friendly techniques to remove the heavy metals from soil and wastewater. It is commonly used to remediate the unfertilized lands to fertile lands for agriculture. Moreover, metal absorbed plants are used for various applications such as tanning and preservative agent in the leather industry. Hence, metal absorbed plants are not dumped as solid waste. Similarly, algae and fungi are used to remove the heavy metals from the tannery waste and can be as metal-polysaccharide auxiliary chemicals during post-tanning processes. Utilization of nonpathogenic bacteria is also used for the absorption of heavy metals. In this case, the handling of biomass is easier compared to other methodologies owing to less time duration and labor friendly, whereas, in the case of phytoremediation, absorption rate directly depends on the growth duration. In the present chapter, detailed case study is carried out to compare the advantages and disadvantages of various bioremediation technologies employed for treating leather wastewater.

Keywords: bioremediation, bacteria, tannery wastewater, chromium, phytoremediation

1. An overview of tanning

Leather industry is one of the fast-growing traditional sectors with high environmental challenges. Leather manufacture deals with the conversion of putrescible material such as skin/hide to non-putrescible material. During the conversion, series of chemical processes and physical operations are carried out to attain the final desired properties of leathers. Pre-tanning, tanning, and post-tanning are the major steps involved in the leather processing. One of the major challenges of leather processing is generation of high solid and liquid wastes that makes it more an environmental concern [1].

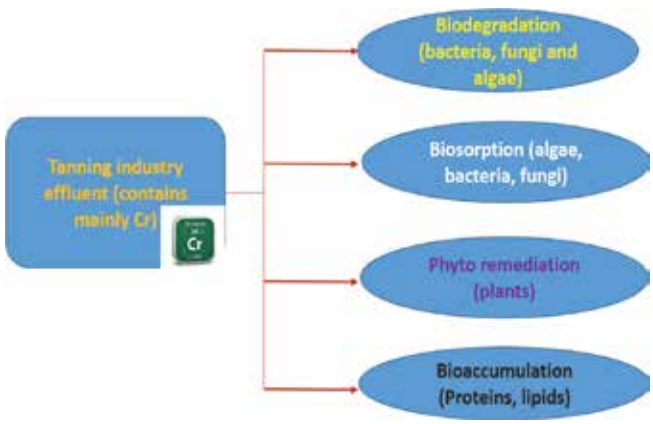

Figure 1. Scheme of bioremediation employed for the removal of heavy metals.

Pre-tanning involves the preparation of skin material tanning; hence raw material undergoes extensive cleaning and removal of unwanted material from the matrix. Soaking, liming, unhairing, reliming, fleshing, deliming, bating, and pickling are various steps involved in pre-tanning. Solid wastes such as hair, lime sludge, interfibrillary materials, and adipose tissues are the major components during this step. Liberation of toxic gases such as hydrogen sulfide and ammonia is also noted. Liquid wastes are generated in all unit processes contributing to the chemicals used in the respective steps. To combat the heavy pollution load, leather researchers have developed several cleaner/greener leather processes to counteract conventional leather process. Enzymatic unhairing and fiber opening, CO_2 deliming, and pickle-less tanning are major alternate processes to reduce the pollution loads. Chrome tanning is widely practiced globally due to its unique leather properties. However, generation of Cr(VI) in the waste stream causes a serious alarm to the environmental engineers due to its high cancer-causing nature (carcinogenic). Next to chrome, vegetable tanning is practiced owing to its nontoxic nature; it is considered as an alternate to chrome tanning. However, the vegetable tanned leathers are unable to match the propertied chrome tanned leathers. Several other tanning agents such as phosphonium, oxazolidine, and silica-based tannings are carried out with their merits and demerits. Bioremediation possibilities and the scheme of tanning process are given in **Figures 1** and **2**.

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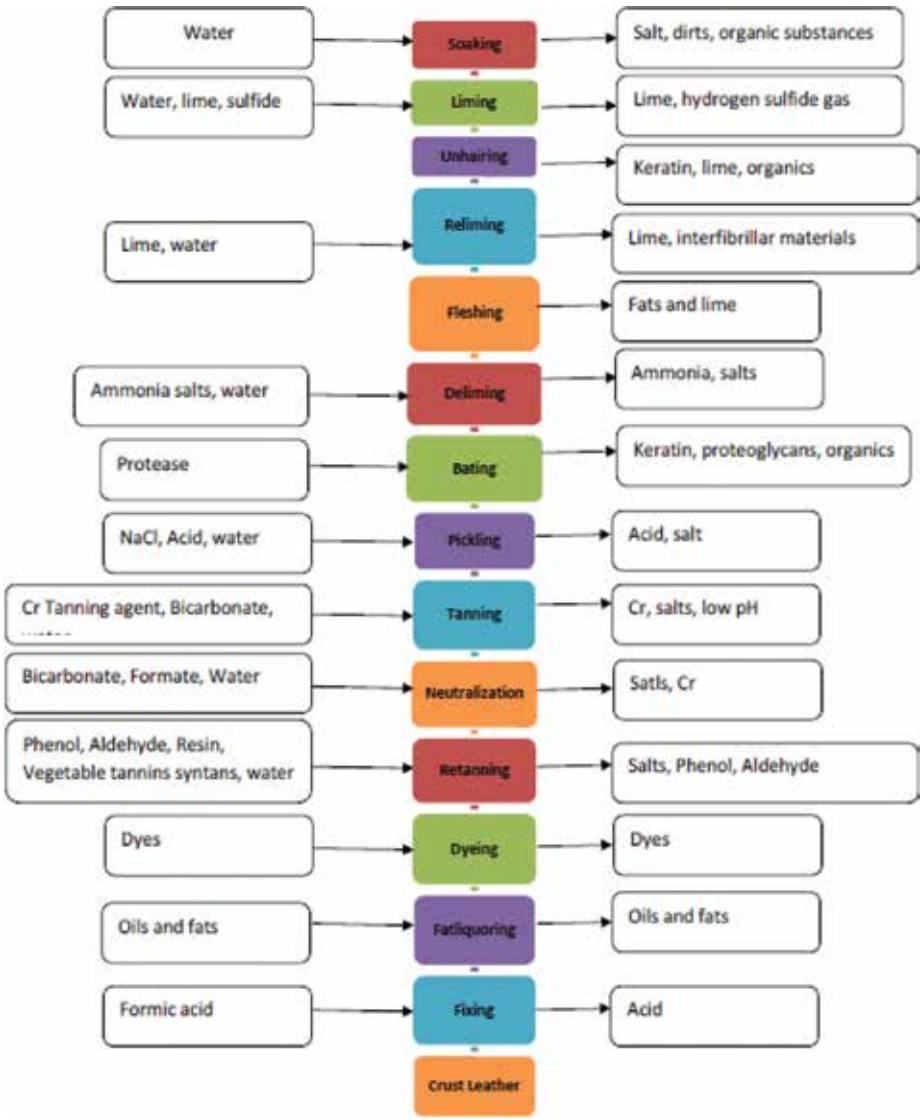

Figure 2. Schematic representation of leather unit processes.

Researchers also work to develop natural-derived materials from plants and microorganisms for tanning such as dialdehyde starch, dialdehyde cellulose, dialdehyde alginic acid, dialdehyde pectin, and scleraldehyde from fungal species (*Schizophyllum commune*). Post-tanning comprises retaining, dyeing, and fat liquoring to impart special properties to leathers as a functional requirement. The waste stream is composed of mixed wastes that make it more difficult during wastewater treatment. The composition of tannery wastewater is provided in **Table 1**.

Pollutants	Values
pH	6.9
BOD_5	50 mg/mL
COD	250 mg/mL
Total suspended solids	50 mg/mL
Sulfide	1.0 mg/mL
Chromium(VI)	0.1 mg/mL
Total chromium	0.5 mg/mL
Chloride	1000 mg/mL
Sulfate	300 mg/mL
Ammonia	10 mg/mL
Oil and grease	10 mg/mL
Total nitrogen	10 mg/mL
Total phosphorous	2 mg/mL
Phenols	0.5 mg/mL
Total coliform bacteria	400 MPN/100 mL
Temperature increase	<3°C

Table 1. Effluent levels for tanning and leather finishing (ref. environmental health and safety guidelines, tanning and leather finishing).

Solvent-based post-tanning is carried out to reduce the pollution and maximize the chemicals exhaustion. Though several alternate cleaner leather processes exist, no holistic approach has been made till date. Furthermore, conventional leather processing methods have found an impeccable place among the tanners due to its unmatching physical and chemical properties to the leathers. Therefore, it would be highly appreciable if wastewater treatment becomes a relevant measure to combat the pollution load. Primary, secondary, and tertiary treatments are practiced to treat the wastes. Biological treatment methods are the fast-growing technology due to their eco-friendly and recycle method. In this chapter, a brief discussion about the various biological systems used in leather wastewater (otherwise known as tannery effluents) treatment is discussed.

2. Application of microorganisms in tannery wastewater treatment

The role of microorganisms is gaining importance in different applications due to their versatile abilities. Availability of single cell microorganism is largely found in the ecology system. These organisms are mainly found in the soil systems, water, and intestinal tract of living system. Microorganisms are classified based on the sources of energy and carbon. The major classifications are autotrophs and heterotrophs. Autotrophs are further classified into photoautotrophic and chemoautotrophic based on energy source as light and inorganic

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oxidation-reduction reaction, respectively. Heterotrophs are further classified into chemoheterotrophic and photoheterotrophic based on their energy source, organic oxidation-reduction reaction, and light, respectively. Researchers have focused to understand the importance and necessities of utilization of microorganisms of human well-being in the different technology fields. One of the unique aspects of their living organisms is their size, regular supplements of nutrition, water, and optimum physiological condition to grow and multiply the cells. During secondary treatment of leather wastes, microorganisms commonly used result in the generation of biomass sludge (see **Figure 3**).

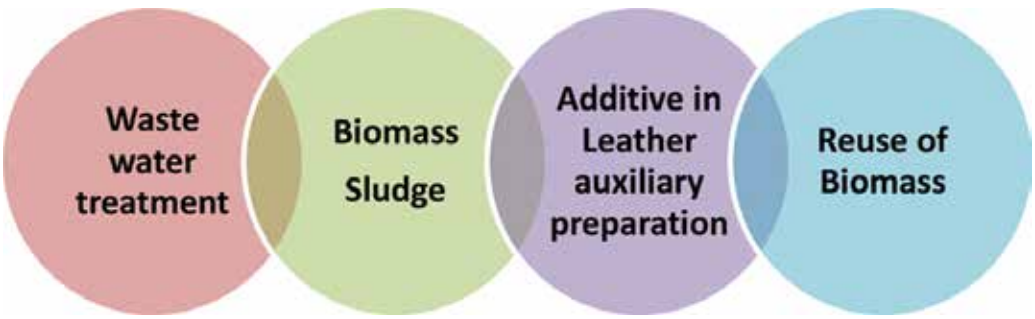

Figure 3. Schematic representation of role of microorganisms in leather wastewater treatment.

2.1. Bacteria

Microbial technology finds an immense application in technological industry owing to its size and handling conditions. Bacteria are single microorganisms and are in small size with different shapes. A list of bacteria involved in the remediation process is given in **Table 2**. Growth of the bacteria requires nutritional supplements like any other living organisms. *Pseudomonas* sp., *Bacillus* sp., and *Lactobacillus* sp. are commonly used bacterial strains. Nutritional requirements are obtained by decomposing organic material and aids in multiplying the cell. Hence, bacteria are efficiently used for decaying organic wastes present in the leather waste stream. Leather wastes during pre-tanning processes are chiefly made up of organic constituents such as collagen, elastin, reticulin, other proteoglycans, keratins, and other nutritional supplements. Aerobic and anaerobic systems are practiced in treating wastewater in the secondary treatment plant. Hydrogen sulfide gas release contributes to gas pollution during liming process. Chemolithotrophic bacteria are used to absorb the toxic gas. After primary treatment of wastewater, i.e., removal of floating materials and particles which can be filtered through screens is sent through biological treatment.

During this process, bacterial cells are purged to undergo biological oxidation. Bacteria tend to degrade the organic component in the waste stream and are used as nutritional supplement. This respiration process utilizes oxygen and exits the carbon dioxide. After the process the wastewater treatment plant will end up with a large amount of activated sludge (bacterial cell

mass). Very often the activated sludge is condensed and treated in large-scale anaerobic digesters designed for further treatment via anaerobic bacteria. However, there should be very careful steps taken toward the disposal of bacterial sludge. Since, it is mainly composed of proteins and carbohydrates which were used as a raw material for preparing leather chemicals. Protein fillers and protein synthetic tanning agents are possible areas to venture for the recycling bacterial sludge.

Bacteria	Process	References
Bacillus subtilis	Biosorption of Cr	[2]
Arthrobacter sp.	Ultrafiltration/microfiltration Lead	[3]
Bacillus sp.	Livestock	[4]
Rhodococcus sp.	Heavy metal absorption	[5]
Escherichia coli	Removal of chlorophenol	[3]

Table 2. List of bacteria used in tannery wastewater treatment.

Mechanism: Biosorption is a process that utilizes the microbial biomass to accumulate the chromium metals. This is based on the process of adsorption. It is based on the nonspecific binding of chromium through addition of polysaccharides. Though, microbial process involves both enzymatic and nonenzymatic mode for the accumulation of microorganisms. During biosorption of chromium, the metal interacts with polysaccharides through nonenzymatic mode. This process is mainly dependent on the physical parameters such as pH and ionic strength. Charge of the polysaccharides is a critical factor that aids in the sorption of metal ions.

2.2. Algae

Algae are significant organisms for biological purification of wastewater because they can be able to accumulate plant nutrients, heavy metals, pesticides, and organic and inorganic toxic substances. **Table 3** lists out the algae species involved in the treatment process. The application of algae in biological wastewater treatment has gained a lot of importance in the recent years. Construction and energy costs are highly lower and the land requirement is not up to that of facultative pond in constructed wet lands. Algae are commonly used to absorb sectional waste from the composite waste liquors. They are chiefly used for the absorption of heavy metals due to their high tolerance and absorption. Moreover, the large surface area/volume ratio makes it as one of the important biological materials for wastewater treatment.

Mechanism: Algae are composed of polysaccharides that make convenient for the absorption of heavy metals. Their high tolerance to heavy metals and growth behavior of autotrophs and heterotrophs make them one of the best choices. Biosorption of chromium, cadmium, and copper ions by other blue green algae *Spirullina* sp. and *Chlorella* sp. is known well. Application

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of algae in absorption of chromium leads to the generation of sectional solid waste. It would be mere conversion of liquid into solid waste; hence, further intensive research needs to be carried out in order to effectively and efficiently reuse the solid waste. Since, solid waste is composed of carbohydrates and metals; it can be used as a raw material for preparing chrome-based retaining agents. It can be further investigated as one of the chemical components during post-tanning processes with chemical modifications.

Algae	Process	References
Phormidium bohneri	Removal of nitrogen and phosphorus	[6]
Spirulina (*Arthrospira*)	Removal of nitrogen and phosphorus	[7]
Spirogyra condensate	Biosorption of chromium	[8]
Scenedesmus sp.	Bioflocculation	[9]
Chlorella vulgaris	Immobilized to remove nutrients (P and N)	[10]

Table 3. List of algae used in tannery wastewater treatment.

2.3. Fungi

Fungi are multicellular organisms unlike bacteria which are unicellular organisms and are abundantly present in wastewater stream. *Aspergillus*, *Penicillium*, *Fusarium*, and other types of fungi are reported to be found in the sectional waste stream. These fungi are found to be effectively used in the removal of nutrient source from the wastewater. It is also reported that certain fungi also have the ability to break down organic matter present in the waste. Critical role of Fungi in the tannery waste water, treatment at very high acidic pH. Though acid-resistance bacteria exist, there is still a challenge for the bacterial treatment at low pH. Hence, fungi can be used in the acidic medium to break down the organic constituents. Moreover, fungi have the ability to trap and absorb the material through their hyphae and aid in the metabolism. Enzymes are also secreted by some fungi that help in the biodegradation of organic compounds present in the waste. Apart from the species mentioned above, many other fungi could degrade the tannery effluent waste; however, their pathogenicity is a big question while it comes to the matter of large-scale applications.

3. Application of plants in bioremediation

3.1. Phytoremediation

Phytoremediation is a branch of bioremediation that deals with the utilization of plants for the wastewater treatment. Halophytes and aquatic plants are more commonly used for the tannery waste treatment. Many researchers have focused on developing phytoremediation technologies for treating the tannery effluent contaminated land sites into reusable lands. The major selections of plants are based on the availability and growth surrounding the tannery plants.

This is based on the hypothesis that plants are resistant to the environmental parameters and resistant to the pollutants discharged from the tanning industry.

Cristina et al., a research group, have investigated four different plants *Canna indica*, *Typha latifolia*, *Phragmites australis*, *Stenotaphrum secundatum*, and *Iris pseudacorus*. These plants are vegetated in wetlands for the treatment of tannery wastewater [11]. The plants are chosen based on the growth and availability surrounding the tannery effluent contaminated lands. Among the chosen five plants *P. australis* and *T. latifolia* are effectively involved in the waste treatment. It might be understood that there would be growth of plants surrounding tannery effluent–contaminated plants. However, it is not mandate that all plants have the ability to effectively participate for the effluent treatment.

Daniel et al. have carried out on water hyacinth (*Eichhornia crassipes*) for the removal of chromium from tannery effluent in constructed pond system. Water hyacinth is free-floating aquatic plants that originated from Amazon, South America. The samplings are collected in the Awash River, Ethiopia, for the treatment [12]. Many other plant species are also involved in the phytoremediation; however, the removal of heavy metals and nutrients should be considered for further applications.

3.2. Constructed wetland

A constructed wetland is an artificial wetland widely used for treatment of municipal and industrial wastewater. Constructed wetland system is a hybrid technology including phytoremediation, soil filtration, and microbial processes and has been reported to enhance overall biological performance by complex interactions inside the ecosystem [11]. In the past decade, researchers have attempted to study the potential of constructed wetland for the treatment of tannery effluent. Cristina et al. conducted the treatment of raw tannery wastewater by horizontal subsurface flow constructed wetlands (HSSFCW) with five different kinds of vegetation (mentioned in Section 3.1). During the one-year experiment, wastewater contained on average 2250 mg/L Chemical Oxygen Demand, 92 mg/L Total Suspended Solids, 188 mg/L Total Nitrogen, 1 mg/L Total Phosphorus, and 0.027 mg/L T-Cr, respectively [11]. All the six HSSFCW systems including control without plant showed significant removal of Chemical Oxygen Demand (833–913 mg/L in effluent), TSS (18–21 mg/L), and Cr (<0.001–0.012 mg/L) under a hydraulic loadings rates (HLR) of 6 cm/d. The *C. indica*-planted Constructed Wetlands showed the best performance in Cr removal (<0.001 mg/L throughout the experiment), while *P. australis*-planted Constructed Wetlands showed the best in Chemical Oxygen Demand removal (833 mg/L). However, all the N and P removal maintained low level.

Kaseva and Mbuligwe tested continuous treatment of tannery wastewater using two kinds of HSSFCW system: one was planted with macrophytes and the other without plant (control) at a hydraulic retention time of 1.6–1.8 days [13]. Although the wastewater contained more than 370 mg/L chromium, the HSSFCW system showed high Cr removing efficiency (99.83%), and even the control system recorded 92.53% removing efficiency. Turbidity was reduced from around 190 NTU to 29–79 NTU (HSSFCW) and 26–83 NTU (control). The study demonstrated

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the potential of constructed wetland systems to be used as an option for treating tannery wastewater with such a high Cr content, removing Cr and turbidity. However, the system was not suitable for reduction of TDS and EC. Saeed et al. tested three-stage hybrid CW system combining subsurface vertical flow (VF) wetland, followed by a horizontal flow (HF) and a VF wetland for tannery wastewater treatment, and demonstrated the high nutrient removal [14]. Although the wastewater contained high organic matter (11,500 mg/L COD, 111 mg NH_4^+, and 30 mg/L PO_4^{3-}), the overall system had 98%, 86%, and 87% of COD, NH_4^+-N, and PO_4^{3-}, respectively, removal efficiency on average.

4. Reuse and recycle of treated waste in tanning industry by-product utilization

Yearly, tannery industries generate more than 600,000 tonnes of waste, posing a major challenge to the environment [15]. It is reported that producing 150 kg of leather would require 1000 kg of raw hide and throw out 850 kg of solid waste to the environment [15]. With the high discharge amounts in leather processing, there is a critical need to treat and recycle the tanning industry by-product. The hierarchy of tanning waste treatment/management is suggested in **Figure 4**.

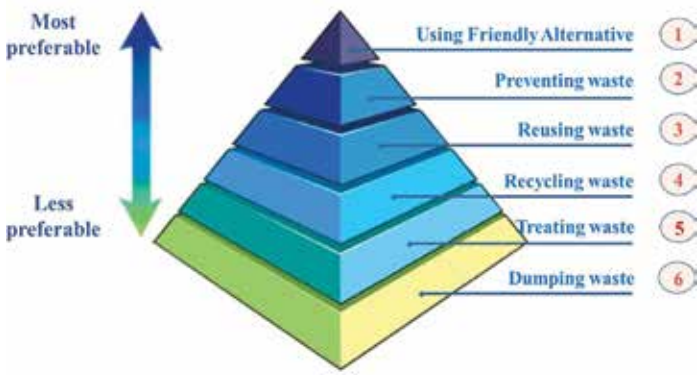

Figure 4. Hierarchy of tanning waste management.

The order of tanning waste management starts from the top of the hierarchy by friendlier option and finish at the bottom of the hierarchy in accordance with "using friendly alternative, preventing, reusing, recycling, treating and dumping waste".

1. *Using environmentally friendly alternatives*: Using biodegradable fat-liquoring alternatives, innovative compounds, which are based on natural materials such as palm oil, are the most preferable option, making them easier to treat wastewater from the tanning sector. Furthermore, the use of innovative technology for leather-tanning process with the goal of reducing hazardous waste should be chosen [16].

2. *Preventing*: Eliminating fat-liquoring agents such as chromium III, arsenic, cadmium, and lead is also recommended in tanning industry in order to prevent hazardous contaminants.
3. *Reusing*: The encouragement of reusing waste and residue for the reproduction or making new products is considered as the third option.
4. *Recycling*: Applying green technologies to treat waste by applying some energy conversion technologies such as biomethanation, bioenergy production, and other feasible technologies such as biosorption, bioaccumulation, biodegradation, and phytoremediation [12, 17].
5. *Treating*: The completed investment of wastewater treatment facility is preferred as end-of-pipe sector of tanning industry. Wastewater treatment standard must be critically followed up to the environmental friendly standards to prevent environmental affection from the untreated waste.
6. *Dumping*: At the bottom of hierarchy, dumpling is the less preferable option due to tanning wastes that cause high environmental pollution and further affect to human health. In fact, due to the costs of treating tanning waste, many tanning manufacturers deal tanning waste with illegal activities. Therefore, treating tanning waste by dumping should be banned in countries such as Bangladesh, Nepal, and Sri Lanka [18].

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Chapter 7

White-Rot Fungi and their Enzymes as a Biotechnological Tool for Xenobiotic Bioremediation

Mariem Ellouze and Sami Sayadi

Additional information is available at the end of the chapter

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Abstract

A huge amount of hazardous organopollutants, often persistent and toxic, is produced annually over the world and may contaminate soil, water, ground water, and air. Being from various sources such as wastewater, landfill leachates, and solid residues, xenobiotics include phenols, plastics, hydrocarbons, paints, dyes, pesticides and insecticides, paper and pulp mills, and pharmaceuticals. Among biological processes for degradation of xenobiotics, fungal ones, being eco-friendly and cost cheap, have been investigated extensively because most of basidiomycetes are more tolerant to high concentrations of pollutants. Fungal bioremediation is a promising technology using their metabolic potential to remove or reduce xenobiotics. Basidiomycetes are the unique microorganisms that show high capacities of degrading a wide range of toxic xenobiotics. They act via the extracellular ligninolytic enzymes, including laccase, manganese peroxidase, and lignin peroxidase. Their capacities to remove xenobiotic substances and produce polymeric products make them a useful tool for bioremediation purposes. During fungal remediation, they utilize hazardous compounds, even the insoluble ones, as the nutrient source and convert them to simple fragmented forms. The aim of this chapter is to elucidate the ability of basidiomycetes to degrade xenobiotics. This is an overview to present the importance of extracellular enzymes for efficient bioremediation of a large variety of xenobiotics.

Keywords: xenobiotics, white-rot fungi, enzymes, bioremediation, biodegradation

1. Introduction

The contamination of soil, water, ground water, and air with toxic chemicals is one of the major environmental problems, faced by the world today. With the intensive industrialization and

the extensive use of pesticides in agriculture and chemicals in various fields, the environmental pollution with organic compounds has become a serious threat. Indeed, pollution of aquatic system and soil is a worldwide problem that can result in accumulation of toxic chemicals in food chains and also harm the flora and fauna. Hence, environmental pollution with hazardous wastes containing recalcitrant chemicals, being often xenobiotic compounds, has become one of the major ecological problems, with an increasing awareness around the world [1, 2]. The quick rise of industrial activities has extremely increased the release of toxic effluents into water bodies along with ground water [3]. The pollution resulting in the release of these compounds causes disturbance to the natural bodies and their ecosystems, leading to climatic changes, water level reduction, and other negative impacts [4]. On the other hand, there are increasing concerns about potential adverse health and ecological effects resulting from the production, the use, and the disposal of numerous chemicals that otherwise offer improvements in human life and economic activities. Thus, a huge amount of hazardous organopollutants is produced annually over the world and only 10% of these are disposed safely. The most hazardous compounds are persistent in the environment and are carcinogenic and/or mutanogenic. Xenobiotics are chemicals that are "foreign to the biosphere" and may become available to microorganisms in different environmental compartments, depending on their fate in air, water, soil, and sediments [5]. Household chemicals, pharmaceuticals, and other consumables as well as biogenic hormones are released into the environment after passing through wastewater treatment processes, which are not designed for their removal [6]. The main sources of xenobiotics are wastewater, landfill leachates, and solid wastes released from the industries directly, such as phenols, plastics, hydrocarbons, paints, dyes from textile mills, pesticides and insecticides from agricultural industries and paper and pulp mills, or indirectly, including pharmaceuticals, especially the group of endocrine disrupters, and pesticide residues. These chemicals include biopolymers (cellulose, kraft lignin, and lignin), synthetic polymers (polyarylate, polyacrylamide, and nylon), polycyclic aromatic hydrocarbons (anthracene, benzo[a]pyrene, chrysene, naphthalene, pyrene, etc.), pentachlorophenols (PCP), polychlorinated biphenyls (PCB), pesticides and insecticides (1,1,1-trichloro-2,2-bis(4-chlorophenyl) ethane [DDT], benzene, toluene, ethylbenzene, and xylene [BTEX]), as well as trinitrotoluene (TNT), dyes (azo dyes, anthraquinone, etc.), and others (including azide, cyanides, aminotriazole, and carbon tetrachloride). Unlike the naturally occurring organic compounds that are readily degraded upon introduction into the environment, some of these synthetic chemicals are extremely resistant to degradation by native microorganisms [7].

Degradation of such compounds by physical and/or chemical processes is costly and often produces undesirable products which are toxic. Biological methods, being eco-friendly and cost cheap techniques, were proposed for xenobiotic degradation purposes in order to overcome these problems. Compared to bacteria, most of the fungi are robust organisms and generally more tolerant to high concentrations of pollutants. It explains why they have been extensively investigated since the mid-1980s for their bioremediation capacities. White-rot fungi (WRF) constitute an eco-physiological group comprising mostly of basidiomycetes and litter-decomposing fungi. Recently, there has been a great interest in white-rot fungi and their ligninolytic enzymes, including laccase, manganese peroxidase (MnP) and lignin peroxidase (LiP), for the degradation of a wide range of xenobiotics.

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These xenobiotics include phenols [2, 8–10], hydrocarbons [11–14], dyes and textile effluents [15–21], pharmaceuticals including the endocrine-disrupter compounds [6, 22], pulp and paper mills [23–25], pesticides, and insecticides [26–29]. Some studies reported the effect of ligninolytic enzymes on degradation of other organopollutants like PCB [1], chlorinated phenols including trichlorophenols [30, 31], and xenobiotics in landfill leachates, solid wastes [32–35], and munitions [26].

Because of the non-specific nature of fungal lignin depolymerization, white-rot fungi can degrade a wide range of persistent environment pollutants even the insoluble chemicals [26]. These microorganisms generally act via the extracellular ligninolytic system showing good potential applications in chemical, agro-food, paper, textile, and cosmetic industries. This group may be a useful and a powerful tool for bioremediation purposes thanks to fungal capacities to degrade many xenobiotic substances.

The expression of these enzymes depends on the strain itself: some white-rot fungi produce LiP and MnP, but not laccase, while others produce MnP and laccase, but not LiP, acting simultaneously or separately on xenobiotics released from the environment. The potential of white-rot fungi can be harnessed thanks to emerging knowledge of the physiology and the morphology of these organisms. This knowledge could be transformed into reliable and robust waste treatment processes. The importance of high extracellular levels of these enzymes to enable the efficient degradation of recalcitrant compounds under in vivo conditions relates to the sorption and complexation of enzymes in soil and the probable loss of their activity once externalized.

The lignin degradation system consists on peroxidases, H_2O_2-producing enzymes, veratryl alcohol (3,4-dimethoxybenzyl alcohol), oxalate, and manganese. LiP and MnP are glycosylated heme proteins that couple the reduction of H_2O_2 to water with the oxidation of a variety of substrates [36]. It was reported that *Phanerochaete chrysosporium*, producing simultaneously both MnP and LiP, was able to degrade many xenobiotics and recalcitrant compounds [26].

Laccases, which are also extracellular enzymes and being blue multicopper oxidases, catalyze the monoelectronic oxidation of a large spectrum of substrates, including phenolic and nonphenolic compounds as well as recalcitrant environmental pollutants [6, 11, 12, 22]. This explains their potential use for xenobiotic degradation, and bioremediation purposes.

2. Origin, threat, and biodegradation of xenobiotics; enzymatic system involved in xenobiotic biodegradation

While a huge amount of hazardous organopollutants is produced annually over the world, only 10% of these are disposed of safely. The most hazardous compounds are persistent in the environment and are known to have carcinogenic and/or mutanogenic effects. The prime source of xenobiotics is wastewater, landfill leachates [33], and solid residual releases from the industries [37]. Solid wastes may contain volatile organic compounds as residues or incorpo-

rated into the structure of materials such as plastic foams, packaging, floor and wall coverings, solvents, paints, and adhesives [38].

Wastewaters, including domestic and industrial wastewaters, contain a variety of compounds. Some of the common compounds present in wastewaters and in other effluents are phenolic compounds, hydrocarbons, dyes, endocrine disrupting compounds, and pesticides [37].

Landfills generate large amounts of leachates containing high levels of organics and ammonia nitrogen [32, 39]. These substances with others like phenols and hydrocarbons can be a major source of contamination of the groundwater. Indeed, the variety of contaminants in landfill leachates, their synergistic and antagonistic effects as well as their physicochemical properties make them serious toxicants, which may survive different treatments [32]. Landfill leachates exhibit consequently high toxicity levels [32, 33, 40, 41]. The efficiency of fungal remediation of landfill leachates has been proved on *Trametes trogii*, *Lentinus tigrinus*, and *P. chrysosporium* [32]. The strains were able, via their extracellular enzymes, to reduce organics (chemical oxygen demand (COD), phenols, and hydrocarbons) as well as toxicity, for twofolds diluted LFL. However, raw LFL caused growth inhibition and enzyme secretion reduction, indicating the sensitivity of these strains to high levels of toxic compounds such as phthalates and phenol derivatives [42]. Tigini et al. reported that autochthonous and allochthonous fungal strains were efficient in LFL treatment, showing a complete spectrum of action and being able to significantly reduce the wastewater toxicity for all the tested strains. Thus, *Porostereum spadiceum* showed the best activity with 40 % of decolorization within 1 week [33].

Solid waste residues can be domestic wastes, including food, paper, and garden wastes; waste from council activities associated with servicing residential areas: street sweepings, tree lopping, parks and gardens, and litter bins; and waste from institutional, commercial, industrial activities, generally containing higher proportions of metals and plastics than domestic wastes. They also can be derived from demolition and building activities, which contain high proportions of inert material (concrete, bricks) and low proportions of other materials. Many xenobiotic compounds are released from municipal solid waste and may be found in the leachates and the gaseous phase of landfills [43]. They include 1,1-dichloroethylene, 2,4,6-trichlorophenol, dimethyl phthalate, phenol, benzoate, and phthalic acid [44].

Phenols and phenolic compounds are widely distributed compounds in the nature, especially in the plants, but also in marine systems, produced by marine plants and animals where they can be degraded by indigenous microbial population [45]. Several types of industries, such as coal refineries, phenol manufacturing, pharmaceuticals, dying, petrochemical, pulp mill as well as agricultural wastes, contain phenols which are considered among the most prevalent pollutants due to their high toxicity even at low concentrations [37, 46, 47]. Phenol is also employed in the production of resins and also used in the manufacture of plastic, biocides, disinfectants, textiles, medicines, explosives, pinks, perfumes, and photographic materials [48]. Consequently, phenols have negative effects on the ozone layer and on the earth heat [47]. Phenol, being a carcinogenic compound, must be removed from industrial wastewaters prior to their discharge, via biodegradation processes resulting in minimum secondary metabolites and harmless end products [49]. Several studies have shown that phenol can be degraded by

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a wide variety of fungi including *P. chrysosporium*, *Trametes versicolor*, *Trametes villosa*, and *Lentinus eodes* [25, 26, 50].

Furthermore, chlorinated phenols are one of the most serious environmental pollutants. Lignin-degrading fungi and their enzymes have been used to detoxify these compounds through their transformation into non-toxic or less toxic substances [51, 52]. Ehlers and Rose found immobilized WRF cultures to be effective in removing phenolic and chlorinated phenolic pollutants [52]. Leontievsky et al. reported that *Panus tigrinus* and *Coriolus versicolor* and their ligninolytic enzyme systems efficiently transform 2,4,6-trichlorophenol (TCP) to 2,6-dichloro-1,4-hydroquinol and 2,6-dichloro-1,4-benzoquinone [51]. However, MnP and laccase differed in their specificity: in *P. tigrinus* culture, primarily the MnP attacked 2,4,6-TCP, whereas in *C. versicolor* culture, predominantly laccase catalyzed the transformation. Besides, *P. chrysosporium* has been the most extensively studied among the ligninolytic fungi, as a model system, and the pathways for degradation of 2,4-di, 2,4,5-, and 2,4,6-trichlorophenols were investigated [26, 53].

Plastics are known to be hazardous materials due to the nature of components that are made of and including polystyrene, polyvinyl chloride, polyethylene, and its derivatives. They are very slowly degraded due to the molecular bonds and interactions. Biodegradation of plastics gained importance in the last few years, but the fragmented compounds released by this biodegradation also lead to other with environmental issues [37]. Cameron et al. reported that *P. chrysosporium* was able to degrade plastics like nylon [26].

Polycyclic aromatic hydrocarbons and saturated hydrocarbons are usually found in petroleum effluents at high concentrations and cause an environmental pollution. Because physical-chemical degradation of such compounds is cost-effective and may lead to further disturbances in the environment, biological treatments offer the alternative to reduce the impact of these pollutants [37]. Hence, bioremediation had a great potential as an alternative method for the rehabilitation of contaminated sites. The use of natural microorganisms, isolated for their ability to degrade a large variety of hydrocarbons [11–14], allows the elimination of such compounds from contaminated sites [54]. Microorganisms that can degrade hydrocarbons are particularly isolated from petroleum-contaminated sites [55]. Indeed, the microbial action depends on aromatics structure since the aromatic fraction is more difficult to degrade [56]. Olusola and Anslem reported that *Pleurotus pulmonarius* was able to degrade crude oil [14]. Other studies reported the effective fungal bioremediation of hydrocarbons [11–13]. Bioremediation of anthracene and pyrene in soil, using mycelia of *P. chrysosporium*, *T. versicolor*, and *Pleurotus ostreatus* was reported as effective, since MnP and LAC were secreted at high levels in the soil. However, these high enzyme levels allowed a more efficient degradation of recalcitrant compounds in liquid media [1].

Volatile organic compounds and additives, such as emulsifiers and texturizers in paint, can be degraded by different tools such as chemicals (water as solvent), hygroscopic stresses, and microbial sources [37]. Some fungi were reported as effective decomposers of paints. The development of microfungi on the surface of painting induces aesthetical, mechanical, and biochemical decay [57].

Textile effluents are one of the principal sources of pollution over the world. In particular, the release of colored effluents into the environment is undesirable, not only due to their color but also because many synthetic dyes with their complex aromatic molecular structure [58, 59] and their breakdown products are toxic and/or mutagenic [59, 60]. Due to the unspecific nature of their lignin-degrading enzymatic system, fungi can also degrade textile dyes [61]. However, the well understanding of the fungal degradation mechanism involved is essential to identify the degradation products and to verify the toxicity removal, consequently. Until now, much research has been done on dye degradation by fungi and or laccases [62, 63] but few studies have focused on the intermediate products toxicity [64]. Many fungal isolates and their enzymes were reported as efficient for the degradation or the decolorization of many polymeric dyes, including blue dextran and Poly R478 as well as the triphenylmethane dyes: cresol red, crystal violet, and bromophenol blue [19, 20]. Ben Younes et al. reported that laccase from the thermophilic fungal strain *Scytalidium thermophilum* catalyzed the decolorization and the detoxification of the azo dye Congo red and the triarylmethane dyes, commonly found in textile industry effluents [20]. The team also reported, in previous studies, that the crude enzyme as well as the purified laccase from *Perenniporia tephropora* was able to decolorize dyes of the textile industries, including neolane pink, neolane blue, and remazol brilliant blue R (RBBR) [18]. The latter was also efficiently decolorized by laccase from *T. trogii* [17]. The ability of *T. trogii* laccase to decolorize azo and triarylmethane dyes was approved in the absence of redox mediators, since MG and BCG were completely degraded with crude laccase within 6 h of treatment. Toxicity evaluation showed a final product detoxification [21]. On the other hand, the fungal decolorization of RBBR has been reported for other strains such as *Dichomitus squalens*, *Ischnoderma resinosum*, *Pleurotus calyptratus* [65], and *P. ostreatus* [66]. Tekere et al. reported the ability of *Trametes cingulata*, *T. versicolor*, *Datronia concentrica*, and *Pycnoporus sanguineus* to decolorize the Poly R478 [67]. Mohorcic et al. found that *Bjerkandera adusta* was able to decolorize the black-blue dye through violet and red to pale yellow via its extracellular enzyme; the MnP which was also reported for its ability to decolorize amaranth and remazol black B [68]. Previously, Swamy and Ramsay reported since 1999 the ability of *Bjerkandera* sp., *P. chrysosporium*, and *T. versicolor* to decolorize remazol orange, remazol brilliant blue, reactive blue, and tropaeolin O in agar plates [69]. Consequently, some strains including *T. trogii* and *S. thermophilum* were reported to be able to decolorize and detoxify textile effluents [19, 20]. Robinson et al. reported that *B. adusta* and *Phlebia tremellosa* provided a good efficiency to decolorize textile effluent in N-limited conditions [62].

Paper and pulp mills are effluents released from paper mill industries and cause serious environmental pollution because they contain chlorinated organic compounds, which are absorbable organic halides, including pentachlorophenols, tetrachlorocatechols, and tetrachloroguaiacols [70]. They are often released to anaerobic conditions, exhibiting high acute and chronic toxicity levels and mutagenicity and/or carcinogenicity. Fungal enzymes were used for bleaching these effluents to obtain high-quality paper pulps [23, 71]. Indeed, it was reported that the laccase from *Coriolopsis gallica* has been implicated in the decolorization of effluents from the pulp and paper industry [25, 72]. Laccases have also been shown to be applicable for the bioremediation of pulp and paper industry wastes by effecting direct dechlorination [73] for the removal of chorophenols and chlorolignins from bleach effluents

[74, 75]. Other uses of laccases for the pulp and paper industry include reduction of the kappa number of pulp [15] and an improvement in the paper-making properties of pulp [76].

A large variety of pesticides and insecticides, including organophosphorous compounds, and benzimidazoles, are intensively used and may contaminate the land due to their slow degradation [37]. Despite the slow process, microbial degradation is considered as a tool to minimize the negative effects of these compounds on the ecosystem. Many studies reported the effective degradation of pesticides by fungal strains, including *P. chrysosporium* and *T. versicolor*, and involving two different enzyme systems: laccase and peroxidases [26–29].

Pharmaceuticals are discharged directly by pharmaceutical manufacturers or in wastewaters from hospitals. These compounds have performed their biologically intended effect, but their degradation into toxic substances in the body is often a cause for concern [77] since they unfortunately get passed into the environment in either their complete or fragmented forms. These pharmaceuticals, used in personal care products (PCPs) or being endocrine-disrupting chemicals (EDCs), mainly include hormones, anesthetics, and antibiotics, and can be accumulated in an organism and passed on to the other through the common food chain [78]. Even though they are the indirect sources, they cause adverse effect on the ecological cycle [37]. Nonsteroidal anti-inflammatory drugs are also a large and diverse chemical group of drugs used on humans and animals for the treatment of inflammation, pain, and fever [6]. The use of diclofenac in animals has been reported to have led to a sharp decline in the vulture population reaching 99% [6]. These compounds, including nonylphenol (4-nonylphenol), bisphenol A (2,2-bis(4-hydroxyphenol) propane), triclosan (5-chloro-2(2,4-dichlorophenoxy) phenol) and others, are frequently detected in receiving waters downstream of intense urbanization [79, 80]. The latter can mimic or interfere with the action of animal endogenous hormones by acting as estrogen agonists, binding to the estrogen receptor or eliminating a normal biological response [6, 81, 82]. The promise of laccase for the transformation or the elimination of PCPs and EDCs from both aqueous solutions and polluted soils has been recently established [6, 83]. Cabana et al. demonstrated that the resulting chemicals do not have any estrogenic activity [84].

It is known that white-rot fungi can degrade lignin in the way that the mycelia of the organisms penetrate the cell cavity and release ligninolytic enzymes to decompose materials to a white sponge-like mass [85]. The ability of fungi to transform a wide variety of hazardous chemicals has aroused interest in using them in bioremediation [86]. Enzymatic treatment, involving mainly peroxidases and/or laccases, is currently considered as an alternative method for the removal of toxic xenobiotics from the environment [87].

2.1. Peroxidase system

The lignin degradation system consists on peroxidases, H_2O_2-producing enzymes, veratryl alcohol, oxalate, and manganese. All of these enzymes are glycosylated heme proteins that couple the reduction of hydrogen peroxide to water with the oxidation of a variety of substrates. The redox potentials of LiP and MnP are higher than for others peroxidases; that is why they have been shown to oxidize chemicals that are not easy to be oxidized by other microorganisms. These chemicals include Polycyclic aromatic hydrocarbons (PAH), phenol

and its derivatives, cyanide, TNT, and others [26]. This finding was reported for the fungus *P. chrysosporium*, which has been shown to degrade many xenobiotics and recalcitrant compounds, both in soil and in liquid cultures, suggesting the attractive use of such fungus in bioremediation.

Lignin peroxidases (LiPs) belong to the family of oxidoreductases [36, 88] and were firstly described in the basidiomycete *P. chrysosporium* in 1983 [89]. This enzyme has been recorded for several species of white-rot basidiomycetes [90]. LiP is dependent of H_2O_2, with an unusually high redox potential and low optimum pH [91, 92]. This enzyme is able to oxidize a variety of substrates including polymeric ones [93] and has consequently a great potential for application in various industrial treatment processes [92].

Manganese peroxidases (MnPs) belong to the family of oxidoreductases [36]. Following the discovery of LiP in *P. chrysosporium*, MnP secreted from the same fungus was found as another lignin-degrading enzyme [94] and was secreted by almost all white-rot fungi. MnP catalyzes the oxidation of phenolic structures to phenoxyl radicals [9]. The product Mn^{3+}, being highly reactive, complex with chelating organic acids, such as oxalate, lactate, or malonate. On the other hand, it was reported that MnP may oxidize Mn(II) without H_2O_2 and with decomposition of acids, and concomitant production of peroxyl radicals [95].

2.2. Laccase system

Laccases which are blue multicopper oxidases, catalyze the monoelectronic oxidation of a large spectrum of substrates, for example, ortho- and para-diphenols, polyphenols, aminophenols, and aromatic or aliphatic amines, coupled with a full, four electron reduction of O_2 to H_2O. Laccases act on both phenolic and nonphenolic lignin-related compounds as well as highly recalcitrant environmental pollutants, and they can be effectively used in paper and pulp industries, textile industries, xenobiotic degradation, and bioremediation and can act as biosensors. Some studies reported the identification of genes that are differentially regulated during fungal growth in the presence of different environmental pollutants. However, abiotic stress caused by many factors including water potential, temperature, and pH can influence the metabolism of the degradation process. Hence, considering bioremediation in soil, the conditions that favor fungal activity in soil, such as temperature, moisture, nutrient status, pH, and aeration, need to be optimized to promote metabolic degradation of xenobiotics. Magan et al. studied the effect of abiotic factors on the fungal degradation of pesticides by *T. versicolor* and *P. chrysosporium* for soil bioremediation purposes [96]. In fact, the potential property of laccase is its highly non-specific nature of substrates [97]. Furthermore, the common presence of one or more substructures in the lignin molecule and in xenobiotics explains the ability of white-rot fungi to degrade such a wide range of environmental organic pollutants, even at high levels [98, 99]. Otherwise, it has been shown that laccase metabolizes these compounds without any net energy gain [100]. Indeed, the oxidation of lignin is performed to access to wood polysaccharides, being their main energy source [101]. This implies that the presence of lignin-cellulosic substrates is required to ensure the degradation of xenobiotic compounds [102].

3. Conclusion

One of the major environmental problems, causing a serious threaten over the world, is the contamination of atmosphere components with toxic chemicals. Unfortunately, the most xenobiotic compounds, produced annually at huge amounts, are persistent in the environment and have carcinogenic and/or mutanogenic effects. The main sources of xenobiotics are wastewater, landfill leachates, and solid wastes. Xenobiotics include phenols, plastics, hydrocarbons, paints, dyes, pesticides, insecticides, paper and pulp mills, pharmaceuticals, and others.

Biological processes, being eco-friendly and cost cheap techniques, were proposed for xenobiotic degradation to overcome these problems. White-rot fungi, especially the basidiomycetes, are the most tolerant microorganisms to high concentrations of pollutants, giving their exceptional abilities for biodegradation in aqueous environments and soil and have been investigated extensively for their bioremediation capacities. Fungal bioremediation is a promising tool since the metabolic potential of such microorganisms converts most of the environmental pollutants to less hazardous or non-hazardous compounds with less input of energy and time. White-rot fungi are the unique organisms that show the capacities of degrading highly toxic organics and recalcitrant compounds. The key enzymes of their metabolism are extracellular ligninolytic enzymes that enable fungi to tolerate high concentrations of toxic substrates. These enzymes have potential applications in a large number of fields, including the chemical, fuel, food, agricultural, paper, textile, and cosmetic industrial sectors. Their capacities to remove xenobiotic substances and to produce others, which are less or non-toxic, make them a useful tool for bioremediation purposes.

The potential of white-rot fungi can be harnessed thanks to emerging knowledge of the physiology and morphology of these microorganisms. This knowledge could be transformed into reliable and robust waste treatment processes. The importance of high extracellular levels of these enzymes to enable the efficient degradation of xenobiotic compounds under in vivo conditions relates to the sorption and complexation of enzymes in soil and the probable loss of much of their activity once externalized.

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Chapter 8

Novel Microbial System Developed from Low-Level Radioactive Waste Treatment Plant for Environmental Sustenance

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Additional information is available at the end of the chapter

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Abstract

A packed bed bioreactor efficiently treated low-level radioactive waste for years with a retention time of 24 h using acetate as the sole carbon source. However, there was generation of dead biomass. This bioreactor biomass was used to develop a bacterial consortium, which could perform the function within 4 h while simultaneously accumulating nitrate and phosphate. The dead mass was negligible. Serial dilution technique was used to isolate the world's first pure culture of a nitrate accumulating strain from this consortium. This isolate could simultaneously accumulate nitrate and phosphate from solution. Its ability to form biofilm helped develop a packed bed bioreactor system for waste water treatment, which could optimally remove 94.46% nitrate within 11 h in batch mode while 8 h in continuous mode from waste water starting from 275 ppm of nitrate. The conventional approach revealed the strain to be a member of genus *Bacillus* but showed distinct differences with the type strains. Further insilico analysis of the draft genome and the putative protein sequences using the bioinformatics tools revealed the strain to be a novel variant of genus *Bacillus*. The sequestered nitrate and phosphate within the cell were visualized through electron microscopy and explained the reason behind the ability of the isolate to accumulate 1.12

mg of phosphate and 1.3 gm of nitrate per gram of wet weight. Transcriptome analysis proposed the mechanism behind the accumulation of nitrate and phosphate in case of this novel bacterial isolate (MCC 0008). The strain with the sequestered nutrients work as biofertilizer for yield enhancement in case of mung bean while maintaining soil fertility post-cultivation.

Keywords: nitrate accumulation, packed bed biofilm reactor, *Bacillus* sp MCC 0008, insilico analysis, transcriptome analysis, radioactive effluent

1. Introduction

All ore mining produce waste rock that in turn may produce acid mine drainage (AMD), due to the presence of sulfides. The waste generated is treated by physico-chemical means and is either stored in engineered containments or in open surface based on the nature of the effluent. Only limited information is available about effects of microbial processes used for similar purposes during large-scale operation. In addition, the mining itself and processing are often associated with a wide range of potential human health risks. Surface and underground mining generate a large volume of waste rock, which may contain only very little uranium but has fission products, for example, radium (radioactive) or lead (highly toxic) that is left behind as a waste. The second step is a process, known as the milling of the ore in which the rocks are crushed and ground. Chemical leaching follows and over 50% of uranium ore is obtained with classic mining methods. Water used in this process that cannot be recycled within a processing plant as well as excess water from a mine needs to be removed or treated to meet environmental requirements. The multistep process of recovery includes neutralization of the effluents, precipitating any metals, and reducing the uranium and radium content. [1–3]. This treatment depends upon the uranium recovery process, chemicals used, and contaminant ores. Water recovered may get recharged as groundwater or is either discharged or used for plant operations. Often this water needs further treatment before it could be reused or discharged for removal of contaminants. The multistep process begin with coagulating or precipitating heavy metals followed by neutralizing acids, or adjusting pH and then precipitating radium with barium chloride. The water treatment process is often followed by additional "clarification" or "polishing" steps using clarifiers, sand filters, and even reverse osmosis. The alternative option might be to use microbial bioremediation using sulfate-reducing bacteria [4].

The foremost source of waste generation occurs during nuclear fuel cycle operations that comprises of facilities to purify, convert, and enrich uranium from mining and milling and to manufacture fuel elements for nuclear reactor and gives rise to a variety of materials and product outputs [2, 3]. Enrichment of radioactive ore involves use of chemicals which lead to high levels of nitrate in the effluent.

The effluent generated cannot be discharged into the environment without treatment. The physicochemical treatment is expensive and economically not feasible during large-scale operation. Hence, biological options were sought. The problem in hand was to develop a microbial process, which could efficiently treat low-level radioactive waste containing nitrate

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generated from ore enrichment. Nitrate being a common pollutant in municipal as well as agricultural waste water, municipal sewage was passed through corrugated sheets of a packed bed reactor to develop a biofilm-based bioreactor that could treat low-level radioactive effluent within 24 h on a continuous basis [5] using acetate as the sole carbon source. However, dead mass was generated during the operation. The biomass was characterized [5] and further enrichment in nitrate broth (HiMedia M439) resulted in isolation of the fastest nitrate removing consortium. This consortium was further characterized to yield the world's first nitrate accumulating pure culture [11] of a *Bacillus* sp. with immense application in terms of waste water treatment, plant growth promotion with seed quality enhancing ability. A combined approach of insilico and conventional analysis revealed the strain to be a novel species of genus *Bacillus*. In this chapter, we present the characterization of this novel isolate involved in bioremediation of soluble nitrate.

2. Consortium development and characterization

Nitrate removal from the medium by the bacteria was the primary step for selecting a consortium for nitrate removal. Either an assimilatory or a dissimilatory pathway results in nitrate removal from solution [6]. An alternative pathway for the nitrate removal was through nitrate accumulation, as evident in isolates from genus *Beggiatoa*, *Thiomargarita*, and *Thioploca*. Mussmann et al. [7] proposed a vacuolar nitrate accumulation mechanism linked to proton translocation in *Beggiatoa* sp. from marine origin. The bioreactor biomass treating low-level radioactive waste was selected as inoculum because it solely treated nitrate as a pollutant and would thus have stronger nitrate reducers/accumulators due to the constant exposure to nitrates in radioactive waste water. The consortium (BN7) developed in nitrate broth under aerobic condition reduced the nitrate within the range of 25–37°C temperature and pH 6–11. The consortium could form a biofilm with an optical density of 0.34. Conventionally, an optical density from 0.2 to 0.35 at 620 nm indicates a structured biofilm formation [8]. The biofilm formation was found to be strengthened (0.64) upon application of phytochemicals from

Figure 1. Picture of the biofilm-based packed bed bioreactor developed using the aerobic consortium BN7.

Mentha spicata. These results were validated by calculating the *t* value (25.23) for 19 df with a 95% confidence level using a two-sample one-tailed *t*-test with equal variance to yield a *p* value of 2.24025 E-16. The consortium was used to develop a packed bed biofilm bioreactor (**Figure 1**) for nitrate and phosphate removal in a continuous system.

The inoculum standardization indicated 10% of the parent culture as optimum for biofilm development. This consortium reduced 97.44% nitrate from the medium within 4 h (**Figure 2**) while simultaneously reducing 48.2% phosphate during incubation in a biofilm-based bioreactor. This consortium could reduce 500 ppm and 1000 ppm nitrate load within 7 and 5 h, respectively. Nitrate concentrations between 1500 and 4000 ppm could be reduced by 99% within 4 h (**Figure 2**), and 5000 ppm nitrate was reduced by 80.5% after treating for 11–12 h and 99.62% after 24 h. The correlation coefficient was −0.53173, which signifies no direct correlation between the initial nitrate load and bioreactor reduction for the range tested in this study. The above data show the aerobic consortium (BN7) to perform the fastest nitrate removal by a microbial system to the best of our knowledge.

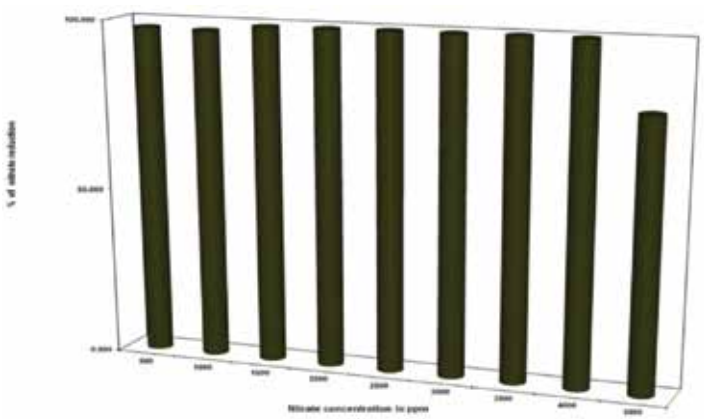

Figure 2. Graph representing the ability of the aerobic consortium BN7 in the packed bed bioreactor (depicted in **Figure 3**) to reduce nitrate from the medium within 4 h of incubation at room temperature with different initial nitrate load.

On further analysis, this consortium was found to accumulate both nitrate and phosphate simultaneously (2.84 gm/gm wet weight for nitrate and 1.14 mg/gm wet weight for phosphate). Cd, Sr, and Ce inhibited the bacterial growth even at a concentration of 0.1 mM, whereas Co and Zn were inhibitory at 0.5 mM. For Cu, Fe, and Zn salts, lower concentrations had minimal impact on the nitrate reduction, and the reduction efficiency in the presence of Pb salts was at par with the control set. After 4 h of growth, 0.5 mM of Pb salts decreased the reduction efficiency by only 3%. Moreover, the nitrate reduction in the presence of Cu salts after 2 h was higher than for the control (37% in Cu-treated cells compared to 8.5% in control), which can be attributed to the presence of *nirK*, a Cu-dependent nitrate reductase gene. The two-sample one-tailed paired *t*-test for means was 21.73 for 2 df and at 95% confidence level; the corresponding *p* value was 0.001. Therefore, the nitrate reduction enhancement in the presence of

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Cu was significant. However, the extent of this reduction decreased with increasing time due to the toxic effect of the metal on the microbes. For metals such as Fe, 0.1 mM and 0.5 mM inhibited the reduction by 4–6%, and similarly, 0.1 mM of Zn reduced the efficiency by 3–5%. The negative impact of metals on the reduction efficiency was significant for Co and Cr salts. For the Co treatment, the reduction after 4 h of growth dropped by 50% and 73% relative to the untreated cells for concentrations of 0.1 and 0.5 mM, respectively. Under similar growth conditions, decreasing the Cr salt concentration decreased the nitrate reduction by 30% while increasing the concentration decreased the reduction efficiency by 46% relative to the control cells. The Energy Dispersive X ray Fluorescence (EDXRF) analysis confirmed the metal accumulation in the biomass with the highest accumulation being for Pb (1200 ppb) followed by Cu (180 ppb), Cr (100 ppb), and Co (15 ppb). A single-factor ANOVA yielded a p value of 1.58 E–05 with an F of 13.90 and critical F of 2.70 for 22 df at a 95% confidence level. Hence, the difference in accumulation upon varied metal treatment was significant for BN7. A consortium capable of growing and accumulating such metals can be used for the bioremediation of nitrate and metal co-contaminants.

Preservation experiment revealed both subculture maintenance and glycerol stock storage at –80°C (two months storage) to be equally efficient with nitrate reduction efficiency of 94% and 92%, respectively, after 12 h of growth for BN7. Preserving the culture as a streak plate or stab reduced the efficiency to approximately 88%. The lyophilized form was less efficient relative to the other three storage methods. Thus, using a glycerol stock could be an efficient strategy for the long-term maintenance of the microbial consortium. At the molecular level, the BN7 harbored members which closely resembled *Pseudomonas* sp. (20%), *Azoarcus* sp. (31%), uncultured bacterium (46%) and *Bacillus* sp. (3%). The GenBank accession numbers were GU644465 to GU644489. A phylogenetic analysis was performed using the neighbor joining method (**Figure 3**) as stated above. The low Shannon diversity index value (0.39) confirms selective enrichment using a specific medium for nitrate reducers. An equitability index value (0.83) near 1 indicates that the different varieties observed were evenly distributed throughout the community. The genus *Pseudomonas* and *Bacillus* could be involved in the phosphate accumulation and nitrate reduction. Hence, a microbial consortium was developed which was acclimatized to low-level radioactive waste and could remove nitrate from it within 4 h of incubation at room temperature while generating little dead mass.

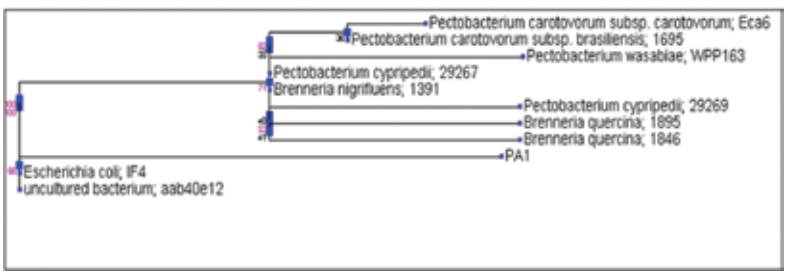

Figure 3. Phylogenetic tree depicting the position of one of the clones from BN7 constructed using the neighbor joining method.

3. Purification of nitrate accumulator and its characterization

Nitrate removal by denitrification and assimilation is well documented for bacterial species. Nitrate accumulation by bacterial genus *Beggiatoa*, Thioploca, and Thiomargarita [9, 10] is relatively a rare phenomenon. Moreover, all reports of such accumulation are in a mixed form or from environmental mixed samples [9, 10]. Before this study, no pure culture of a nitrate accumulator was reported. Serial dilution and streaking on nitrate agar plates were used to isolate the only pure culture of *Bacillus* sp. MCC0008 [11]. Among the pure strains isolated, MCC0008 was found to be a Gram-positive *Bacillus* (**Figure 4a**). Fatty Acid Methyl Ester (FAME) (0.733) as well as Phospholipid-Derived Fatty Acid (PLFA) analysis revealed similarity with *Bacillus cereus*. MCC0008 shows terminal endospore formation like *Bacillus subtilis* and unlike *Bacillus cereus* (which shows central endospore). Paraspore is absent, while the size is 1.85 μm by 0.899 μm. It has a generation time of 21.4 min and shows terminal endospore location. **Table 1** shows the characteristics of the isolate.

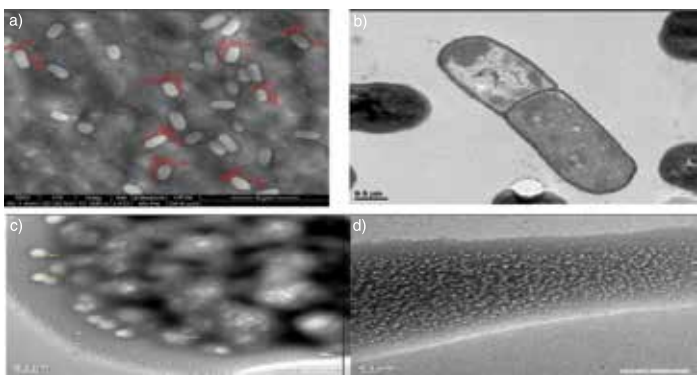

Figure 4a. (a) ESEM micrograph taken using ESEM, FEI QUANTA 200 MARK 2. **(b)** TEM micrograph taken using TEM, 120 kV, 5000× magnification. **(c)** Phosphate granules of 0.13–0.59 μm in the periphery of cells when grown in low phosphate concentration. **(d)** Phosphate accumulation throughout the cell when grown at high phosphate concentration.

Enzyme production	Catalase, oxidase, protease, amylase, lipase, DNAse positive, lecithinase negative
Carbohydrate utilization	It utilizes dextrose, trehalose, esculin, glycerol, maltose
Plant growth promotion traits	Phosphatase and ammonia production positive, indole acetic acid, hydroxymate siderophore and hydrogen cyanide production negative
Antibiotic sensitivity	Sensitive to ciprofloxacin, norfloxacin, cephadroxil, neomycin, gentamycin, doxycycline hydrochloride
	Resistant to metronidazole, rifampicin, ampicilin, trimethoprim, roxythromycin, cloxacin, ceftazidime

Table 1. Characteristics of *Bacillus* sp. MCC0008.

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The transmission electron micrographs clearly revealed the presence of vacuoles (**Figure 4b**) which has earlier been reported for nitrate accumulators. This indicates the possibility that the isolate is a nitrate accumulator. The nitrate accumulation study following sonication-based lysis of the harvested pellet and measurement of released nitrate from the intracellular cell free supernatant as per the method of Cataldo et al. [12] exhibited nitrate accumulation of up to 1278.66–1302.122 ppm/gm (0.021 M) of wet weight. It is less than the extent of accumulation reported for *Beggiatoa* but is the first pure isolate of a nitrate accumulator and also the first *Bacillus* reported to perform such function. The isolate accumulated 1115.25 μg/gm of wet weight of phosphate. The extent of phosphate accumulation was higher than that reported by type strain of *Acinetobacter baumanii*. The reason behind this enhanced efficiency was revealed by Transmission electron microscopy of whole cells which showed through and through accumulation of polyphosphate granules in this strain when grown in nitrate broth overnight with high phosphate concentration (**Figure 4c** and **d**).

The strain showed polysaccharide formation starting from the fourth hour that continued till the eighth hour. This property might provide the benefit of attachment to suitable surfaces to the strain. Active log-phase culture was used to determine whether the isolate could form biofilm according to the method of Martin et al. [8].

Different percentages (1%, 2%, 4%, 6%, 8%, 10%, 15%) of actively growing culture were inoculated in nitrate broth into small falcon containing identical number of plastic rachig rings. The performance in terms of nitrate and phosphate removal was checked for repeated recharges with sterile nitrate broth. The isolate showed good biofilm formation with 10% inoculum being the optimum. The biofilm formation showed saturation by eleventh hour. Optimum performance in terms of nitrate reduction was also observed in the eleventh hour (**Figure 5**). This optimization was further utilized for immobilization of the isolate in the reactor.

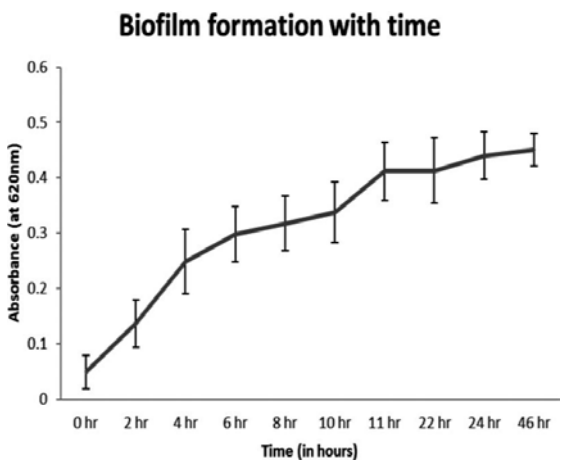

Figure 5. Extent of biofilm formation with time. The saturation was observed after 11 h of incubation.

Accompanied by this, the isolate's ability for active biofilm formation was checked by assaying the supernatant in the tissue culture plate for nitrate and phosphate removal. By this, the time needed for biofilm formation along with optimal functioning in terms of nitrate and phosphate removal was determined to be 11 h for nitrate and 22 h for phosphate (**Figure 6**).

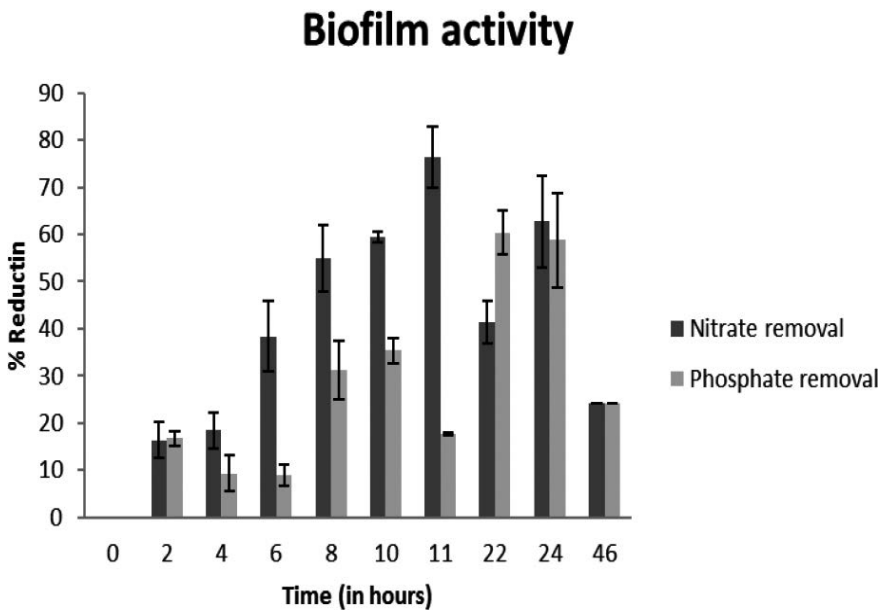

Figure 6. Optimization of time of incubation for biofilm performance.

Since the isolate grows as biofilm, it could be used for setting up of a biofilm-based bioreactor for continuous waste water treatment in terms of nitrate removal. However, a prerequisite for it was to design the minimal growth condition for the same. This would ensure that enrichment culture components would not be needed to run the process and in turn the influent would not add to the COD load of the effluent. Dextrose, glycerol, and citric acid were chosen to check the growth of MCC0008 in minimal condition. The isolate showed the best growth in glycerol, and hence, it was further utilized as the carbon source to determine the optimum percentage of carbon source for growth as well as performance. One percentage of glycerol showed the optimum growth as well as nitrate and phosphate removal under minimal condition. Hence, 1% of glycerol was standardized as the carbon source for the isolate for further studies in packed bed bioreactor.

The comparison of the isolate's activity under different oxygen availability in the eighth hour after inoculation revealed that the isolate performed optimally in aerobic condition followed by anaerobic condition. Oxygen depletion in anaerobic state resulted in a decrease in activity. Highest amount of nitrate reduction and subsequent conversion to ammonia was also in aerobic state due to the assimilatory pathway. Substantial accumulation also occurred in

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aerobic state so that the accumulated nitrate could be used as terminal electron acceptor in oxygen-depleted state.

In the 5 L suspended bioreactor, the strain grew exponentially up to 5 h with 65% denitrification and phosphate removal taking place within the fourth hour (**Figure 7**).

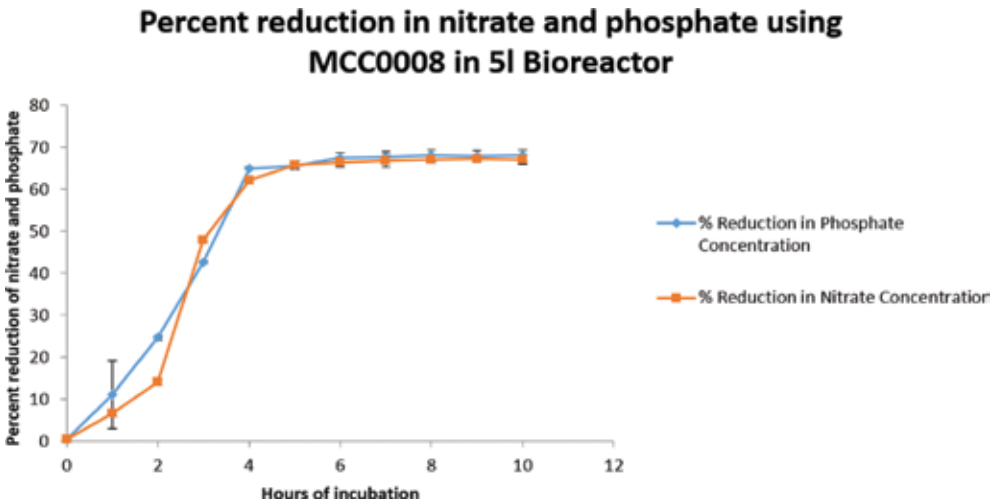

Figure 7. Percent reduction in nitrate and phosphate concentration with time using MCC0008 in 5 L suspended bioreactor.

4. Immobilization and acclimatization in a packed bed bioreactor

Fixed packed bed configuration has high surface area to volume ratio, thereby increasing the microbial density and improving the conditions necessary for nutrient removal. Biofilm-based reactors also have the advantage over other types of bioreactors with respect to ease of operation, high-density accumulation of microbe, resistance of the system to environmental stress [13] and do not require any additional measure to retain biomass in culture [14]. Rotating biological contractors (RBC), trickling filters and biofilm membrane bioreactor are some of the widely used biofilm-based bioreactor. Thus, in order to make the system more cost-effective along with better nutrient sequestration rate, the abilities of the isolate were further exploited. In order to exploit these biofilm forming, nitrate, and phosphate sequestration abilities, a reactor packed with suitable matrix with a fixed bed was developed. The bioreactor was designed of glass with steel mesh as immobilization matrix (**Figure 8**). The isolate could bind equally well to steel and plastic. The total capacity of the bioreactor was 9 L with a working volume of 5 L post-filling up with steel matrix up to sixty percent capacity. The steel mesh acted as the matrix for the formation of MCC0008 biofilm. Ports were designed at different heights of the bioreactor as shown in the **Figure 8**.

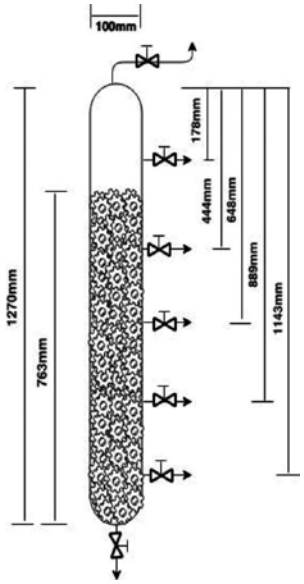

Figure 8. Schematic diagram of 9 L packed bed bioreactor.

The graphical representation shows the initial acclimatization period for proper biofilm development. The initial rise and fall in the performance correlate well with the biofilm character of slough off and growth to achieve stability. It required about 30 loadings to attain stability. After the 39th loading, approximately full nitrate reduction was obtained in one hour only in nitrate broth which was retained for more than 90 days (**Figure 9a**).

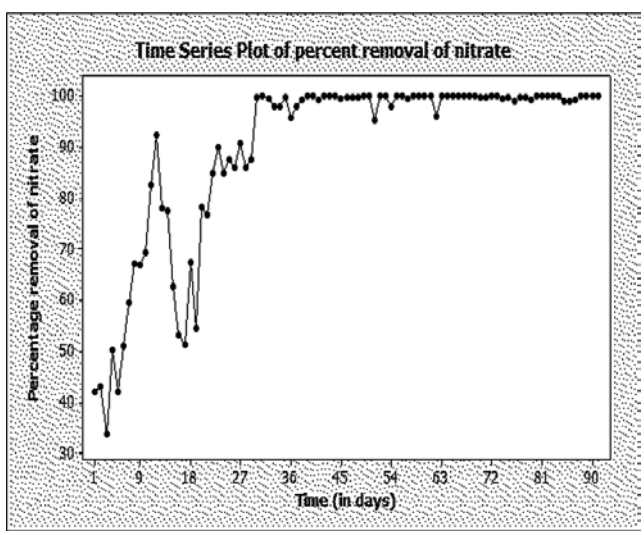

Figure 9a. Performance in terms of nitrate removal plotted as a time series.

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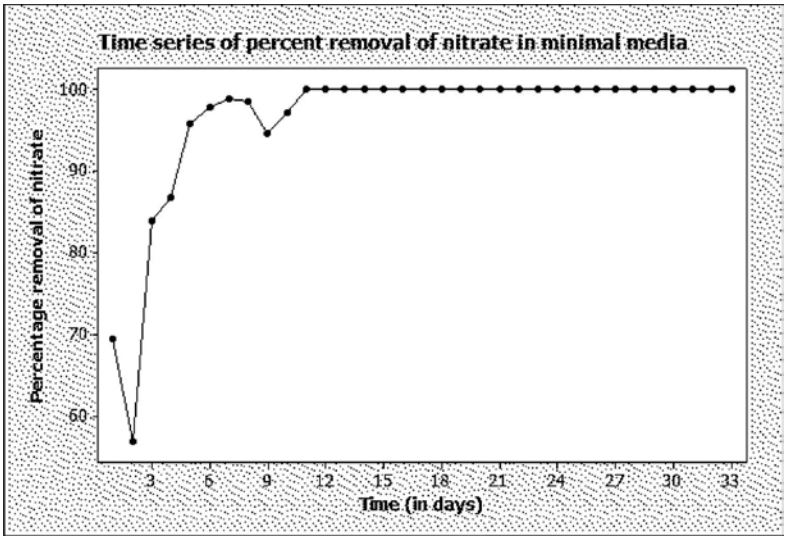

Figure 9b. Performance in terms of nitrate removal in minimal medium with time.

After stable performance of the bioreactor in enriched media, next the performance of the reactor was monitored in minimal media (**Figure 9b**). This was done in order to acclimatize the reactor to minimal conditions before exposure to waste water. It contained 495ppm nitrate and 1% glycerol.

The biofilm was observed to be dense with thick layer of polysaccharide during environmental scanning electron microscopy (**Figure 10**).

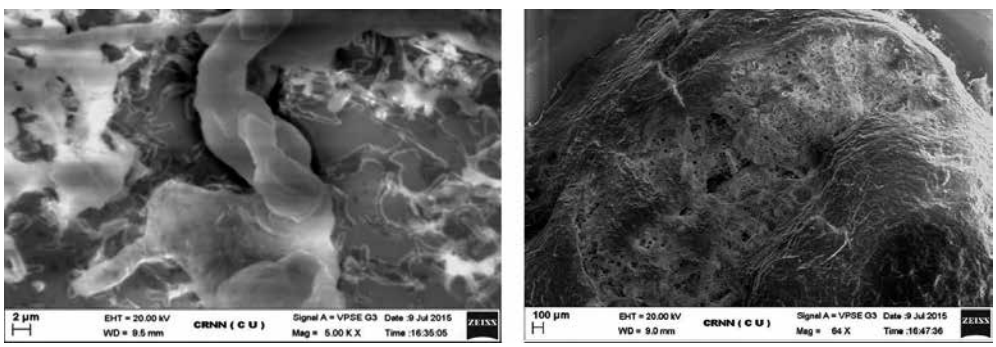

Figure 10. Environmental scanning electron micrograph taken using Zeiss EVO-MA 10 of the biofilm on the inert matrix of a packed bed bioreactor.

Post-acclimatization of the biofilm to minimal media, non-radioactive wastewater was charged. The dynamics of nitrate removal in batch mode is reflected in **Figure 11**. Since the isolate is from a consortium acclimatized to radioactive waste water, it is expected to show similar performance with low-level radioactive waste.

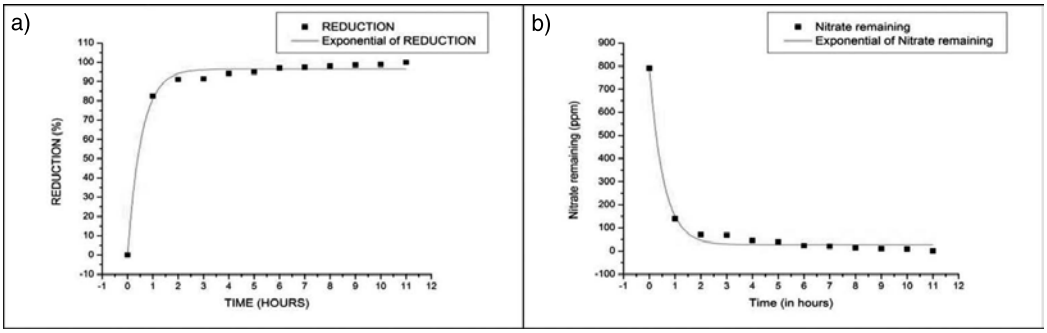

Figure 11. Kinetics of nitrate removal from waste water in batch mode. (a) Nitrate reduction kinetics following nonlinear curve fit (exponential). (b) Kinetics of remaining nitrate in the medium from 0 h (time of charging).

The equation, statistics, summary, and ANOVA for nitrate reduction kinetics (depicted in **Figure 11a**) are as follows:

$$y = y_0 + A * e^{(R0*x)}$$

where y = % Reduction, y_0 = initial nitrate concentration, x = time (in hours).

Statistics:

	Reduction
Number of points	12
Degrees of freedom	9
Reduced Chi squarer	7.39081
Residual sum of squares	66.51727
Adj. *R*-square	0.99046
Fit status	Succeeded(100)

The equation, statistics, summary, and ANOVA for remaining nitrate in the medium with time (depicted in **Figure 11b**) are as follows:

$$y = y_0 + A * e^{(R0*x)}$$

where y = Remaining nitrate in the medium, y_0 = initial nitrate concentration, x = time (in hours).

Complete nitrate removal from wastewater took place in 11 h. The longer retention time for waste water treatment as compared to that in minimal media in terms of nitrate removal may

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be due to the presence of other contaminants to which the biofilm is sensitive. Multivariate analysis using response surface methodology revealed higher nitrate removal at higher initial concentration of nitrate with little effect of the flow rate (within the range tested) on the system performance (**Figure 12**).

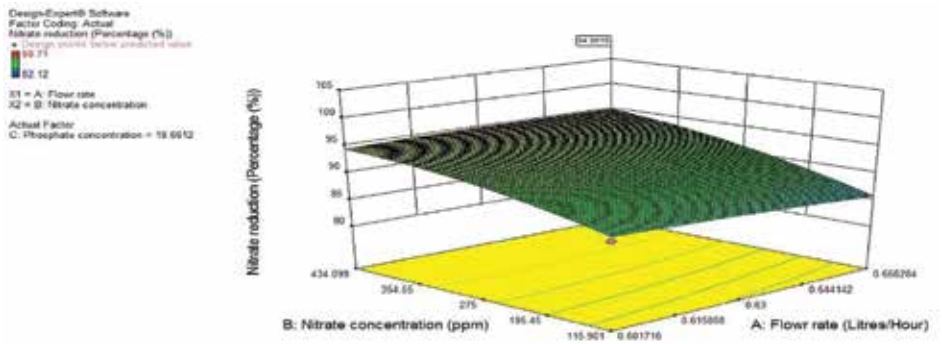

Figure 12. The figure shows the response of nitrate concentration and flow rate on nitrate reduction in the bioreactor.

Response	**1**			**Nitrate reduction**		
ANOVA for response surface quadratic model						
Analysis of variance table [Partial sum of squares—Type III]						
Source	**Sum of Squares**	**df**	**Mean Square**	**F Value**	**p-value Prob > F**	
Model	279.17	9	31.02	17.36	0.0029	Significant
A-Flow rate	14.96	1	14.96	8.37	0.0340	
B-Nitrate concentration	76.14	1	76.14	42.61	0.0013	
C-Phosphate concentration	1.25	1	1.25	0.70	0.4414	
AB	0.93	1	0.93	0.52	0.5034	
AC	4.56	1	4.56	2.55	0.1711	
BC	0.39	1	0.39	0.22	0.6586	
A2	0.42	1	0.42	0.24	0.6477	
B2	12.37	1	12.37	6.92	0.0465	
C2	14.61	1	14.61	8.18	0.0354	
Residual	8.93	5	1.79			
Lack of fit	7.84	1	7.84	28.59	0.0059	Significant
Pure error	1.10	4	0.27			
Cor total	288.11	14				

Std. dev.	1.34	R-squared	0.9690
Mean	92.69	Adj R-squared	0.9132
C.V. %	1.44	Pred R-squared	−1.9466
PRESS	848.93	Adeq precision	15.118

	Coefficient		Standard	95% CI	95% CI	
Factor	**Estimate**	**df**	**Error**	**Low**	**High**	**VIF**
Intercept	94.22	1	0.58	92.74	95.70	
A-Flow rate	−1.93	1	0.67	−3.65	−0.22	2.00
B-Nitrate concentration	4.36	1	0.67	2.64	6.08	2.00
C-Phosphate concentration	0.56	1	0.67	−1.16	2.28	2.00
AB	0.68	1	0.95	−1.75	3.11	2.00
AC	−1.51	1	0.95	−3.94	0.92	2.00
BC	0.44	1	0.95	−1.99	2.87	2.00
A^2	−0.23	1	0.48	−1.47	1.00	1.00
B^2	−1.27	1	0.48	−2.50	−0.029	1.00
C^2	−1.38	1	0.48	−2.61	−0.14	1.00

The final equations obtained through RSM-based optimization are as follows:

$$\text{Nitrate reduction} = -81.1 + 424.3 * \text{Flow rate} - 0.049 * \text{Nitrate concentration} + 4.74 * \text{phosphate concentration} + 0.15 * \text{Flow rate} * \text{Nitrate concentration} - 6.04 * \text{Flow rate} * \text{Phosphate concentration} + 3.13e^{-004} * \text{Nitrate concentration} * \text{Phosphate concentration} - 292.25 * \text{Flow rate}^2 - 5.002e^{-005} * \text{Nitrate concentration}^2 - 0.018 * \text{Phosphate concentration}^2$$

The packed bed bioreactor system could treat waste water optimally removing 94.46% nitrate within 11 h in batch mode while 8 h in continuous mode from waste water containing 275 ppm of nitrate at 0.63 L/h flow rate.

5. Application as biofertilizer

Singh et al. [15] conducted experiments using *Advenella species* (PB-05, PB-06, and PB-10) and *Cellulosimicrobium* sp. PB-09 to analyze the IAA production, HCN production, ammonia production, and phosphate solubilization and correlated the results to the isolates' capability to promote plant growth. For them the isolates positively affected all characteristics except HCN production [15]. Since the isolate MCC0008 could accumulate both nitrate and phosphate simultaneously and also produce phosphatase, its effect on plant growth promotion was checked in case of mung bean (Vigna radiate var Samrat). **Table 2** representing the germination

percentage, germination index, and vigor index for Mung bean (*Vigna radiata)* seeds with and without treatment with isolate (soil and seed application) revealed better germination upon soil application. It was expected since the isolate produces plant growth hormones.

Sample	Control	MCC0008 (coated)	MCC0008 (soil)
Germination percentage	74.074	83.333	87.037
Germination index	39.772 ± 9.39	62.298 ± 12.234	75.313 ± 9.44
Vigor index	1639.056	2390.688	2006.801

Table 2. The table shows the germination parameters in case of mung bean upon application of MCC0008.

Soil application gave better result, and so further experiments were conducted by sowing soaked seeds, followed by soil application of the isolate. The germination in the presence of antifungal agent (Saaf) was better upon application of the isolate to soil.

		Changes in %
Elements	**MCC0008**	**Chemical**
Zn	16.04	−7.99
Fe	2.84	−7.20
Mn	14.49	7.08
Cu	25.41	8.97
P	12.82	−66.60
K	4.39	−19.16
S	12.57	−26.24
Ca	5.59	−12.59

The control was taken as reference and that for biofertilizer and chemical fertilizer was calculated accordingly.

Table 3. Chance in elemental content of seed grown without fertilizer (control), with chemical fertilizer and with biofertilizer.

Pot trial and field trial were carried out. For field trial, randomized block design with four replicates was carried out. The sowing was done in the north–south orientation. The seeds' post-germination was subjected to thinning such that each 1 m^2 area contained a total of 40 plants (4 rows of 10 plants each). The inoculum for the germination trial was 4.2×10^6 cells per 125 gm soil in a thermo coal glass/germination tray, 1.39×10^7 cells per 8 kgs soil in each pot and 3.68×10^9 cells per 1 m^2 plot for field trial. The yield per hectare of land was calculated for the consortium when compared with control (without fertilizer) and chemical fertilizer application. The yield per hectare for control, MCC0008 application, and chemical fertilizer application was 1277.5 kg, 1974.5 kg, and 1685 kg, respectively. The elemental content improved post-application as compared to control as measured through EDXRF analysis

(**Table 3**). This shows that not only the yield improves as compared to chemical fertilizer but also the elemental content was better as compared to control as well as chemical treatment in MCC0008-treated seeds. The data revealed that upon treatment with MCC0008, there was desirable change in the nutritional quality parameters. There was increase in energy value (4.3%), total carbohydrate (4.5%), total sugar (0%), total dietary fiber (4.5%), protein (4.9%) content while a decrease in moisture content (23.4%), total ash (6.4%), and crude fat (7.5%). The decrease in moisture would ensure better storage of the grains, decrease in ash content means less non-utilizable component, while decrease in fat improves the quality further.

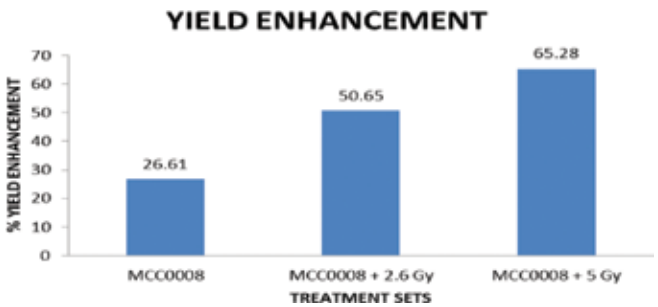

Figure 13. Yield enhancement of mung bean in the presence of biofertilizer with and without gamma irradiation.

According to the previous reports, gamma irradiation of seeds brings about faster germination [16–18]. This is due to increased levels of transcription. The antinutrient as well as elemental levels following irradiation (presowing) is also reported to be lower. Thus, a combined effect of low-dose gamma irradiation of mung bean seeds along with biofertilizer application was tested. The effect of combined application of low-dose gamma irradiation (2.6 Gray and 5 Gray) on germination, yield enhancement, and elemental content of mung bean seeds were tested. The cell structure and viability of the irradiated seeds were studied following ESEM analysis and microtomy using standard techniques. There was mild improvement in germination following irradiation at 5 Gray while significant yield enhancements in irradiated seeds as shown in the **Figure 13**.

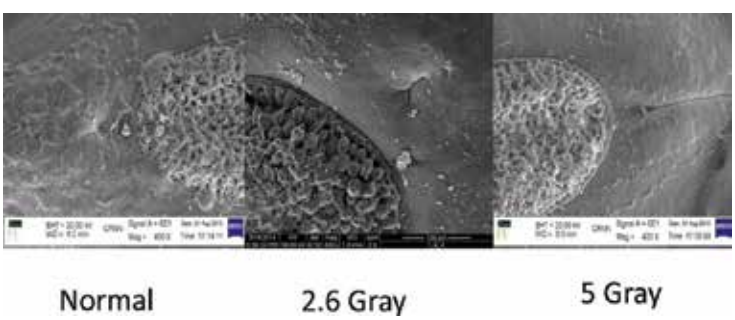

Figure 14a. ESEM image of control and irradiated seeds showing part of the seed coat and hilum.

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In order to explore the reason behind improved germination, detailed analysis of seed structure and hilum morphology was carried out using ESEM as shown in **Figure 14a** and **b**.

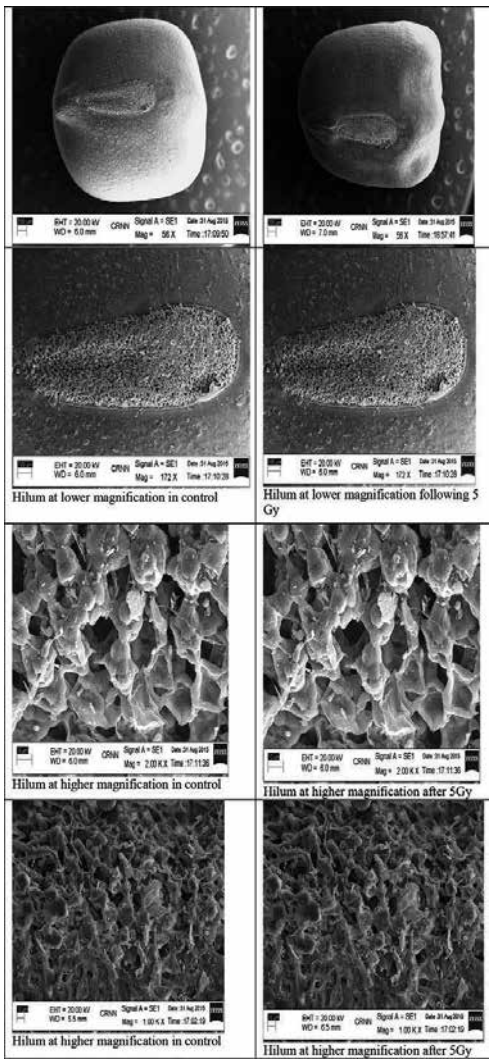

Figure 14b. ESEM analysis seed coat and the hilum of un-irradiated and irradiated seeds.

However, this depth of analysis could reveal just dehydration and nothing beyond that. Dehydration is expected to delay germination, while here we observe faster germination. Hence, there must be some other phenomenon which is induced during irradiation. Since germination is initiated through hilum and it is the point of contact for imbibition of water, further analysis with conventional microtomy was carried out. It revealed loosening of the compact arrangement of protein sheets with starch granules upon irradiation (**Figure 15**). Since irradiation might inactivate the germplasm hence viability staining was carried out for the

same set, it was revealed that the vitality of the seeds was maintained for the irradiated seeds within the range tested. Hence, low-dose gamma irradiation does not destroy the seed but makes the hilum loose to enable better uptake of water and nutrients and hence faster germination and enhanced yield. Further analysis at the transcription level will be required for better understanding of the phenomenon.

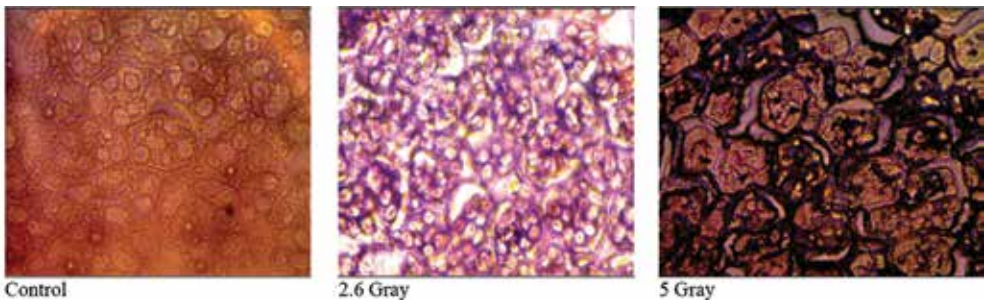

Figure 15. Microtomy images of hilum at 40× magnification of Dewinter Trinacular Microscope (New Crown) showing disintegration of compact protein sheets with irradiation.

The application of this strain as biofertilizer to enhance yield while maintaining nutritional quality of the grain and soil fertility has been filed as patent application in India [19]. To protect the intellectual property associated with this discovery, a PCT has also been filed [19].

6. Bioinformatics-based strain identification

The genus "*Bacillus*" has a long history of importance, both from an economic point of view and as a source of experimental microorganisms. Bacteria of the genus *Acinetobacter* were originally thought to be the major PAOs (polyphosphate accumulating organism). The pure isolate of nitrate accumulating *Bacillus* sp. MCC0008 showed potential for waste water treatment as well as biofertilizer application, hence of immense commercial importance. Knowing the identity of the strain becomes essential for better understanding of the system. This study was undertaken to decipher its species identity as per standard procedure [20] while exploring its underlying phenomenon of nitrate and phosphate accumulation. ANI (Average Nucleotide Identity) was calculated using ANI calculator for this strain with respect to the type strains of *Bacillus cereus, Bacillus thuringiensis*, and *Bacillus anthracis*. The ANI calculator estimates the average nucleotide identity using both best hits (one-way ANI) and reciprocal best hits (two-way ANI) between two genomic datasets [21]. Inter-genomic distances between this strain and its closest neighbors were determined using Version 2.0 of the DSMZ Genome-To-Genome Distance calculator, an *insilico* version of DNA-DNA hybridization [20]. The draft genome of each isolate was compared to the genome sequence of the type strains of *Bacillus cereus, Bacillus thuringiensis*, and *Bacillus anthracis* using dot plot analysis through a genomic similarity search tool, YASS [20, 22], to understand the similarities between the isolates over the length of their genomes. The contigs were uploaded to the Rapid Annotation using

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Subsystems Technology (RAST) server, which is a fully automated service for annotating bacterial and archaeal genomes and provides high-quality annotation for these genomes across the phylogenetic tree. The annotated genomes in the seed viewer depicted the metabolic patterns for the strain and the four reference *Bacillus* strains. The gene arrangements on each chromosomal segment were compared for the strain with that of the other *Bacillus* sp. for phosphate metabolism as per earlier studies [20]. Furthermore, metabolic pathway reconstruction was performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database through RAST. The genomes were compared in terms of the number of genes involved in different metabolic pathways and in phosphate metabolism as compared with the type strains.

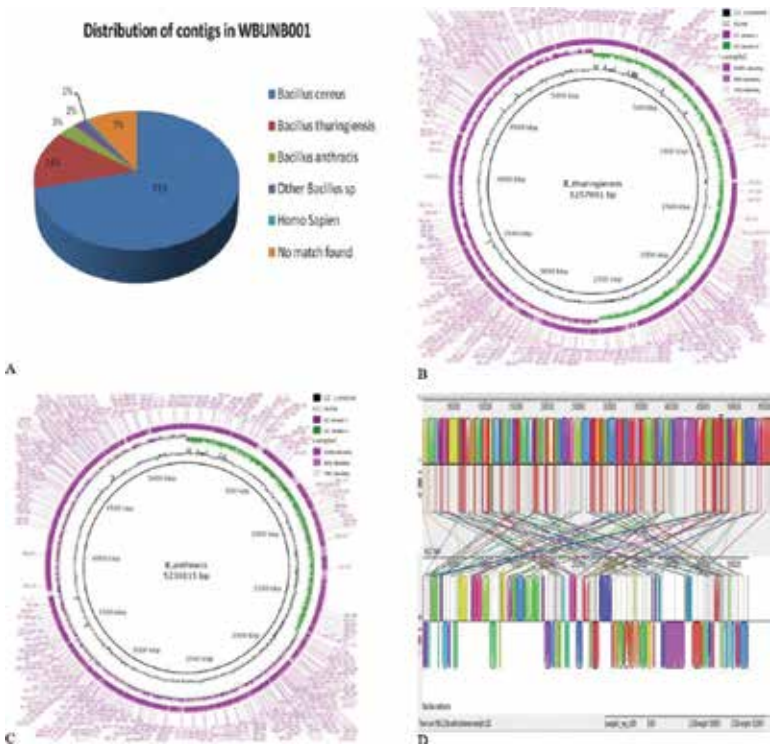

Figure 16. Genomic comparison of the draft genome of MCC0008 (also named WBUNB001) with other members of *Bacillus* species. **(A)** Pie chart of the data generated following blast analysis of the contigs revealing maximum similarity with different organisms. Maximum similarity of major portion of the contigs is with *Bacillus cereus*. **(B)** Represents the comparison of the genome of MCC0008 with *Bacillus thuringiensis*. The graphs depicted gene transfer within the genome (GC content) while the GC skew data which should be 50% positive and 50% negative under ideal condition showed 45–50% +ve with *Bacillus thuringiensis*. **(C)**. Represents the comparison of the genome of MCC0008 with *Bacillus anthracis*. The graphs depicted gene transfer within the genome (GC content) while the GC skew data which should be 50% positive and 50% negative under ideal condition showed about 30%+ve with *Bacillus anthracis*. The gap in the genome sequence was also revealed through this analysis. This analysis also revealed maximum identity with *Bacillus* cereus at the nucleotide level. **(D)** Mauve analysis to determine genome rearrangement when compared with *Bacillus anthracis*. There is extensive rearrangement in all three cases emphasizing the isolate to be a novel species different from these three type strains of *Bacillus* sp.

The phylogenetic analysis of the novel strain was done with its closest neighbors using the 16SrRNA sequence as well as using seven housekeeping genes, namely RNA polymerase B (rpo B), gyrase B subunit (gyrB), pyruvate carboxylase A(pyc A), malate dehydrogenase (mdh), rod shape determining protein(MreB), DNA mismatch repair protein (MutS), and transcription regulator (pIcR). The software used was MEGA (Molecular Evolutionary Genetics Analysis) Version 6.0. MEGA is an integrated tool which is used for constructing sequence alignment, inferring phylogenetic trees, estimating divergence times, mining online databases, estimating rates of molecular evolution, inferring ancestral sequences, and testing evolutionary hypotheses. MEGA is used by biologists in a large number of laboratories for reconstructing the evolutionary histories of species and inferring the extent and nature of the selective forces shaping the evolution of genes and species.

The draft genome sequence of *Bacillus* sp. MCC0008 had a total number of assembled reads of 1,740,538, with 331 contigs (43 × coverage), in which 315 were large with 35.1% G + C content. A total of 307Mb were sequenced with 202Mb having quality values >20 [11]. Average nucleotide identity and phylogenetic analysis revealed that *Bacillus* sp. MCC0008 is closest to *Bacillus anthracis* while FAME (Fatty Acid Methyl Esters), PLFA (Phospholipid-Derived Fatty Acids), BLAST (Basic Local Alignment Search Tool), Dot plot and BRIG (BLAST Ring Image Generator) analysis and partial 16SrDNA revealed maximum identity with *Bacillus cereus*. However, MAUVE analysis performed on the draft genome of MCC0008 (GenBank Accession Number: ANAU00000000) with the type strains of *Bacillus cereus, Bacillus thuringiensis*, and *Bacillus anthracis* revealed extensive genomic rearrangements while RAST analysis revealed 40% subsystem coverage whereas remaining 60% did not have identity with any known sequence stretch. From the combined interpretation, it is apparent that the strain under investigation is novel species of genus *Bacillus* (**Figure 16**).

House-keeping genes	Closest neighbor
DNA gyrase subunit B	*Bacillus anthracis str Ames* and *Bacillus anthracis str Sterne*
DNA-directed RNA polymerase beta subunit	*Bacillus thuringiensis serovar konkukian str* 97-27
Malate dehydrogenase	*Bacillus anthracis str* A1055
DNA mismatch repair protein mutS	*Bacillus anthracis str Ames* and *Bacillus anthracis str Sterne*
Phosphatidylinositol specific phospholipase C	All the strains of *Bacillus anthracis*
Rod shape determining protein MreB	*Bacillus anthracis str Ames* and *Bacillus anthracis str Sterne*
Pyruvate carboxyl transferase	*Bacillus thuringiensis str. Al Hakam*
Partial 16S rRNA	*Bacillus cereus*

Table 4. Closed neighbor of MCC0008 in case of the house-keeping genes.

DNA–DNA hybridization which calculate the inter-genomic distances between the strains with the score of >70% indicates the same species. The strain was compared with the type strains of *Bacillus anthracis* (Ba), *Bacillus thuringeinsis*(Bt), and *Bacillus cereus*(Bc) revealing a value of 81.8 ± 2.72%, 79 ± 2.82%, and 61.3 ± 2.83% respectively. Hence *Bacillus* sp. MCC0008

was closest to *Bacillus anthracis* (Ba), followed by *Bacillus thuringeinsis* and had the least identity with *Bacillus cereus*. The phylogenetic analysis of the different housekeeping genes at the nucleotide sequence level showed similarity with different species of genus *Bacillus* as revealed in **Table 4** indicating it to be a novel species of genus *Bacillus*.

7. Phylogenetic analysis of putative protein

The nucleotide sequence stretches: ANAU01000001, ANAU01000016, ANAU01000020, ANAU01000033, ANAU01000036, ANAU01000046, ANAU01000052, ANAU01000062, and ANAU010000274— each containing several genes – from the draft genome of MCC0008 [11] were translated in MEGA6 [23] using the standard genetic code. The protein sequences generated from these stretches were submitted to HAMAP [24], Interproscan [25, 26], EMBL-Fasta [26, 27], Prositescan [26, 27], and NPSA blast [28] for predicting their functions. The largest amino acid sequence stretch derived from ANAU01000036 was divided into parts, and the protein blast search of NCBI [29] was used to decipher the function of its individual proteins. The consensus predictions from the tools used, were selected for further detailed analysis. Each of the prediction was verified by scanning the proteins for function specific sequence signatures, using the Scanprosite [30, 31] tool. Alternatively, conserved patterns were identified from the HAMAP seed alignment [24] and uniprot protein cluster —UniRef [32] of the said functional protein category.

Nucleotide sequence stretch of MCC0008	Putative protein	Closest species	Prosite entry	HAMAP entry	UniRef entry	Sequence motif in MCC0008
ANAU01000001	Malate synthase	*Bacillus cereus*	PS00510			KDHSAGLNCGRWDYIF
ANAU01000001	NAD kinase	*Bacillus anthracis*		MF_00361		GGDG
ANAU01000001	FabH	*Bacillus cereus*		MF_01815		AACAGF
ANAU01000001	ATP dependent helicase	*Bacillus cereus*		MF_01452		LIA
ANAU01000001	Peptide ABC transporter permease	*Bacillus anthracis*	PS50928			TRVSLYIALLAAAIDLVIGVAYGGISAF
ANAU01000001	spx transcription regulator	*Bacillus thuringiensis*		MF_01132		IDEKRLQVGY, SCTSC
ANAU01000016	Quinone oxidoreductase	*Bacillus cereus*	PS01162			VLIHAAAGGIGTT
ANAU01000020	Zinc containing alcohol dehydrogenase	*Bacillus thuringiensis*	PS00059			GHEFSGEV
ANAU01000020	Transaldolase	*Bacillus cereus*	PS01054			GVTTNPSLV
ANAU01000020	Phosphate uptake ABC transporter permease	*Bacillus anthracis*	PS50928			RLCIETMASLPSIVVGLFGLLVFVTMTGW

Nucleotide sequence stretch of MCC0008	Putative protein	Closest species	Prosite entry	HAMAP entry	UniRef entry	Sequence motif in MCC0008
ANAU01000020	FAD dependent oxidoreductase	*Bacillus cereus*	PS00862			IRVVGSGH
ANAU01000020	GerLA	*Bacillus anthracis*			UniRef50_Q93N70	PAMYVALVSYHQGLI
ANAU01000020	GerLB	*Bacillus cereus*			UniRef50_Q93N69	GTYLAW
ANAU01000033	Phosphoglycerate kinase	*Bacillus anthracis*	PS00111			RVDFNVP
ANAU01000033	Uvr domain A	*Bacillus cereus*	PS50151			EKTIAKMEAEMKEAAKALDFERAA
ANAU01000033	Uvr domain B	*Bacillus anthracis*	PS50151			EKTIAKMEAEMKEAAKALDFERAA
ANAU01000033	Central glycolytic genes regulator	*Bacillus thuringiensis*			UniRef90_A0RKS8	SASLGMT
ANAU01000033	Murein hydrolase export regulator	*Bacillus anthracis*			UniRef50_Q6HR39	TTVAIASD
ANAU01000033	Transcription regulator WhiA	*Bacillus anthracis*			UniRef50_O06975	TLKELGEMV
ANAU01000033	Autotransporter	*Bacillus cereus*	UniRef90_B7HGW2			LKREV
ANAU01000036	Acetyl ornithine deacetylase	*Bacillus cereus*			UniRef50_K0IAN5	YGRG
ANAU01000036	Acyl co-A dehydrogenase	*Bacillus anthracis*	PS00072			ALTEPNAGSDALS
ANAU01000036	Alpha beta hydrolase	*Bacillus cereus*		MF_00832		YDQR
ANAU01000036	Aminotransferase classIII	*Bacillus thuringiensis*	PS00600			FIADEVMTGLGRTGAW
ANAU01000036	Aspartate semialdehyde dehydrogenase	*Bacillus cereus*	PS01103			MAATCVRVPVISGHS
ANAU01000036	ATPase AAA	*Bacillus cereus*			UniRef90_A0A0A0WLW6	NFNEN
ANAU01000036	Chloramphenicol acetyltransferase	*Bacillus cereus*			UniRef90_A0REA1	GETMG
ANAU01000036	Choloylglycine hydrolase	*Bacillus cereus*			UniRef90_Q81H11	GVNEHG
ANAU01000036	Citrate synthase	*Bacillus thuringiensis*	PS00480			GFGHRVY
ANAU01000036	Cold shock protein	*Bacillus anthracis*			UniRef50_Q45096	NLIFADTS
ANAU01000036	D-alanyl D-alanine carboxypeptidase	*Bacillus cereus*			UniRef90_Q6HBP6	SYAAGI
ANAU01000036	Diguanylate cyclase	*Bacillus cereus*			UniRef90_A0R9R1	NITLA
ANAU01000036	DNA binding protein	*Bacillus thuringiensis*	PS50943			LKTIREKEKLSLEKVSQLTGVSKTMIGQ
ANAU01000036	Glucokinase	*Bacillus cereus*			UniRef90_Q738U1	YQLFSRYVVD

Nucleotide sequence stretch of MCC0008	Putative protein	Closest species	Prosite entry	HAMAP entry	UniRef entry	Sequence motif in MCC0008
ANAU01000036	Membrane protein	*Bacillus cereus*			UniRef50_C3BJ03	LGITV
ANAU01000036	MFS transporter	*Bacillus cereus*	PS50850			MIRILAIVAFFVGLDSLLVAP
ANAU01000036	Multidrug ABC transporter	*Bacillus cereus*	PS50893			GPTGSGKTTIINLLTRFYD
ANAU01000036	NADH ubiquinone oxidoreductase	*Bacillus cereus*			UniRef90_Q81K10	ARGVYANA
ANAU01000036	Serine threonine protein kinase	*Bacillus cereus*	PS50011			IGMGSYGVTYVV
ANAU01000036	Threonyl tRNA synthetase	*Bacillus cereus*		MF_00184		GFYYD, GAYWRGD
ANAU01000046	FenI	*Bacillus cereus*			UniRef90_Q6HIP8	NTTYKKHELRAVW
ANAU01000052	Nitrite/Nitrate response regulatory protein	*Bacillus cereus*	PS50110			SVLVVDDHVAVGLGTKALIEKYDDMNVEVVHDST
ANAU01000052	ABC transporter	*Bacillus cereus*	PS50893			ILKQGETLGVVGKTGSGKTTLVRQ
ANAU01000062	Non homologous End joining protein Ku	*Bacillus cereus*		MF_01875		WKG
ANAU01000062	Spore coat cotJA	*Bacillus cereus*			UniRef50_Q45536	HSPQDPCPPIGKKYY
ANAU010000274	Hypothetical protein					

Table 5. Comparison of contigs of MCC0008 with closest neighbor at the putative protein sequence level.

Hence from the combined interpretation it is concluded that due to extensive genomic rearrangement, *Bacillus* sp. MCC0008 has emerged to be a novel species.

The members of the Genus *Bacillus* comprising of *Bacillus cereus*, *Bacillus thuringiensis*, *Bacillus anthracis*, *Bacillus mycoides*, *Bacillus pseudomycoides*, and *Bacillus weihenstephanensis* [33–35], which share high degree of sequence similarity with MCC0008, were chosen for the functional annotation and the phylogenetic study of MCC0008. The protein sequences of the above group members, having the functions as predicted in MCC0008, were retrieved from the protein database of NCBI [36] as available. The sequences which could be acquired were aligned in MEGA6 [23] with the corresponding translated nucleotide stretches of MCC0008, using the clustalW program [37]. The protein weight matrix was set to BLOSUM [38]. The prosite motifs/conserved patterns from HAMAP seed alignment/UniRef, pertaining to the relevant function, were searched in the alignments.

The consensus predictions for the translated nucleotide sequence stretches of the draft genome of MCC0008 are summarized in **Table 5**. The putative proteins showed sequence specific characteristics of the predicted functions, as validated through sequence motifs in the prosite database /HAMAP family profile/UniRef. The sequence alignments of the MCC0008 proteins

with the corresponding proteins of the Genus *Bacillus* and the presence of signatures from Prosite/HAMAP/UniRef therein brought out the sequence motifs of the group and the MCC0008 strain. The database entries along with the corresponding exact motif in MCC0008 are tabulated in **Table 5** again. The high degree of sequence similarity amongst MCC0008 and the members of Genus *Bacillus* resulted in these sharing the same protein sequence motif, with a few exceptions of diverging sequences of *Bacillus* sp. These hint that the isolated strain being reported could belong to the Genus *Bacillus* but not any of these known species. The phylogenetic trees computed for the different proteins show that in most of the cases, *Bacillus cereus*, *Bacillus anthracis,* and *Bacillus thuringiensis* gets clubbed with MCC0008, with *Bacillus mycoides*, *Bacillus pseudomycoides,* and *Bacillus weihenstephanensis* being clad out. The study indicates that MCC0008 is closest to *cereus*, *anthracis,* and *thuringiensis.* Further, *Bacillus cereus* emerges nearest to MCC0008 for ATP-dependent helicase, FabH, Malate synthase, Quinone oxidoreductase, FAD-dependent oxidoreductase, Transaldoase, GerLB, Uvr system domain A, Autotransporter, D-alanyl D-alanine carboxypeptidase, Glucokinase, NADH ubiquinone oxidoreductase, Membrane protein, Acetyl ornithine deacetylase, ATPase AAA, Serine threonine protein kinase, Diguanylate cyclase, Threonyl tRNA synthetase, Alpha beta hydrolase, Chloroamphenicol acetyltransferase, MFS transporter. Aspartate semialdehyde dehydrogenase, Choloylglycine hydrolase, Multidrug ABC transporter ATP binding protein, FenI, ABC transporter, Nitrite/nitrate response regulatory protein, End joining protein ku, spore coat protein CotJA. *Bacillus anthracis* on the other hand appears closest to MCC0008 for peptide ABC transporter permease, NAD kinase, Phosphate uptake ABC transporter, Phosphoglycerate kinase, GerLA, Uvr system domain B, Murein hydrolase export regulator, sporulation regulator WhiA, Acyl co-A dehydrogenase, Cold shock protein. Spx transcription regulator, Zn-containing alcohol dehydrogenase, Central glycolytic genes regulator, Aminotransferase class III, Citrate synthase, and DNA-binding protein show MCC0008 getting clubbed with *Bacillus thurigiensis*. The picture that emerges here is that the strain in question seems to be a novel species mostly toward *Bacillus cereus*, with traces of *Bacillus anthracis* and a dash of *Bacillus thurigiensis.* The probable novel strain MCC0008 which shares traits from *Bacillus cereus*, *Bacillus anthracis,* and *Bacillus thurigiensis* could have emerged from genetic rearrangements between these species of the *Bacillus* group. *Bacillus anthracis* which is not reported to be a phosphate accumulator appears nearest to MCC0008 for phosphate uptake ABC transporter permease and phosphoglycerate kinase. *Bacillus cereus* is in closest proximity to MCC0008 for the nitrite/nitrate response regulatory protein. It appears from these observations that the genetic components from *cereus*, *anthracis,* and *thurigiensis* have given rise to this novel strain which has acquired the unique property of phosphate and nitrate accumulation.

8. Transcriptome analysis (BioProject PRJNA222597)

From transcriptome analysis (**Figure 17** and **Table 6**), it is concluded that there is significant upregulation of sporulation genes, which can be due to the accumulation of poly-P in the bacterial cells [39]. The sporulation of *Bacillus* species initiates with the asymmetric division of

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cellular compartment into two parts: the mother cell and the forespore. In the model organism *B. subtilis* (Bs), this process is temporally and spatially regulated by a set of sigma factors of RNA polymerase: the main vegetative sigma factor SigA and SigH in the pre-asymmetric division cell; SigE and SigK in the mother cell; and SigF and SigG in the forespore. The DNA-binding protein Spo0A is the master regulator for entry into sporulation in *B. subtilis* [40]. Further there is significant upregulation of serine protein kinase which also play a role in sporulation [41] and also there is upregulation of histidine kinase which also play a significant

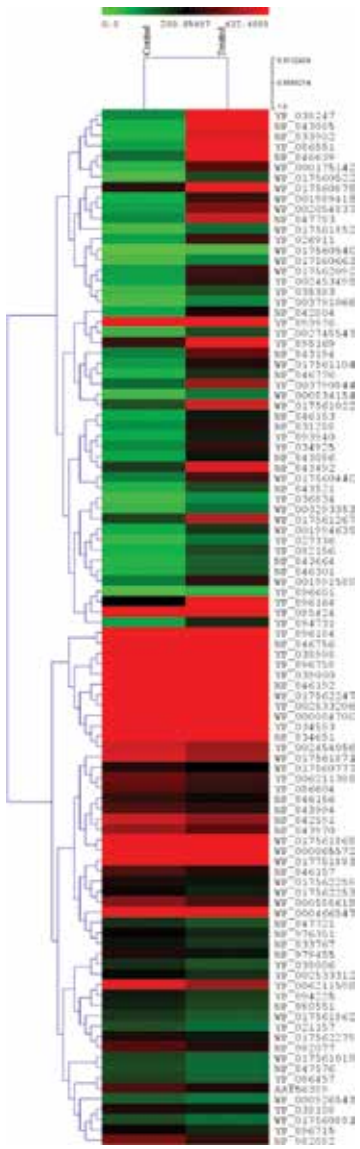

Figure 17. Heat map of top 100 differentially expressed transcript contigs in control and treated samples.

role in sporulation. The initiation of sporulation in *Bacillus subtilis* and most likely in aerobic *Bacillus* species in general is controlled by the phosphorelay signal transduction system [42]. The ultimate goal of the phosphorelay is to activate by phosphorylation the Spo0A transcription factor, which represses certain genes and promotes the transcription of a large number of genes for stationary-phase functions as well as sporulation [40, 43]. The signals that initiate the phosphorelay reactions are recognized and interpreted by several sensor histidine kinases [44–47]. The initiation of sporulation in *Bacillus subtilis* is controlled. *Bacillus* sp. MCC0008 synthesizes poly-p granules as revealed from the significant upregulation of phasin and also through polyphosphate staining and TEM analysis. Phosphorus (P) is an essential element for all cells as it is a component of, for example, DNA, RNA, and membrane lipids. The common phosphorus source is inorganic phosphate (P*i*), which is taken up by bacteria either via secondary transporters or via ATP-driven ABC transporters. Extracellular phosphate esters can serve as an alternative P source. Phosphate esters are hydrolyzed by bacterial phosphatases and the resulting P*i* imported into the cells. In addition, some bacteria utilize specific uptake systems for the transport of *sn*-glycerol-3-phosphate as organophosphate. The intracellular P*i* is assimilated into cellular metabolites by reactions such as F1F0-ATP synthase or glyceraldehyde-3-phosphate dehydrogenase. Moreover, polyphosphate can be formed as a readily available intracellular P*i* source. From the transcriptome analysis, it is revealed that there is downregulation of glyceraldehyde-3-phosphate dehydrogenase; hence, there is no assimilation of phosphate in the form of poly-P.

For nitrate accumulation, it is hypothesized that the nitrate accumulation occurs due to electrochemical gradient (Δp) [48]. In plants, typically vacuolar-type H^+ATPases and H^+pyrophosphatases (HPPases) catalyze a proton translocation over endomembranes to generate a Δp for solute transport and likely also nitrate transport [49]. Vacuolar-type ATPases also occur in plasma membranes of some Archaea, but they are rarely encountered in Bacteria [50, 51]. A vacuolar Hþ-pyrophophatase (hppA) and an uncommon Ca^{2+} translocating ATPase, may also contribute to generation of a Δp/ΔPh [52]. From transcriptome analysis it is revealed that there is 3.95-fold change in cation-transporting ATPase, which can be responsible for electrochemical gradient and nitrate accumulation.

Genes	Protein encoded	Fold Change	log2Fold Change	Function
BCK_14255	**Stage III sporulation protein AH**	**12.53**	**3.65**	**Involved in forespore engulfment**
BCK_05395	**Serine protein kinase**	**11.43**	**3.51**	**Kinase enzyme that phosphorylates the OH group of serine**
BCK_14250	**Stage III sporulation protein AG**	**10.31**	**3.37**	**Sporulation resulting in formation of a cellular spore**
BCK_17375	**Hypothetical**	**12.84**	**3.68**	**Unknown**

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Genes	Protein encoded	Fold Change	log2Fold Change	Function
	protein			
BCK_14245	Stage III sporulation protein AF	8.94	3.16	Leading to endospore formation
BCK_08950	Stage II sporulation protein	8.80	3.14	Sporulation resulting in formation of cellular spore
BCK_17370	Uncharacterized protein	8.08	3.01	Unknown
BCK_23315	Uncharacterized protein	9.89	3.31	Unknown
BCK_12895	Stage VI sporulation protein D	6.18	2.63	Required for assembly of a normal spore coat. May be a component of the innermost layer of the spore coat that aids in its adherence to the prespore.
BCK_02050	2-oxoglutarate dehydrogenase E1 component	5.89	2.56	The 2-oxoglutarate dehydrogenase complex catalyzes the overall conversion of 2-oxoglutarate to succinyl-CoA and CO_2. It contains multiple copies of three enzymatic components: 2-oxoglutarate dehydrogenase (E1), dihydrolipoamide succinyltransferase (E2) and lipoamide dehydrogenase (E3).
BCK_02290	Uncharacterized protein	5.81	2.54	Unknown
BCK_08430	Uncharacterized protein	5.84	2.55	Chromatin binding
BCK_17365	Hypothetical protein	5.67	2.50	Unknown
BCK_02225	Spore coat protein Z	5.92	2.57	Sporulation resulting in formation of a cellular spore
BCK_04810	Ribose import ATP-binding protein RbsA	5.07	2.34	Part of the ABC transporter complex RbsABCD involved in ribose import. Responsible for energy coupling to the transport system.
BCK_03255	Group-specific protein	6.24	2.64	Catalyses the phosphorylation of incoming sugar substrates concomitant with their translocation across

Genes	Protein encoded	Fold Change	log2Fold Change	Function
				the cell membrane
BCK_03260	**Histidine kinase**	**4.02**	**2.01**	**Phosphorelay sensor kinase activity**
BCK_04960	**Amino acid ABC transporter ATP-binding protein**	**3.38**	**1.76**	**–**

Table 6. Table showing fold change in expression of different genes during transcriptome analysis of MCC0008.

9. Conclusion

The isolate MCC0008 is an extracellular protease, amylase, lipase, catalase, oxidase, phosphatase, and DNAse secreting strain which can form well-structured biofilm. It was isolated from a consortium developed from low-level radioactive waste treatment plant biomass. The strain is a novel species of genus *Bacillus* which falls within the group of *Bacillus cereus*. It could sequester nitrate within one hour from nitrate broth while took 11 hours to do the same from waste water under minimal condition in batch mode. This could be due to the antagonistic effect of the natural microflora of waste water or non-biological inhibitors. The well-developed biofilm ensured sustained performance of the system. The isolate during soil application retains phosphate and nitrate in the root zone ensuring better access to the plants. This was the reason behind approximately twofold and 1.2-fold yield enhancement as compared to no fertilizer and chemical fertilizer application respectively. Hence, microbial isolate from the low-level waste water treatment plant could help sequester essential plant growth nutrients from waste water. This would help reuse these nutrients while purify the water which in turn can be reused for agriculture and aquaculture, hence preventing wastage of potable water for non-potable application. This solution leads to environmental protection (prevention of eutrophication) and sustenance (organic farming).

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Chapter 9

Insights into Ionizing-Radiation-Resistant Bacteria S-Layer Proteins and Nanobiotechnology for Bioremediation of Hazardous and Radioactive Waste

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Additional information is available at the end of the chapter

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Abstract

S-layers are crystalline arrays formed by proteinaceous subunits that cover the outer surface of many different kinds of microorganisms. This "proteinaceous cover" is particularly important in the case of ionizing-radiation-resistant bacteria (IRRB) that might be used in bioremediating hazardous and radioactive wastes (HRW). Despite the exponential growth in the number of comparative studies and solved proteic crystal structures, the proteic networks, diversity, and bioremediation-useful structural properties of IRRB S-layers remain unknown. Here, aided by literature, a tentative model of *Deinococcus radiodurans* R_1 S-layer proteins (SLPs) and the network of its main constituents were proposed. The domain analysis of this network was performed. Moreover, to show the diversity of IRRB S-layers, comparative genomics and computer modeling experiments were carried out. In addition, using *in silico* modeling, assisted by previously published data, the outermost exposed segments of *D. radiodurans* SlpA (surface layer protein A) that were predicted to interact with uranium were mapped. The combination of data and results pointed to various prospective applications of IRRB S-layers in nanobiotechnology for bioremediation of radioactive waste.

Keywords: bioremediation, ionizing-radiation-resistant bacteria, nanobiotechnology, radioactive waste, S-layer proteins, uranium

1. Introduction

Hazardous and radioactive wastes (HRW)—containing organic contaminants, toxic metals, and/or actinides as well as other radionuclides—released, for example, from nuclear weapons production, mining, and nuclear accidents can be legitimately considered as pools of ionizing-radiation-resistant bacteria (IRRB)—vegetative bacterial cells with D_{10} (acute ionizing-radiation dose for 90% reduction in colony-forming units (CFUs)) greater than 1 kilogray (kGy) [1]. Indeed, previously, IRRB were isolated from a high-level radioactive environments (*Kineococcus radiotolerans* SRS30216 [2]) and were engineered for metal remediation in radioactive mixed waste environments [3–5]. Yet, it is important to remember that, for instance, for the most studied "gold medalist" for radiation resistance, *D. radiodurans*, its ionizing-radiation resistance seems to be uncorrelated with its metal or actinide tolerance except in cases where the metal/actinide directly damages DNA [6].

Since the outer envelope—regular crystalline highly porous (glycol)protein meshworks ((s(urface)-layers)—of IRRB ([7, 8] and references therein) represents the first front that might encounter HRW and damaging radiation, it is expected to possess pertinent characteristics such as the ability to interact with actinides. These latter (e.g., americium-241 (^{241}Am), neptunium-237 (^{237}Np), plutonium-241 (^{241}Pu), and thorium-230 (^{230}Th)) might also be present in most radioactive wastes; however, uranium-238 (^{238}U) is the priority pollutant ([9] and references therein). Analyses of the uranium bound to S-layer proteins (SLPs) of vegetative cells of IRRB to form complexes were reported in previous studies [10–12]. Although it is assumed that bacteria-carrying SLPs use S-layer variation—changing proteic expression through rearrangements of DNA, etc.—to adapt to different stress factors ([12] and references therein), the role of S-layers in ionizing-radiation resistance has not yet been demonstrated, but a role in response to radiation damage has been proposed [13].

SLP lattices are composed of a single protein or glycoprotein monomers with apparent relative molecular weights ranging from 40 to 200 kDa depending on the particular bacterial species [14]. The presence of SLPs has been reported in hundreds of different species belonging to all major phylogenetic groups of (Gram-positive and Gram-negative) Eubacteria [15], e.g., *Aeromonas salmonicida* VapA; *Bacillus anthracis* Sap, EA1, and the BSL family; *Campylobacter fetus* SapA and SapB; *Caulobacter crescentus* RsaA, *Clostridium difficile* SlpA, and the CWP family; *D. radiodurans* SlpA and Hpi (Hexagonally packed intermediate-layer surface); *Geobacillus stearothermophilus* SbsA, SbsB, SbsC, SbsD, and SgsE; *Lactobacillus crispatus* CsbA, SlpA, and SlpC; *Synechococcus* spp. SwmA; *Tannerella forsythia* TfsA and TfsB; etc. ([16] and references therein). For review, a survey of SLPs with known amino acid (AA) sequence was conducted by Sára and Sleytr [14]. Among bacterial SLPs, glycosylated proteins have been observed [17], for instance, for different species belonging to Firmicutes like two lactobacilli [18], *Aneurinibacillus thermoaerophilus* [19], etc.

A major challenge is to uncover structure-function relationships of SLPs to obtain many clues, for instance, about radioresistance and tolerance to toxic molecules. During decades following their discovery [20], various high-resolution techniques have been used for the observation of bacterial SLPs. Briefly, the biophysical methods of choice for studying the

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structure of SLPs might be categorized into three groups: (1) electron-microscopy-based techniques (e.g., electron diffraction (e.g., SLPs of *Acetogenium kivui* [21]), freeze-etch electron microscopy (e.g., SLPs of *Thermoanaerobacter thermohydrosulfuricus* [22]), atomic force microscopy (AFM) (e.g., SbpA of *Lysinibacillus sphaericus* [23], Hpi layer of *D. radiodurans* [24])); (2) scanning probe microscopy techniques (e.g., TfsA-GP TfsB-GP of *Tannerella forsythia* [25]); and (3) X-ray scattering techniques (e.g., X-ray crystallography (e.g., SbsB of *Geobacillus stearothermophilus* [26])). Except the latter, all the other methods are only useful to determine the overall topologies of SLPs and the symmetrical associations between the different molecular partners involved in their formation. The X-ray crystallography however is able to get insight into the atomic arrangements between the AAs of the SLP. This kind of data might be very useful for structure-function relationship studies as well as for developing biotechnological applications [27]. Yet, the tendency of SLPs to form two-dimensional lattices is considered as a major issue for growing crystals.

Spectroscopic methods such as nuclear magnetic resonance (NMR) spectroscopy, electron paramagnetic resonance (EPR) spectroscopy (combined with site-directed spin-labeling), and Fourier-transform infrared (FTIR) spectroscopy could also complement the structural and biochemical techniques to study the dynamics of SLPs and their interaction with other molecules (proteins, radionuclides, etc.). Indeed, for example, analyses of the secondary structure of *Lactobacillus* SLPs and their behavior upon heating were studied by combining FTIR and differential scanning calorimetry (DSC) methods [28]. In addition, instrumental methods such as X-ray photoelectron spectroscopy and matrix-assisted laser desorption ionization or electrospray ionization mass spectrometry have been introduced for the analysis of the protein and glycan portions of SLPs ([29, 30] and references therein). Also, immobilized *C. crescentus* S-layers on zincite-coated nanoparticles of iron oxide were investigated using FTIR spectroscopy, powder X-ray diffraction (XRD)—the diffraction pattern is obtained from a powder of the material rather than an individual crystal, AFM and field-emission scanning electron microscope (FESEM) [31]. Recently, Madhurantakam et al. [32] revised the methods employed to analyze the properties and structural characteristics of the S-layer lattice, demonstrating the increase in the usage of X-ray-based methods, spectroscopy, cryo-electron microscopy, and other high-throughput data-processing techniques to study the structure of SLPs during the last two decades.

It goes without saying that theoretical methods, including homology modeling, threading, and *ab initio* methods, cannot substitute experimental techniques to accurately determine the molecular structure of proteins. Nevertheless, even inaccurate, models can be useful especially to evaluate the overall fold of the proteic macromolecule, to map certain properties on molecular surface or to probe the three-dimensional patterns and the spatial repartition of certain AAs.

In this chapter, we present a survey of IRRB S-layers based on literature with a special focus on *D. radiodurans* and SlpA. Moreover, we introduce an automated computational pipeline adapted for the ease of use for the identification and analyses of SLP structures. The proposed pipeline was applied to completely sequenced genomes of previously known IRRB available in the genomes online database (GOLD) [33] and the radioresistant prokaryotes database

(RadioP) [34]. Accordingly, we suggest prospective applications of IRRB SLPs in nanobiotechnology for bioremediation of hazardous and radioactive wastes.

2. Survey of S-layer proteins (SLPs) in *Deinococcus radiodurans* R_1

The order of *D. radiodurans* envelope layers and their nature has been investigated in many studies ([8] and references therein). Using electron microscopy, Rothfuss et al. [8] have identified five layers: (1) the inner membrane, (2) the peptidoglycan cell wall, (3) the interstitial layer, (4) Hpi and the backing layer, and (5) the carbohydrate coat. *D. radiodurans* envelope has an unusual structure and composition with the outermost surface of this formation is the "pink envelope" containing carbohydrates, proteins, carotenoid (deinoxanthin [7]), lipids, and most likely the outer membrane [8]. SlpA (DR_2577) and Hpi (DR_2508) are the most abundant components responsible for the envelope maintaining [35]. The S-layer regular topology is distinguished in electron microscopy even when *hpi* gene is altered; however, the deletion of *slpA* results in the incapacity of Hpi to form the hexagonal porous structure on the S-layer outer surface and in losing the integrity of the pink envelope [8].

Fagan and Fairweather [16] estimated that the *C. difficile* S-layer contains up to 500,000 subunits, synthesized at a rate of 140 subunits per second per cell during the exponential growth phase. Several distinct mechanisms have evolved to cope with this high-protein flux. In many Gram-negative species, S-layer secretion relies on a specific type I secretion system; and in some studied Gram-positive bacteria, the secretion of the S-layer precursor is dependent on the accessory Sec secretion system. Interestingly, there is a striking degree of genetic linkage between the genes that encode SLPs and their dedicated secretion systems in many bacteria ([16] and references therein). This genetic linkage was not observed in the case of *D. radiodurans slpA* and *hpi* (**Figure 1**).

Figure 1. Gene local context and predicted *Deinococcus radiodurans* R_1 *slpA* and *hpi* operons [36]. Locus tags and abbreviations: DR_2576, putative manganese-dependent inorganic pyrophosphatase; DR_2577, *slpA; tgt*, Queuine tRNA-ribosyltransferase; DR_2507, medium-chain fatty-acid-CoA ligase; DR_2508, *hpi*; DR_2509, hypothetical open reading frame.

The S-layer is anchored to the cell surface via non-covalent interactions with cell surface structures, usually with lipopolysaccharides (LPSs) in Gram-negative bacteria and with cell wall polysaccharides in Gram-positive bacteria [14]. For example, in *C. crescentus*, the 225 N-terminal AAs from a total of 1026 residues of RsaA SLP is required for the binding to LPS on the cell surface [16, 37, 38]. Experimental evidence that the surface layer homology (SLH) domains recognize a cell envelope by binding to peptidoglycan was further provided for the

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SLPs Sap and EA1 of *B. anthracis* [39, 40], SlpA of *C. thermocellum* [39, 41], and the SLP and cell-wall-associated xylanase of *Thermoanaerobacterium thermosulfurigenes* EM1 [39]. Importantly, it was shown that pure peptidoglycan was unable to bind the SLH domains [39] situated at the N-terminal region of the SLP of *C. crescentus*. However, it recognizes distinct oligosaccharides of the LPS as binding partners [42, 43]. On the other hand, it has been proposed that hydrophobic interactions are responsible for attachment of the S-layer to the outer membrane in the backing layer, as well as for the association of the S-layer units in *D. radiodurans* [44]; while Hpi is involved in *in vivo* cleavage and is closely associated to SlpA. The SLH domain of SlpA was shown to bind deinococcal peptidoglycan-containing cell wall sacculi [45]. Indeed, it has

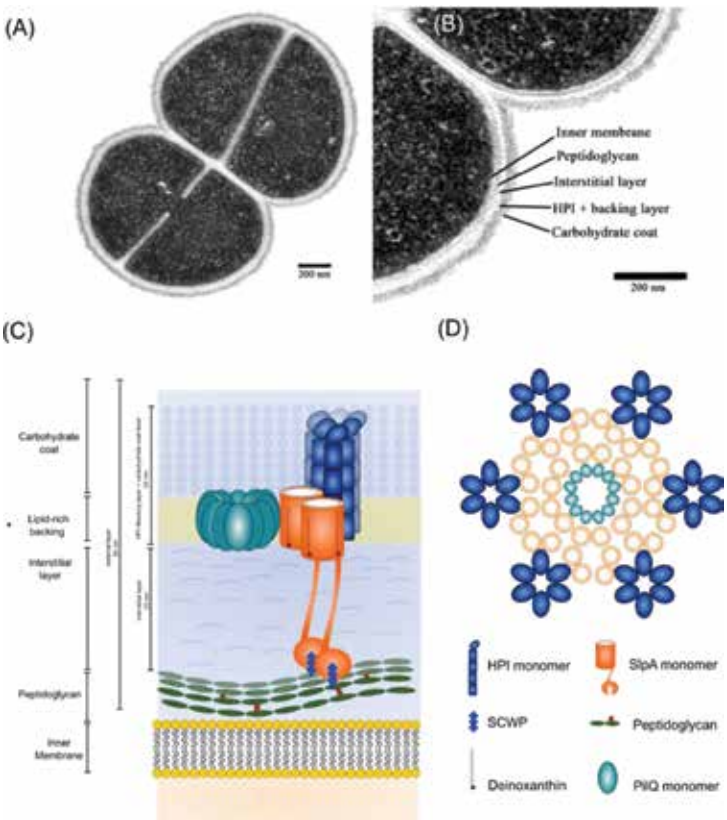

Figure 2. A tentative model of the S-layer of *Deinococcus radiodurans* R_1 cell envelope based on literature (see the text for details). (A) Thin section electron micrograph of *D. radiodurans* tetrad. (B) Magnification of the membrane with indication of different layers (Photos credit (A and B): Prof. Mary E. Lidstrom and co-authors [8], *Frontiers* (www.frontiersin.org) and Microbiology Journal (http://mic.microbiologyresearch.org/content/journal/micro/10.1099/mic.0.28971-0#results-1)). (C) The five layers in *D. radiodurans* cell envelope [8], PilQ (DR_0774) as a component related with the S-layer [35] and the predicted association between: (i) SlpA and deinococcal peptidoglycan-containing cell wall [45], (ii) SlpA and Hpi [45], and (iii) SlpA and deinoxanthin [7]. The association of SlpA with peptidoglycan on one side and Hpi on the other localizes this protein in the "interstitial" layer of the deinoccocal cell wall [45]. (D) The predicted association between the dimeric structure of SlpA and the hexameric structure of Hpi [47]. The blue, orange, and purple shapes represent Hpi, SlpA, and DR_0774 (secretin), respectively.

been previously determined that the backing layer of the pink envelope, rather than the Hpi layer, provides the rigidity and the curvature of the cell envelope [46], suggesting that the SlpA protein interacts with the backing layer [8].

Based on structural, functional, and proteomic data collected from literature, we propose in this chapter a tentative model describing the interaction between SlpA, deinoxanthin, Hpi, and PilQ (DR_0774) within *D. radiodurans* cell envelope (**Figure 2**).

The description of *D. radiodurans* SlpA and Hpi proteic network and domains is vital to our understanding of the function(s) of SLPs in this extremophile. **Figure 3** shows *D. radiodurans* Hpi and SlpA known and potential functional partners predicted using the STRING database available at http://string-db.org/ [48].

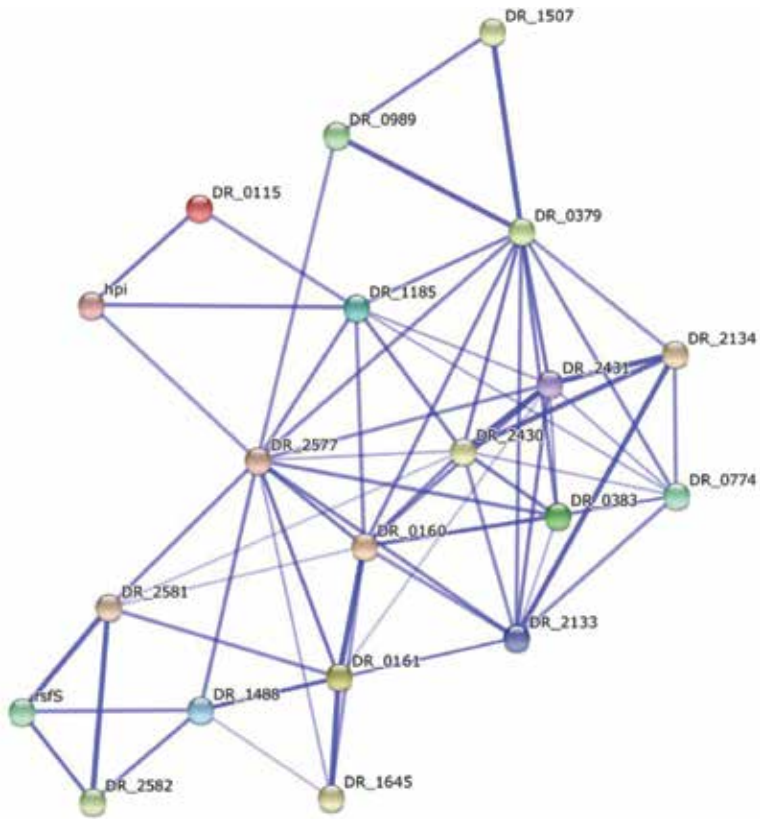

Figure 3. Network of Hpi (DR_2508) and SlpA (DR_2577) predicted functional partners. Prediction methods are based on neighborhood, co-expression, gene fusion, experiments, co-occurrence, databases or text mining via the STRING database [48] and pertinent literature [35, 45].

Proteic domains of *D. radiodurans* S-layer and S-layer-like proteins, presented in **Figure 3**, were investigated using the SMART database [49], available at http://smart.embl-heidelberg.de/, and listed in **Figure 4**. Several proteins showed the presence of an SLH domain.

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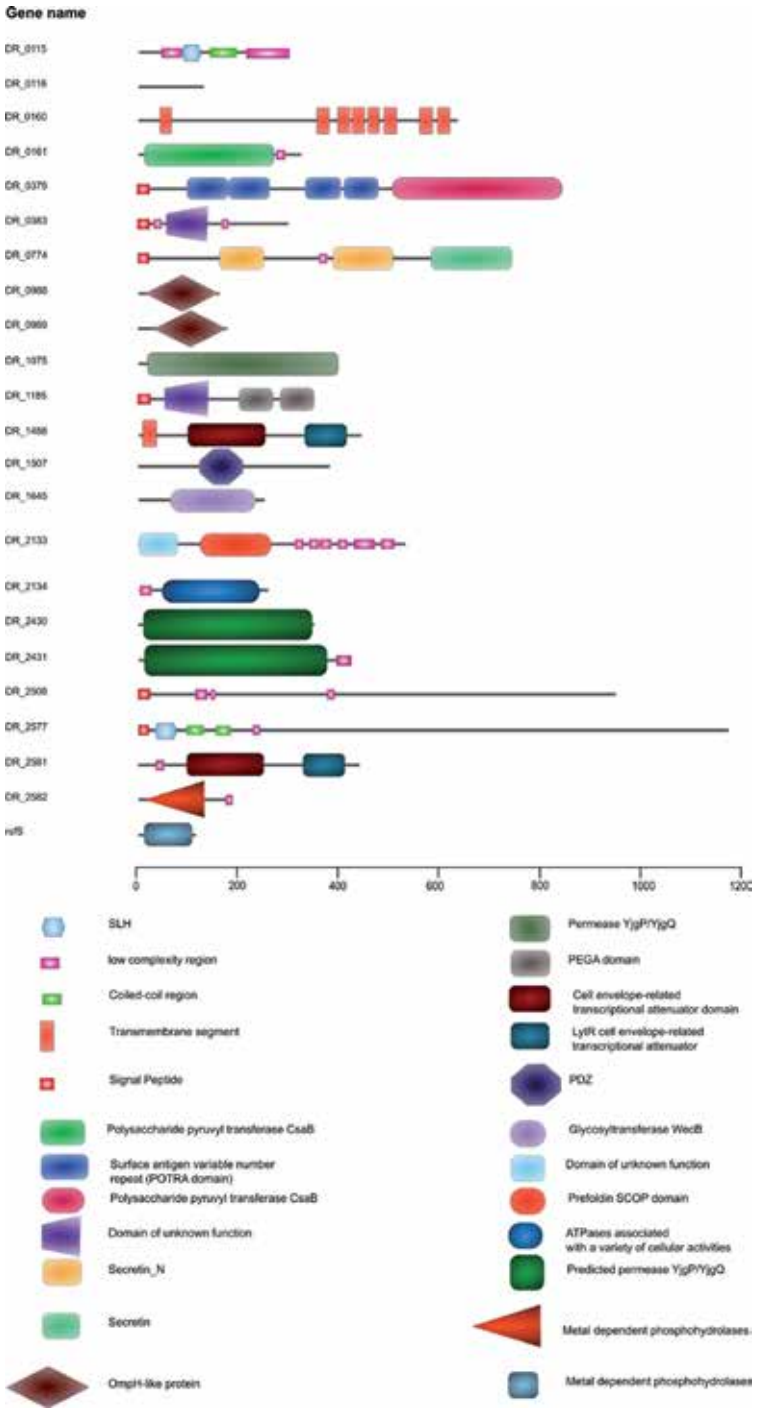

Figure 4. Proteic domains of *Deinococcus radiodurans* S-layer and S-layer-like proteins.

3. Automated computational pipeline for identification and analyses of S-layers in ionizing-radiation-resistant bacteria (IRRB)

IRRB were previously regrouped in the RadioP database [34]. In *Deinococcus geothermalis* and *D. radiodurans*, SLP is among the top 20 predicted highly expressed (PHX) genes; whereas *K. radiotolerans* and *Rubrobacter xylanophilus* have no similar genes [50]. In this work, the bioinformatics pipeline, as illustrated in **Figure 5**, was developed to perform an integrated analysis of IRRB SLPs (domains, interaction network, orthologs, etc.) including the prediction of the exposed residues in their structures to be explored for a potential use of IRRB (e.g., nanobiotechnology for bioremediation of radioactive waste). SlpA and Hpi were taken as working examples.

In an attempt to identify SLP (highlighted in **Figures 3** and **4**) orthologs in the genomes of IRRB species for which D_{10} is known [1, 34, 51], computational analyses using BLASTP program [52] were performed based on the best reciprocal hits (BRH) approach (*e*-value threshold ≤ $1e^{-05}$). While *D. radiodurans* SLP SlpA has orthologs in related IRRB species, Hpi protein did not show any significant similarity with proteins in other related completely sequenced IRRB using our submentioned approach. As *D. radiodurans* Hpi lacks homology to any predicted peptide from other genomes of similar species, *hpi* can be considered as an orphan gene. In addition, analysis of phyletic similarity patterns—patterns of presence or absence, using similarity measures, of

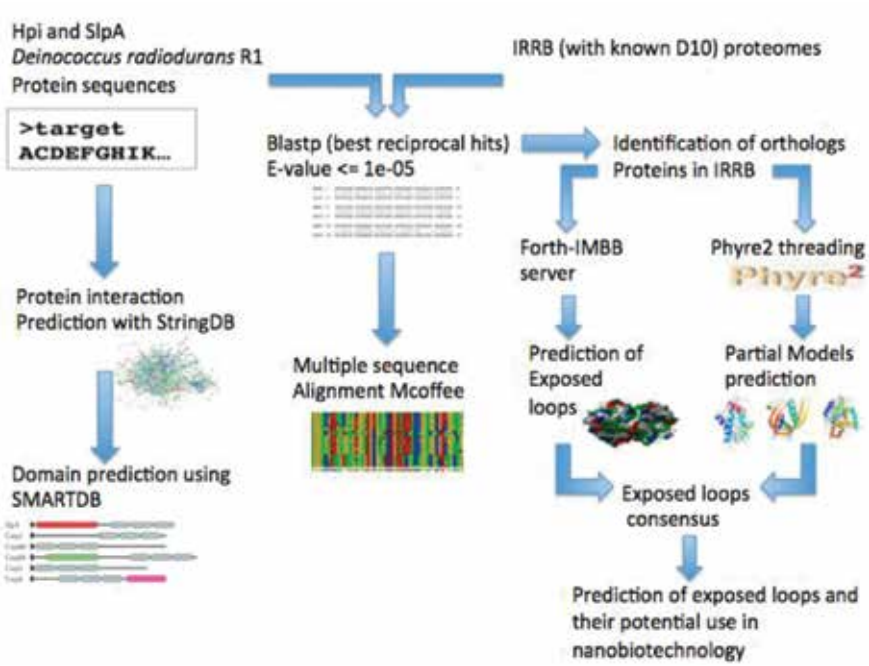

Figure 5. Computational pipeline for an integrated analysis including structural predictions of S-layer proteins (SLPs) as illustrated with *Deinococcus radiodurans* R_1 SlpA and Hpi.

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given proteins in the analyzed proteomes—suggests that several IRRB proteins might be key ancestral surface-layer players as they are not taxonomically restricted (**Figure 6**).

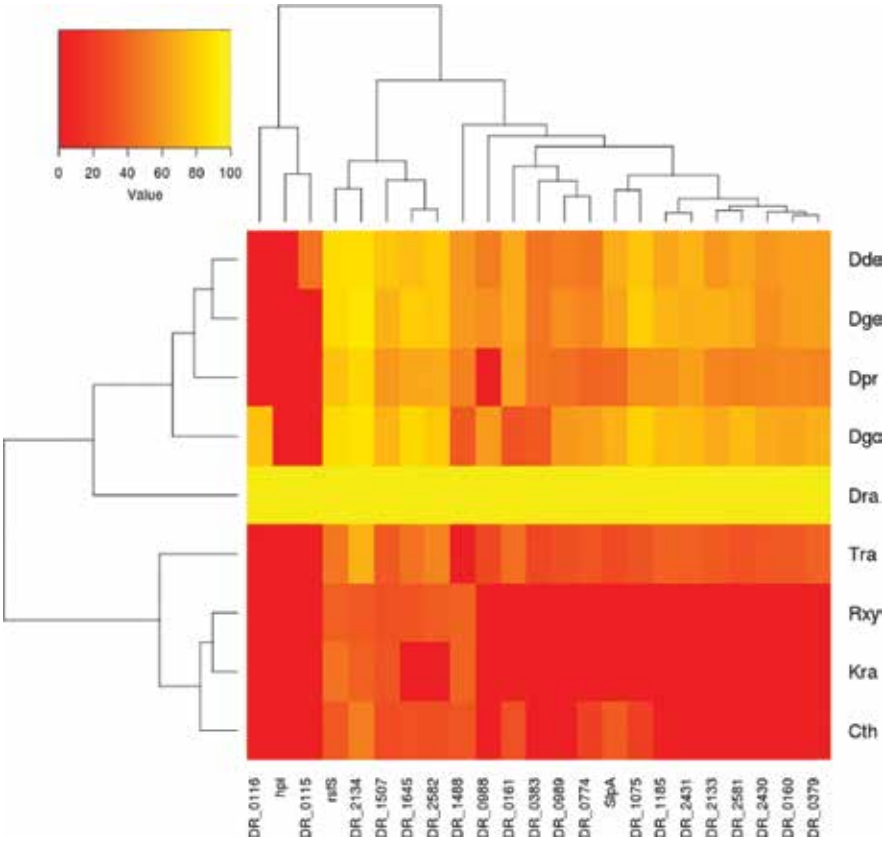

Figure 6. Heatmap showing phyletic similarity patterns of *Deinococcus radiodurans* R_1 SlpA-Hpi-associated proteins in analyzed IRRB proteomes. *D. radiodurans* proteins were chosen based on STRING interactions with SlpA and Hpi and pertinent literature (see **Figures 3** and **4**, and text for details). Patterns were derived from best reciprocal hits BLASTP results. Colors indicate phyletic similarity measures. The species names are abbreviated as follows: Dde, *Deinococcus deserti*; Dge, *Deinococcus geothermalis*; Dpr, *Deinococcus proteolyticus*; Dgo, *Deinococcus gobiensis*; Dra, *Deinococcus radiodurans*; Tra, *Truepera radiotolerans*; Rxy, *Rubrobacter xylanophilus*; Kra, *Kineococcus radiotolerans*; Cth, *Chroococcidiopsis thermalis*.

Based on the BRH approach, a *D. radiodurans* SlpA ortholog could not be confidently identified in the actinobacteria *K. radiotolerans*. To further validate this result, the conserved *D. radiodurans* SlpA SLH domain sequence (AAs 32 to 75) was extracted via the SMART (Simple Modular Architecture Research Tool, smart.embl-heidelberg.de) web server [49]. Subsequently, an iterative (five iterations) PSI-BLAST [53] analysis against a specific database (*K. radiotolerans* (taxid:131568)) did not reveal any hit above the 0.005 threshold. Indeed, a statistically non-significant similarity (expect value of ~0.44) to a peptidase M20 (441 aa, WP_011981201) was observed for the first hit. The same analysis realized with *Kineococcus* group sequences (taxid:

83778) as a database identified an hypothetical ortholog (320 AAs, WP_056676327) from *Angustibacter* sp. Root456 with four SLH domains, as a statistically significant (expect value of ~$2e^{-15}$) hit. Further investigations should be done on genomes of "SLH-negative" IRRB, such as *K. radiotolerans*, using sensitive methods for sequence similarity detection of highly diverged homologs.

The multiple sequence alignment of SlpA orthologous proteins using M-Coffee program [54] showed that conserved AAs between IRRB species (unpublished data) are extremely rare and can be summarized in a high sequence identities limited to the N-terminal region probably corresponding to the SLH domain, which is responsible for binding of the S-layer subunits to the underlying cell envelope layer [17]. The middle and C-terminal S-layers parts, comprising domains involved in the self-assembly process and exposed inside the pores and on the S-layer surface, showed very low sequence similarities.

Domain architecture of *D. radiodurans* SlpA and of its statistically significant hits were investigated using SMART database [49]. The structure of *D. radiodurans* SlpA vindicates previous results—SLH domain(s) [21] at the N-terminal part of many SLPs [41]—that all SlpA sequences display at least one SLH domain (**Figure 7**).

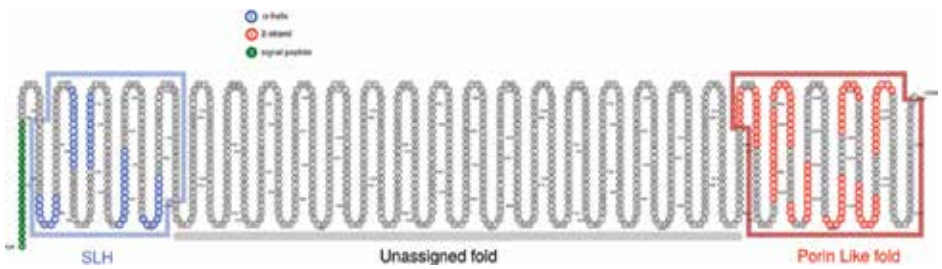

Figure 7. Structural domain topology of *Deinococcus radiodurans* R_1 SlpA predicted by the Phyre2 web server [55].

Only 35% of the total sequence of the SlpA protein was assigned to a protein fold without counting the signal peptide predicted by SignalP server [56]. There are two main protein segments which were modeled with high degree of confidence returned by Phyre2 server [55].

The first segment corresponds to the SLH domain (99.6% degree of confidence) at the N-terminal portion of the sequence and was built based on the *B. anthracis* homologous protein domain. The domain adopts the form of a pseudo-trimer presenting a helix-bundle fold. A central groove of the domain was previously proposed as the binding site of the secondary cell wall polysaccharide (SCWP) molecules [57].

The second segment corresponds to C-terminal of SlpA that was associated with a β-barrel-like fold, typical to porin structure (see **Figure 7**), with a degree of confidence of 88.4%. In fact, it has been shown that the β-barrel assembly machinery (BAM) complex [58] is required for the assembly of SlpA from *Thermus thermophilus* HB27 [59] a closely related bacteria to *D. radiodurans*. Indeed, the signal sequence responsible for the efficient binding of main protein from the BAM machinery to SlpA was also found in *D. radiodurans*. In particular, the highly

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conserved last phenylalanine (Phe/F) residue of the C-terminal is required for the protein-protein interaction to ensure the proper folding of SlpA. Mutants of *T. thermophilus* lacking the terminal Phe of SlpA showed defective folding of the SlpA protein that was more sensitive to proteases than in a wild-type strain [59]. The amphipathic character of the β strands from the C-terminal segment supports also the presence of the β-barrel-fold type. Phylogenetic relationship between porins and S-layer has been already suggested and was supported by the electron microscopy imaging from *T. thermophilus* S-layer [60, 61].

The threading prediction was also applied for the constructed sequences set of IRRB species (**Figure 8**).

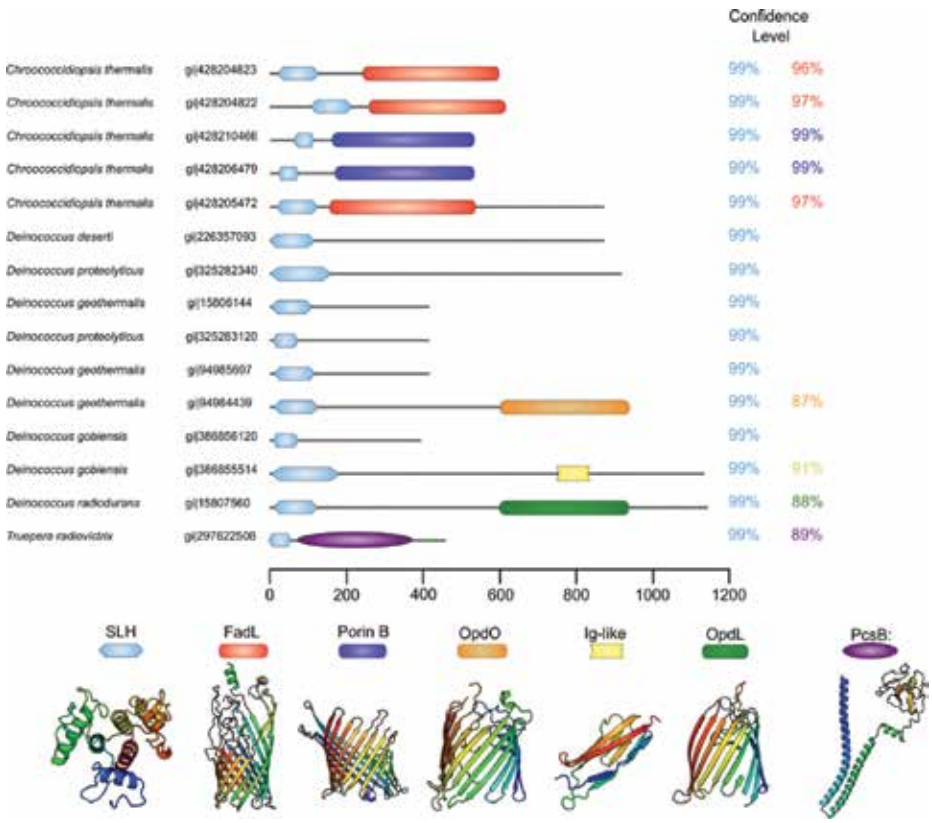

Figure 8. Prediction of the structures of multiple SlpA proteins from different radioresistant bacteria using Phyre2 server. The confidence level represents the probability (from 0 to 100%) that the match between the submitted sequence and the template used to generate the model is weak/strong. In the case of *D. radiodurans*, for example, the template used is the structure of OpdL protein (PDB code: 2Y0H). We reported only the structural domains with more than 90% of confidence or with less than 90% but with extended range over the protein sequence. Each confidence level is associated with one domain highlighted with the same color.

The first information to notice is the high conservation of the SLH domain in all taxa which is in concordance with previous findings using sequence analysis tools [41, 62]. However, the

length of the SLH domain differs and this is due to a variation in the numbers of the SARP subdomain units even among paralogs (e.g., *Chroococcidiopsis thermalis* and *Deinococcus proteolyticus*). This result suggests that the length of SlpA SLH domains does not affect its binding to the peptidoglycan layer.

Also, it was observed that the SlpA presents different folding categories for the non-SLH segments. This result suggests that IRRB SLPs have evolved different molecule preferences and specific biological functions although evolution of molecule preferences does not necessarily follow proteic divergence. In all the cases, the Phyre2 server succeeded to model either one or two structural domains for the orthologous sequences. Among them, six does not present any structural assignment except for the SLH domain. Except for *Deinococcus gobiensis* (gi|386855514), all domains are assigned to a bacterial porin like structure built by the server using different templates. Among studied IRRB species, *Truepera radiovictrix* ($D_{10} > 5$ kGy [63]) is the only one with SlpA non-SLH domain presenting elusively an all α-fold type. Eight sequences showed an all β-barrel fold type for this non-SLH domain. In this context, it is important to note that previous findings have pointed out that Hpi contains a β-strand rich region [64]. Moreover, the main secondary structure of the domain PEGA (e.g., in DR_1185)—found in both archaea and bacteria and presenting similarity to SLPs—is predicted to be β-strands [65]. Taken together, these observations strongly suggest that the β-barrel fold type is "under positive selection" within the non-SLH domain of SLPs.

Based on the proposed model, it should be emphasized that the C-terminal part of the protein including the region with no assigned fold is the most interesting part being probably the outermost segment of the SLP (unpublished results). The orientation of the protein toward the outer membrane is reported in **Figure 8**. The residues fulfilling the criteria to chelate radionuclides (exposed, charged and forming structural clusters) generated by our pipeline are highlighted in the next section.

4. Prospective applications of ionizing-radiation-resistant bacteria (IRRB) S-layers in nanobiotechnology for bioremediation of hazardous and radioactive wastes

Many protocols related to nanobiotechnology with SLPs were previously highlighted by Schuster et al. [66]. Indeed, SLPs possess mesmerizing features that have attracted attention for their various promising applications as building blocks in nanobiotechnology (1–100 nm) for bioremediation of hazardous and radioactive wastes:

1. self-assembly *in vivo* and *in vitro* into crystalline arrays on many surfaces ([67] and references therein);

2. highly repetitive surface structures—oblique (p1, p2), square (p4) or hexagonal (p3, p6) array structures—with nanometric lattice spacing with different sizes (center-to-center spacing: 3–30 nm; thickness: 5–10 nm) [17];

3. enhanced properties (e.g., optical nonlinearity) suitable for bottom-up fabrication of novel types of active nanoelectronic devices ([68] and references therein);
4. localization of functional proteins at cellular surface [45];
5. negative charges and strong interaction with cations ([67] and references therein);
6. binding noble metal ions and cleaning up of uranium contaminated soils and waters with selective and reversible binding of high amounts of U (up to 20 mg U/g protein), Cu, Pb, Al, and Cd ([12] and references therein); and
7. diverseness (e.g., position of SLH domains).

Bacterial interactions with uranium have been documented extensively ([12, 69] and references therein): (i) biosorption and bioaccumulation; (ii) biotransformation of organic and inorganic uranium complexes; and (iii) enzymatically catalyzed reduction of U(VI) to U(IV), resulting in the precipitation as uraninite (UO_2). To the best of our knowledge, the molecular interaction of uranium with an SLP has been reported only for *L. sphaericus* JG-A12 (also known as *B. sphaericus* JG-A12) isolated from a uranium mining waste pile [12, 70]. The percentage AA identity between the known S-layer proteins of *B. sphaericus* strains was shown to be between 71 and 98% for the N-terminal parts and 20 and 87% for the C-terminal segments [71]. Interestingly, ortholog of *D. radiodurans* SlpA in *L. sphaericus* (*e*-value = $7e^{-08}$, 1258 aa, WP_010861478.1, hypothetical protein) shows three SLH domains at its C-terminal as depicted in **Figure 9**.

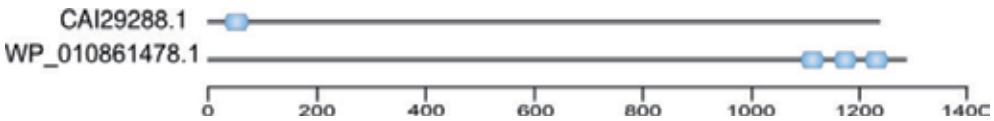

Figure 9. Surface layer homology (SLH) domains (blue shapes) at the N-terminal and C-terminal of two S-layer proteins (SLPs) of *Lysinibacillus sphaericus* JG-A12.

However, there remains the question of which and how specific SLPs AA sites interact with actinides, particularly uranium? In general, the relevant functional groups involved in the interaction between bacteria and metals are reported to be –COOH, –OH, $–NH_2$, and $–PO_4$, etc. [72, 73]. The segments at the outermost surface of SLPs are harboring –COOH, –OH, and $–NH_2$ groups [74]. In addition, using analyses of post-translational modifications, it was highlighted that SLPs can be phosphorylated [10]. Moreover, it was observed that, at acidic pH (4.3), SLPs formed complexes with U(VI), where uranium (U(VI)) is coordinated to carboxyl groups, in a bidendate binding fashion, and to phosphate groups in a monodendate binding mode [10–12]. Using our pipeline, exposed residues of *D. radiodurans* SlpA were predicted from the model we generated with Phyre2 [55]—[YVPTTATATTGDFSG], [TQLDSRPA], [YTADNRVAAGNYNANA], [AQNRQYTPFDGD], [SYG], and [KVN]; and interactions with uranium were modeled based on previous literature [10–12] (**Figure 10**).

This model (**Figure 10**) highlights the importance of the acidic residues (aspartic acid (Asp/D) and glutamic acid (Glu/E)) and the phosphorylation of the SLPs which can be carried at serine

(Ser/S) and threonine (Thr/T) groups. In this context, it is important to remember that the interaction between uranium and SLPs is dependent on the oxidation state of the actinide which in turn depends on the extracellular pH. Indeed, previously, the speciation of uranium associated with *Idiomarina loihiensis* strain MAH1 was shown to depend mainly on the pH [11]. Taking together, the S-layer could serve as a binding matrix to introduce new functional properties like metal sorption [75].

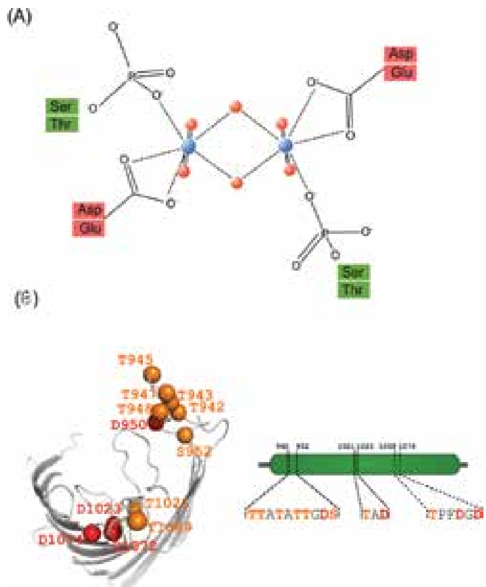

Figure 10. Model for the interaction between an S-layer protein (SLP) and an actinide (uranium) at pH 4.3 [10–12]. (A) Model of coordination between uranyl $[UO_2]^{2+}$ (red: O, blue: uranium) with residues (aspartic acid (Asp/D), glutamic acid (Glu/E), serine (Ser/S) and threonine (Thr/T)) from *Bacillus sphaericus* SLP as proposed by Merroun et al. [10]. (B) Mapping of negatively charged (red) and putatively phosphorylated Ser and Thr residues (orange) on the outermost exposed segments of SlpA predicted using the presented workflow. The residues are indicated by spheres corresponding to Cα atom. The model is truncated because the structure was not fully predicted by Phyre2 [55].

There is plenty of room for improvement of the proposed computational pipeline to enhance its predictive power of SLPs within IRRB. Text mining, a careful analysis of the sequence data, and structure prediction methods were revealed to be useful in probing pertinent functional groups of SLPs. Overall, this chapter is expected to incite *in vitro* research that might elucidate various aspects related to IRRB SLPs and bioremediation of hazardous and radioactive wastes.

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