NOTE TO USERS

ENHANCED DEGRADATION OF POLYCHLORINATED BIPHENYLS BY SURFACTANTS AND A DIOXYGENASE ENZYME COMPLEX

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Abstract

ENHANCED DEGRADATION OF POLYCHLORINATED BIPHENYLS BY SURFACTANTS AND A DIOXYGENASE ENZYME COMPLEX

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There is a worldwide initiative to remove polychlorinated biphenyls (PCBs) from the environment because of their potential hazard. Animal experiments have shown they can produce liver damage, developmental defects and even cancer. Thirty per cent of the 310,000 tons of PCBs that have been produced globally are now considered as persistent toxicants in the environment. To date, only 4% have been successfully treated. Although a number of technologies are in existence for the treatment of persistent organic pollutants (POPs), they do have several drawbacks including the production of toxic volatiles in incinerators and high cost associated with these procedures. On the other hand, successful bioremediation is relatively inexpensive, as shown for polyaromatic hydrocarbons (PAH) degradation, with the added advantages of being able to be carried out *in situ*.

In this study, preliminary experiments demonstrated that certain surfactants enhance PCB degradation in aqueous solution in the presence of enzymes, in a

concentration-dependent manner. These surfactants have not been previously reported as enhancers in PCB degradation. Conditions were optimized before experiments with soil samples were performed. A soil sample (free of PCBs) was spiked with varying amounts of PCBs and internal standards. Samples were mixed at various speeds, temperatures, incubation periods and concentrations of surfactants, before the metabolites were extracted and mass analyzed. An aged PCB-contaminated soil sample was also studied.

PCBs extraction recoveries for the spiked samples two hours after exposure to the toxicants were 93% whereas average recovery from the samples extracted after 60 days of toxicant exposure was 67%. Within 2 hours of incubation, 47, 28 and 14% of PCBs were biodegraded from the contaminated sample by the addition of surfactin, rhamnolipids and octyl glucoside respectively, at concentrations above their critical micelle concentrations. If the soil composition and mechanism for removal at a contaminated site are fully understood, *in situ* biodegradation can be exploited.

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List of Abbreviations

AOP	Advanced oxidative process
APEG	Alkaline polyethylene glycolate
BPDO	Biphenyl dioxygenase
CYP	Cytochrome P450 enzymes
EPA	Environmental Protection Agency
IARC	International Agency for Research on Cancer
NADH	Nicotinamide adenine dinucleotide
PCBs	Polychlorinated biphenyls
PAHs	Poly aromatic hydrocarbons
PCDD	Polychlorinated dibenzo-p-dioxins
POP	Persistent organic pollutants
VOC	Volatile organic compounds

Chapter 1

Introduction

1.1: Background

Polychlorinated biphenyls (PCBs) are toxic pollutants that threaten the environment. Their popular use in industry (in transformers, dielectric fluids, flame retardants etc) has resulted in widespread release in the environment for several decades. They are very stable, persistent and are even found in the food chain. They adhere tightly to soil, a key factor that limits their degradation.

High temperature incineration is one of the most applied technologies for the remediation of several contaminants including PCBs, pesticides, and explosives (Rahuman et al., 2000). Elevated temperatures (870°C to 1200°C) are used for the *ex situ* destruction of pollutants in soil. The process involves introducing the contaminated material into the incinerator high temperatures and the presence of oxygen, resulting in the volatilization and combustion of the pollutants with conversion into nontoxic materials. PCBs and dioxins can be destroyed using this technique, with high removal efficiencies (OHM Remediation Service, 1995).

However, recent work has demonstrated that certain non-combustion technologies can be more efficient. Moreover, the incineration of persistent organic pollutants (POPs) and other waste chemicals can spread undestroyed and other POPs that

are formed during the combustion process resulting in pollution of the environment. (Costner, 1998).

Cost-effective technologies are under development for the degradation of PCBs and polyaromatic hydrocarbons (PAHs) where bioremediation is being investigated. Because of current environmental laws, bioremediation processes are the logical options for PCBs disposal. These processes have been used successfully in treatment of PAHs in the petroleum industry. Successful biodegradation of PAHs depends on the availability of nutrients and oxygen, the soil composition, the inherent soil microbial population, and the type and concentration of hydrocarbons present. The technology is relatively inexpensive and can be done *in situ*, avoiding transportation of toxicants. A PAH mixture was successfully degraded by biosurfactants from *Pseudomonas aeruginosa* (Jain et al., 1992) after an incubation period of two months. An evaluation of PCB degradation in waste, contaminated with Aroclor 1248, a well known PCB, shows that *P. aeruginosa* could degrade most of the PCBs content in 90-130 days of incubation in a reactor (Hamdy et al., 1986). This system might be more efficient with the addition of surfactants.

1.2: Statement of the problem

Although there are eight known major dechlorination pathways, the main and most understood pathway is known as the catabolic " biphenyl" or bph pathway (Bedard, 2003). This is a four-step enzymatic process where the final products are chlorobenzoates and chlorocathechols, compounds which are far more water soluble and less toxic than the parent compounds. The first stage of this cascade utilizes an enzyme complex which

consists of (a) an biphenyl dioxygenase (b) ferridoxin reductase (c) ferridoxin and an electron donor NADH (nicotinamide adenine dinucleotide hydride) to convert the PCBs into cis-2,3-dihydro-2,3-dihydroxychlorobiphenyls. This reaction is one of the limiting factors in this pathway and its acceleration would enhance biodegradation.

The method was initially tested successfully on the degradation of commercial lyophilized PCBs in an aqueous environment. A reference soil (free of PCBs - manufacturer's specification and author's analysis) was then spiked with varying amounts of PCBs and internal standards. Samples were mixed, incubated at different agitation rates, temperatures and times. They were extracted with different solvents and recoveries calculated. In addition to the sample treatment described above, some of these spiked samples were also treated with enzymes and various concentration of a surfactant. The metabolites were also extracted and quantified.

The biosurfactants have not been previously reported as enhancers in PCBs degradation. If the soil composition at a contaminated site, and its mechanisms are fully understood, *in situ* biodegradation should be exploited.

This work focuses primarily on the acceleration of the first step in the catabolic biodegradation of PCBs in soil samples. Although the use of surfactants in PAHs degradation and oil recovery is well known, their role in PCB degradation is unclear. Biosurfactants and a chemical surfactant were utilized in conjunction with the purified enzyme complex.

1.3: Objectives

The objectives of this work were as follows:

- To evaluate surfactants for the enhanced degradation primarily for the first catabolic step in PCBs degradation.
- To evaluate surfactants / enzymes for the bioremediation of contaminated soil samples.
- To optimize process design by varying environmental parameters such as temperature, pH, incubation period, enzyme / contaminant ratio and degree of mixing required.
- To determine changes in the substrates and products during the remediation processes by high-resolution electrospray quadrupole time-of-flight mass spectrometry.

1.4: Organization of the thesis

The thesis is divided into five chapters. The contents are as follows:

- Chapter 1 includes the introduction of the subject, description of the problem, the objectives and organization of this particular study.
- Chapter 2 describes the background information for the various concepts covered in the thesis and a literature review of similar studies.
- Chapter 3 includes the materials, methods and procedures used in the experiments.

- Chapter 4 summarizes the results obtained from different experiments and discussion.
- Chapter 5 includes the conclusions, recommendations for further work and contributions.

Chapter 2

Literature review

2.0: Introduction

Polychlorinated biphenyls (PCBs) are a class of small ultra hydrophobic organic molecules that have infiltrated our environment for the past seventy-five years. They were first discovered as a by-product of coal tar in 1865 but were first synthesized by Monsanto in 1930 where they found industrial and commercial applications (Table 2.1) because of their chemical stability, high boiling point, low vapor pressure and extremely low flammability. PCBs show extreme thermostability (up to 350°C) and were less likely to explode than the mineral oils they replaced. In the electrical industry, they were particularly useful as dielectric fluids in transformers (Figure 2.1), as heat transfer fluids, flame-retardants and organic diluents. Another important use of PCBs was in the aluminum industry. Aircraft components and motor vehicular wheels are shaped by large hydraulic presses. These machines use massive volumes of lubricating oil whose temperature becomes elevated, creating potential fire and explosive hazards. The oils were replaced by PCBs and they were believed to be the most effective fire retardants.

They were also used as plasticizers in paints, plastic and rubber products, in dyes and carbonless copy paper, as additives in pesticides, in sealants and several other applications. Approximately 0.7 billion kg were manufactured in the United States before production was halted in 1977 (United States EPA, 2004).

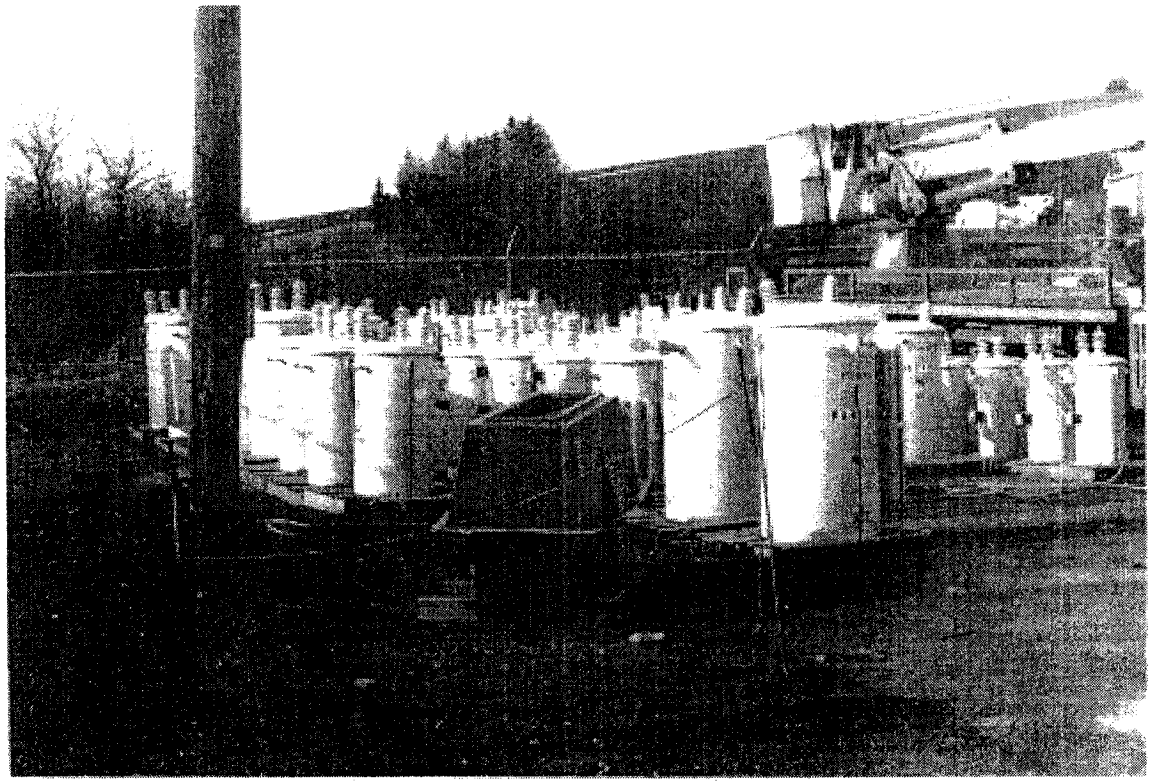


Figure 2.1: Transformers in a maintenance storage yard (Watts, 1997)

Table 2.1: Industrial use of PCBs (US EPA, 1994)

PCBs use	Percentage
Capacitors	50.3
Transformers	26.7
Plasticizer uses	9.2
Hydraulics and lubricants	6.4
Carbon-less copy paper	3.6
Heat transfer fluids	1.6
Petroleum additives	0.1
Miscellaneous industrial uses	2.2

The desired properties that render PCBs so exceptionally important in industrial applications have also made them persistent in the environment. Thirty years after being in use, it was discovered that these compounds tend to adhere tightly to soil and sediments at the bottom of lakes and wells rather than degrade. Preliminary medical data suggested they were a health hazard in 1968 (BAN, 2003) and consequently global attention became focused on their destruction.

2.1: Synthesis and structures of PCBs

The starting material, biphenyl (Figure 2.2) was subjected to progressive chlorination until the desired percentage of chlorine by weight was obtained. The process uses anhydrous chlorine and the temperature is above 150° C to keep the biphenyl molten and prevent clogging. Ferric chloride is used as a catalyst. This procedure cannot be

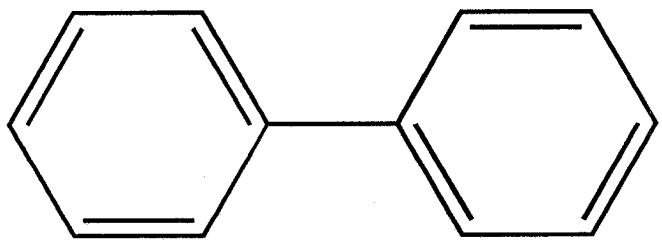


Figure 2.2: Biphenyl structure

rigidly controlled, and the crude product is purified with diluted alkali (0.3-0.5%) and distilled under vacuum to produce a mixture of several congeners. The reaction time (12 to 36 hours) will determine the percent chlorination retained.

In PCB terminology, a congener is defined as a particular chemical formula for a PCB, where the position and content of chlorine atoms are defined. There are 209 congeners in theory (Table 2.2) but 20 congeners are not present in commercial samples

Table 2.2: PCB Homologs and maximum dissolved concentrations in water for PCB compound groups

Homolog	Chlorine Atoms per Biphenyl	Number of Isomers (PCB congeners)	Aqueous Solubility (mg/L)[a]
Monochlorobiphenyl	1	3	5.90
Dichlorobiphenyl	2	12	2.03
Trichlorobiphenyl	3	24	0.64
Tetrachlorobiphenyl	4	42	0.18
Pentachlorobiphenyl	5	46	0.03
Hexachlorobiphenyl	6	42	0.01
Heptachlorobiphenyl	7	24	0.006
Octachlorobiphenyl	8	12	0.001
Nonachlorobiphenyl	9	3	0.0002
Decachlorobiphenyl	10	1	0.00008
	Total	209	

[a] Highest measured solubility value at 25°C for individual compounds in the compound group. (Opperhuizen et al., 1988).

due to statistical, mechanical and chemical constraints (Hutzinger et al., 1974). Their physical appearance can be an oily liquid (low chlorination) to waxy solids (high chlorination). They are colorless to light yellow in color and are odorless and tasteless. The "Aroclor" series, with a number identifying the percentage of chlorine, was the most common product name used. As an example Aroclor 1242 (Table 2.3) defines a PCB with 12 carbons containing approximately 42 % chlorine by weight. Arochlor 1242 was the most widely used PCB in the world. The composition of the Aroclor in Table 2.3 was

determined by gas chromatography with electron captor detection. PCBs have been manufactured and sold under many names including Aroclor, Arochlor, Clorphen and Chlorphen (Table 2.4).

Table 2.3: Percentages of mono, di, tri and tetra chloro PCBs in some Aroclors (Ultra Scientific, North Kingston, RI USA)

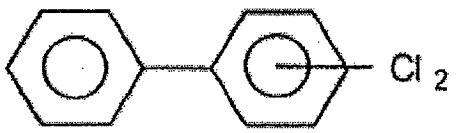
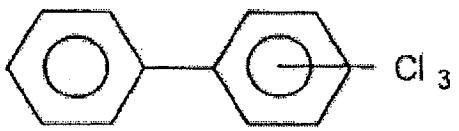
STRUCTURE	PERCENTAGE IN		
	1232	1242	1254
	12	3	2
	28	13	3
	32	28	9
	24	30	18
	4	22	68

Table 2.4: Commercial names used in the marketing of PCBs (Rahuman et al., 2000)

PCBs common trade names		
Aroclor	Chlorinol	Fenclor
Arochlor B	Chlorphen	Hyvol
ALC	Clophen	Inclor
Apirolio	Clorinol	Inerteen
Asbestol	Diaclor	Keneclor
ASK	DK	Kanechlor
Askarel	Dykanol	Magvar
Adkarel	EEC-18	MCS 1489
Capacitor 21	Elemex	No-Flamol
Chlorextol	Eucarel	Nepolin

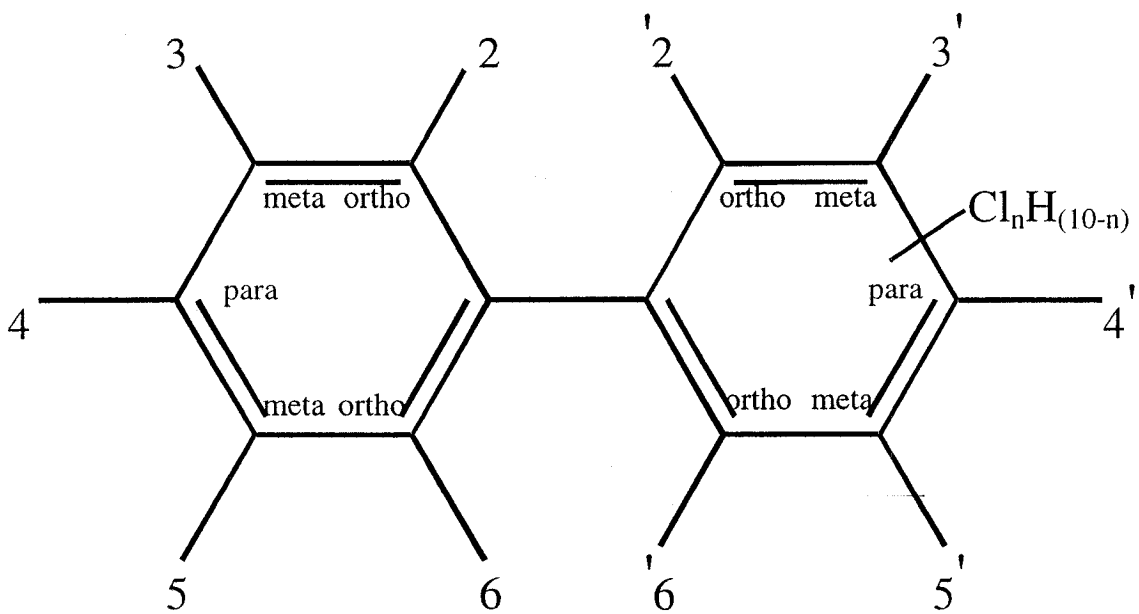


Figure 2.3: Polychlorinated Biphenyls (PCBs)

As shown in Figure 2.3, ten positions on the biphenyl molecule are available for chlorine substitution. These positions are numbered 2,2', 3,3', 4,4', 5,5'6, 6' with the 1,1' positions serving as the chemical bond between the two phenyl rings. Congeners with one chlorine atom are monochlorobiphenyls. There are three monochlorobiphenyls and they are called isomers – same molecular weight and same number of chlorine, carbon and hydrogen atoms. They are structurally different due to alternately positioned chlorine atoms. Those with two chlorine atoms are bichlorobiphenyls, three chlorine atoms (trichlorobiphenyls) etc. until the biphenyl is fully substituted with 10 chlorine atoms (decachlorobiphenyl). Positions 2,2' are known as ortho, 3,3' meta and 4,4' para.

Several possible names can be used to identify a given congener due to the equivalence of the phenyl rings and the ability of the rings to rotate freely about the 1,1' connecting bond, with the exception of decachlorobiphenyl. To avoid redundancies, naming conventions were established by IUPAC and CAS. Names begin with the lowest number(s) with a "primed" number considered greater than the corresponding "unprimed" number e.g. 2,2',3-trichlorobiphenyl. An additional rule states that the "primed" numbers may not exceed "unprimed" numbers in the name of given congener (Ballschmiter et al., 1992). Of the 209 congeners, 10 naming differences exist between IUPAC and Ballschmiter nomenclatures. To establish a convenient and systematic way for naming the 209 PCB congeners, a sequential numbering system was proposed known as the "BZ" numbers (Ballschmiter and Zell, 1980). The scientific community has largely embraced this system.

2.2: Health concerns

Recently several industrial chemicals, including PCBs, have shown their abilities to disrupt endogenous hormones. For example, the ortho-substituted (also known as non-planar) PCBs displayed estrogenic effects in animal experiments (Arcaro, 1999) whereas the non- and mono-*ortho* congeners exhibited a host of more serious biological concerns. This latter class, also called co-planar congeners, was initially believed to exhibit toxicity by binding to an aryl hydrocarbon receptor located in the cytoplasm of the cell, then transformed and relocated into the nucleus. It then occupies a binding site in the nucleolus modulating gene expression, which results in severe toxicity which can be manifested as, carcinogenicity, teratogenicity, immunotoxicity and reproductive dysfunction (Safe, 1994).

The identical physiological responses were seen by a similar class of compounds known as polychlorinated dibenzo-*p*-dioxins, also known as PCDD (Figure 2.4). Members of the cytochrome 450 family, the most important group of metabolic enzymes, are induced by PCBs to varying degrees. The non-ortho-PCB congeners have been shown to induce CYP1A1/1A2 through the aryl hydrocarbon receptor mechanism whereas di-ortho PCBs, having a low affinity for the Ah receptor and induce other members of the CYP family through other mechanisms. Mono-*ortho* PCB congeners, having dioxin like properties, can induce both CYP1A and CYP2B enzymes.

Thus various PCB congeners can induce enzymes involved in metabolism of carcinogens and hormones including estrogen (Wolff and Toniolo, 1995). Studies performed with purified liver P450 from rats induced with phenobarbital and β-napthoflavone have demonstrated the effects of PCB substitution on hydroxylation of

various dichlorobiphenyls. The two P450 enzymes induced by phenobarbital and β-napthoflavone respectively differed in the rates and regioselectivities of the dichlorobiphenyl metabolism raising the possibility that the toxicity and carcinogenicity of the PCBs may depend on the P450 enzymes induced (Kaminsky et al., 1981).

PCBs can also affect neurotransmitters in adults (Seegal, 1996). Furthermore, a study of the effects of 43 individual congeners found that they decrease dopamine concentrations *in vitro* whereas the coplanar congeners had no activity (Shain et al.,

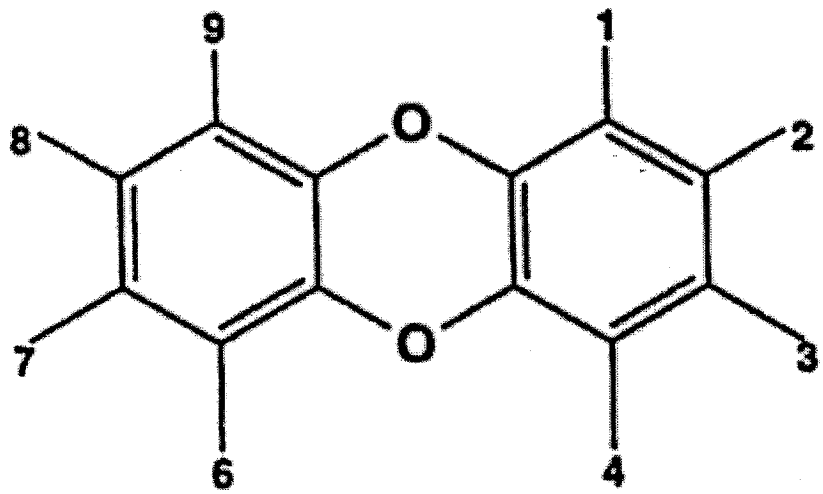


Figure 2.4: Polychlorinated dibenzo-*p*-dioxins

1991). The PCB Aroclor 1254 produces a concentration dependent reduction of basal and cellular levels of dopamine (Tilson et al., 1988). *Mono-* and di-*ortho* congeners reduced the amount of dopamine released from PC12 cells, while the non-*ortho*-congener

3,3',4,4',5-pentachlorobiphenyl affected dopamine release only at cytotoxic concentrations (Lee et al., 2004).

Several other neuroendocrine and neurochemical effects have been associated with PCBs. Studies have shown that exposure of rats to PCBs results in reduced 5-hydroxytryptamine in certain regions of the brain. Furthermore a single oral dose of Aroclor 1254 induce a decrease of 5-hydroxytryptamine in specific certain regions of the rat brain by inhibiting tryptophan hydroxylase activity. Similar results were observed in the Atlantic croaker. These authors concluded that TPH is a key target in PCB neurotoxicity in mammals and fish (Khan and Thomas 2004). Basal levels of corticosterone are increased in response to PCBs exposure. This compound also alters neurite elongation *in vitro*. The ontogeny of rat monoamine oxidase, acetylcholinesterase, testosterone and estradiol are altered by exposure to PCBs (Vincent et al., 1992). Several reports have described the interference of PCBs on thyroid function. PCBs are structurally related to thyroid hormone and recently it was shown they can induce suppression of transcription to cause partial dissociation of the thyroid hormone receptor and the retinoid receptor complex from the hormone response element (Miyazaki et al., 2004). Gestational and lactational exposure to Aroclor 1254 causes persistent changes to glial and neuronal cell markers in rats (Morse et al., 1996). In general, non-dioxin like *ortho*-substituted PCB congeners affect brain neurochemistry, while non-*ortho*-substituted PCBs with dioxin like activity do not significantly affect the nervous system.

The most commonly observed health effects in people exposed to large amounts of PCBs are skin conditions including rashes and acne. The deleterious effect of this xenobiotic family was demonstrated in an unfortunate accident in Japan. In 1968, rice

bran oil was accidentally contaminated with Kanechlor 400 (US equivalent of Arochlor 1248). The contamination was estimated to be 1500-2000 ppm. Over 1000 people orally consumed the oil for several months, which led to an outbreak of what became known as "Yusko disease". The disease manifested as nausea, lethargy, brown coloration of the skin and nails, edema, chloroacne, excessive eye discharge, visual impairment, swelling of the eyelids, jaundice and gastrointestinal disturbance (EPA, 1976). Individuals with ages ranging from 13 to 29 were most affected. The manifestation continued for as long as six years after the original ingestion. Other damages include decreased hepatic function, lower birth weight in kids whose ingested contaminated food (Safe, 1994). There was also retarded growth in affected children.

Similar symptoms occurred in another accident in Taiwan in 1979, although less severe since the contamination was found to be about an order of magnitude lower. Generally, the average population is not likely to be exposed to sufficient levels of PCBs for the effects on skin and liver to manifest (Wikipedia, 2005). Knowledge of the effects of PCBs on the general population is primarily derived from studies of children of mothers who were exposed to PCBs (Tilson and Kodavanti, 1997).

Animals exposed acutely to PCBs through contaminated foods had liver damage with some fatalities. Those exposed to lower concentrations of PCB - contaminated food for several weeks or months developed various health effects including anemia, skin conditions resembling acne, and injuries to liver, stomach and thyroid. PCB exposed animals also exhibited changes in the immune system, behavior and impaired reproduction.

Several health organizations including the Department of Health and Human Services, the Environmental Protection Agency (EPA) and the International Agency for Research on Cancer (IARC) have concluded that PCBs may be considered as carcinogens. Rats fed with PCB contaminated food for two years eventually developed cancer of the liver. Some studies have demonstrated that PCBs exposure causes the development of certain human cancers, including liver and bilary tract cancer (Wikipedia, 2005).

Significant levels of PCBs in flounder fish caught in Boston harbor have been

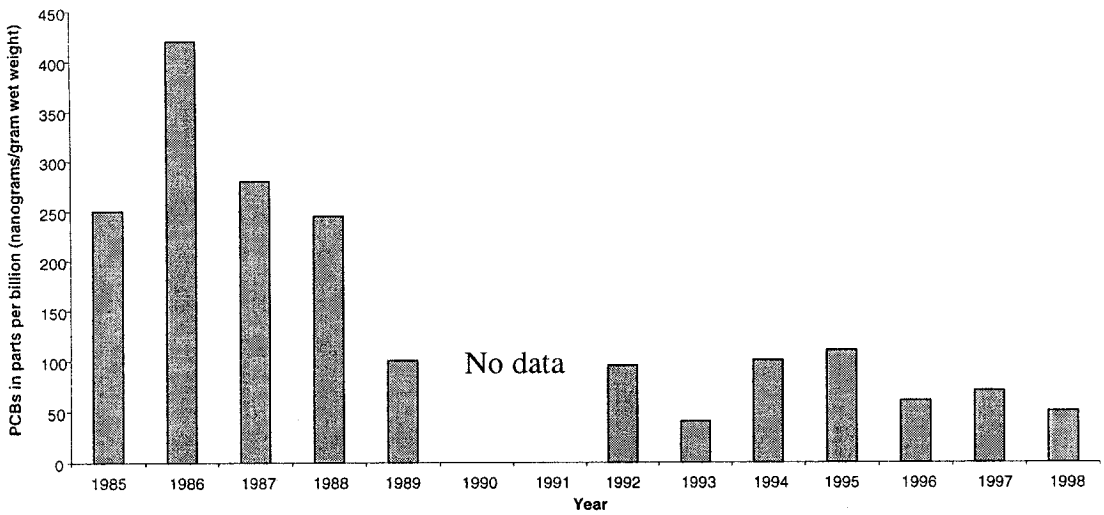


Figure 2.5 PCB concentrations in winter flounder (filet) caught near Deer Island, Boston Harbor, 1985-1998 (MWRA, 1999)

detected (Figure 2.5). Fortunately, the levels have been decreasing since 1986.

Mothers exposed to high levels of PCBs in the workplace or from contaminated fish had underweight babies. These children were prone to abnormal responses to behavioral tests. Several of the behavioral problems such as short-term memory loss and

motor coordination lasted for several years. Children exposed to PCBs via their mother were intellectually impaired (Harada, 1976). Prenatal PCBs exposure was correlated with poorer visual recognition memory according to the Fagan test (Jacobson et al., 1985). Later it was demonstrated that children with increased serum PCB levels had poorer performance on McCarthy Verbal and Memory Scales (Jacobson et al., 1990). Hence, research in humans suggests that developmental exposure to PCBs affects brain development with impaired cognitive function. Research conducted on laboratory

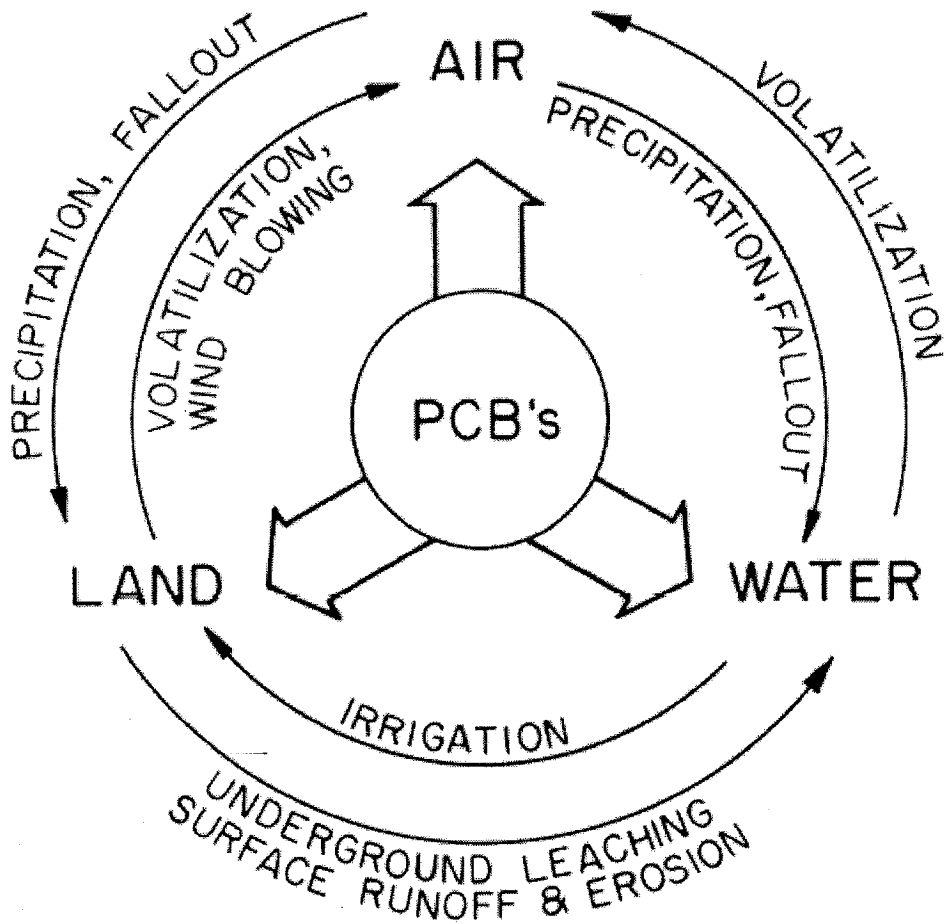


Figure 2.6: PCB environmental cycle (Lauber, 1986)

animals supports the results observed in humans (Tilson et al., 1990).

A recent study correlated PCBs exposure to an increase risk of Non-Hodgkin's Lymphoma (NHL) (Rizzo, 2005). The reasons for the rise in NHL occurrence over the past several years are unclear. These compounds concentrate in carpet dusts where they remain for several years. The concentration of PCBs in the dusts was used as the indicator and NHL incidence were higher with owners of older carpets.

PCBs deposited in a particular site can migrate to other environments.

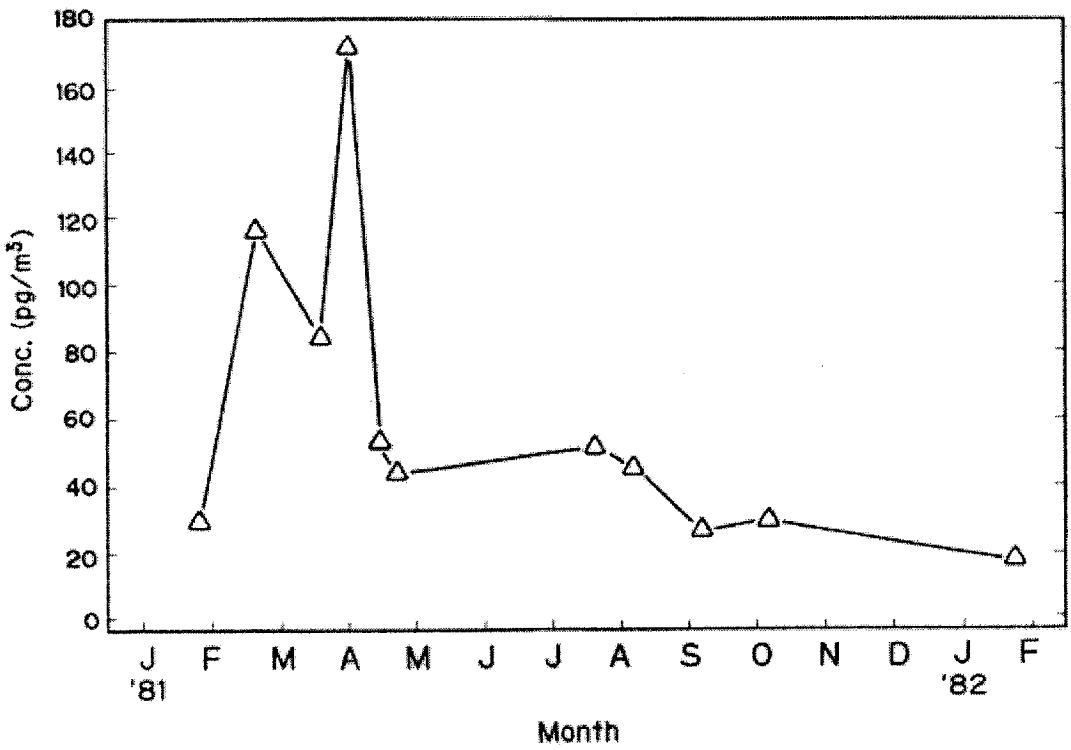


Figure 2.7: Seasonal variation of PCB concentrations in the Antarctic atmosphere (Tanabe et al., 1983)

Contaminants can volatilize, bind atmospheric particles and return to distant land and water surfaces in a cyclic fashion (Figure 2.6). The variation in the atmospheric concentration of PCBs at Syowa station Antarctica from (January 81' to January 82') is shown in Figure 2.7. High concentrations of PCBs were found in the austral summer.

2.3: PCBs in the environment

PCBs being denser than water tend to sink to the bottom of pools and wells, thereby contaminating the sediments. They are very lipophilic molecules and prefer to concentrate in fine-grained sediments that are abundant in native organic matter (NOM). They prefer to associate with carbon-containing molecules like themselves rather than molecules that do not contain carbon. They are very slightly soluble in water (Table 2.2); congeners with the highest amount of chlorine being least soluble. Isomers show slightly different solubilities. PCBs exhibit very low vapour pressures. They attach themselves to soil, which contains carbon. Neutral compounds attract other neutral compounds, hence the strong interaction between animal fatty tissues and PCBs. PCBs have an affinity for neutral hydrophobic soil components. Over 99% of the PCBs in the environment are soil-bound (Travis and Hester, 1990). Remediation of soils containing PCBs or other persistent chemicals e.g. polyaromatic hydrocarbons (PAHs) is currently a major environmental problem.

2.4: Management technologies for PCBs

In general, dredging methods are commonly used to remove contaminated sediments. In principle, these methods are inefficient since the contaminated sediments are re-suspended in the water column and can never be completely removed. These methods have not been successfully implemented for PCBs removal (Cassidy et al., 2002). PCBs levels found in fish after dredging were higher than before dredging (Bremle, 1997). Presently, the two most popular disposable methods for sediments containing PCBs are incineration and landfilling (Bracewell et al.,1993). Both techniques

Table 2.5: Electrochemical peroxidation reactions (Rahuman et al., 2000)

Fenton's reagent creates free radicals (^{o}OH)

Eq. 1: $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + {}^{o}OH$

Free radicals produced can participate in reactions which indiscriminately oxidize organic matter

Eq. 2: $RH + {}^{o}OH \rightarrow H_2O + R^{o}$

Organic radicals (R^{o}) can also be oxidized, dimerized or reduced, resulting in a variety of possible reaction pathways and products

Eq. 3: $R^{o} + Fe^{3+} \rightarrow Fe^{2+}$ **+ Oxidized product**

Eq. 4: $2R^{o} \rightarrow$ **Dimer product**

Eq. 5: $R^{o} + Fe^{2+} \rightarrow Fe^{1+} + RH$

Eq. 3 Allows for the regeneration of Fe^{2+} from Fe^{3+} which allows for the propagation of redox chain reactions

RH = Organic contaminant

are inherently problematic and are very expensive. Ideally, *in situ* methods are preferred, and are currently being investigated by several groups.

Capping is a very common management technology. The cap provides a cover for the buried wastes, preventing contact with the environment. This system is used to effectively manage the risks associated with a remediation site. The most critical aspects of a cap are its barrier and drainage layers. The cap layer can be a simple layer of soil or a complex multilayer system of soils and geosynthetic materials. The nature of the environment and type of waste will determine the type of cap to be used. For example, dry climates may only require a simple cap while wet climates may require a more complex system to prevent infiltration of precipitation. Caps can be used for waste masses that are too large to be treated by other methods. The method is not suitable for the disposal of liquid pesticides or very mobile wastes. In addition, capping is not a destructive technology for persistent substances such as PCBs.

Landfilling PCBs can provide a temporary means of containment. Nevertheless the containment provided may not be effective as the pollutants can escape through groundwater by leaching and other means (Chiarenzelli et al.,1998; United Nations Food and Agriculture Organization, 1996).

Another approach to enhance degradation of PCBs in contaminated soil is chemical oxidation before biological treatment. Fenton reagent (hydrogen peroxide and iron) can produce free radicals to oxidize PCBs (Aronstein and Price, 1995) (Table 2.5).

Hydrogen peroxide has been shown to enhance oxygen delivery in many cases. Because oxygen is sparingly soluble in water, its delivery is very difficult for in *situ* treatment. In contrast, hydrogen peroxide is soluble in water and breaks down into oxygen and water to yield 0.47 g of oxygen per g of hydrogen peroxide. Injection of this oxidizing agent requires materials of special construction. These authors achieved a seven-fold increase in subsequent biodegradation with the use of hydrogen peroxide. This process is only feasible below pH 3, and pH adjustment was required for subsequent biological treatment. The reaction produces excessive heat, which can release volatile components into the atmosphere. On the contrary, a novel technique called ozone sparging can be done at neutral pH prior to degradation by bioaugmentation. In this procedure, ozone reacted with the PCBs to remove the chlorine atoms stoichiometrically. Ring cleavage did occur and the final products were organic acids (Cassidy et al., 2002).

Historically, persistent organic pollutants (POPs) including PCBs that are not easily destroyed have been managed by several methods including storage, landfilling, and combustion in incinerators, boilers or cement kilns. Of these approaches, only the combustion methods achieve destruction of pollutants, but to a limited extent (Rahuman et al., 2000).

Combustion methods such as high temperature incinerators and cement kilns can be effective for the destruction and removal of PCBs and dioxins. Waste materials are heated to very high temperatures with process designs allowing for long residence times with high destruction efficiencies. In particular, the alkaline conditions of the cement kiln are well suited to the destruction of chlorinated organic wastes. Neutralized chlorides and sulfates are formed from chlorinated liquids, chlorine and sulphur. Small quantities of

inorganic and mineral elements added to the chlorinated waste are sufficient for chlorine neutralization. Ideally, waste materials for combustion methods can add energy as a substitute fuel. Both liquid waste and low ash wastes can be easily burned in the cement kiln. The material can be added as a fuel supplement and fed into the combustion zone, which is heated to 1450°C. However, combustion methods can produce fumes and volatiles that are much more toxic than the original compounds (Costner, 1998). As a result, growing concerns about the emissions from incinerators, boilers and kilns have resulted in public opposition to the use of the technology for waste remediation. Alternatives to incineration for the destruction of pollutants have been sought.

2.5: Other treatment technologies for dechlorination

The use of commercial ZHI powder for PCB dechlorination has also been investigated in subcritical water raised to very high temperature and pressure (Yak et al., 1999). Under such conditions, water acts as a solvent resulting in significant dechlorination of aroclor 1260. Highly chlorinated PCBs were converted to more lightly chlorinated congeners. Lightly chlorinated congeners were more resistant to dechlorination but did reduce to biphenyl. Research performed by the same group has shown that the position specific dechlorination pattern was similar to that observed by microbial remediation (Yak et al., 2000). The positional facility of dechlorination was para>meta>ortho. The difficulty in dechlorinating ortho substituted PCBs may step from their non-coplanar orientation which prevents the free rotation about the C1 carbon. The electron cloud is thought to hover over the opposite phenyl ring, preventing its reduction (Yak et al., 2000).

Experiments have shown that ZHI can also rapidly and extensively dechlorinate PCBs in contaminated sediments (Gardner, 2002). When micro scale ZHI particles were injected into PCB rich sediments taken from two sites, exciting results were obtained, with 84 % of PCBs dechlorinated in one day in the sample taken from the Housatonic River. Sediments taken from the New Bedford Harbor showed a 56% decrease in PCB content with the same incubation period. In both instances, biphenyl was observed as a reaction product, indicating the complete dechlorination of certain congeners. The difference between the two results obtained with the two samples is attributed to the nature of the sediment. The Housatonic River sediment is loose and sandy with a weak adsorption of PCBs, while the sediment taken from New Bedford Harbor contains more clay with a decreased rate of PCB desorption.

2.6: Solubilization of PCBs and their entry into the cell

The hydrophobicity of PCBs may cause them to accumulate in the cell membrane, the most hydrophobic region of the cell. It is possible that the sequestering of the PCBs in the membrane may result in decreased bioavailability and degradation. Excessive accumulation of PCBs in the cell membrane may disrupt cellular function and lead ultimately to toxic effects (Sikkema et al., 1995). PCBs have limited water solubility and bind tightly to soil particles, which makes their biodegradation inefficient. The enzymes expressed by microorganisms capable of degrading PCBs are localized intracellularly, which requires that the contaminant be solubilized in order to enter the cell. Optimizing the solubilization of PCBs can facilitate their entry into the cell, with an increased rate of

PCB degradation. Surfactants can be used to enhance PCB solubility. However, it has been demonstrated that increased PCB solubilization does not necessarily increase the rate of their degradation (Billingsley et al., 1999). Explanations for this observation may include toxic effects of the surfactant to the cells, or the sequestering of PCB with micelles with a decrease in bioavailability. Several reports have verified the beneficial characteristics of biosurfactants in an extremely wide variety of industrial processes (Fava et al., 2003; Golyshin et al., 1999). One drawback to the use of biosurfactants is their high cost. Given these considerations, the use of a biosurfactant producing strain for PCB degradation would be ideal (Bodour et al., 2003). Identifying biosurfactant-producing strains that thrive in a given contaminated environment may be useful as such an organism may be transformed with genes encoding desired PCB degrading enzymes.

Progress has also been substantial in understanding the chemical, hydrologic and biotic factors involved in *in situ* remediation. Methods for delivering oxygen have also been improved. It has also been discovered that hydro-fracturing can improve the permeability of various media. Understanding biological processes is also important in determining intermediate products, and environmental requirements.

Bioremediation, in comparison to other chemical processes, is less expensive and is low in energy requirements. It is effective and complete mineralization is possible. For bioremediation, one should consider using a biosurfactant rather than a synthetic surfactant given the decreased toxicity and biodegradability of biosurfactants (Makkar and Rockne, 2003). For in situ remediation, the waste is treated on site without the liability associated with hauling and disposal. The risk that the contaminants will be spread further is reduced, particularly since microorganisms reduce soil permeability

(U.S DOE, 1995). In addition, the presence or absence of appropriate bacteria is another factor in determining if biodegradation is an appropriate method. Biological treatment is a cost-effective method that is less likely to produce toxic by-products.

Bioremediation is the second most widely used innovative technology. Bioremediation projects have increased from 1982 to 1996. Petroleum hydrocarbons, BTEX and PAHs are treated most frequently. In terms of soil treated, bioremediation (ex situ) has been used to treat more soil than another process (38,000 m^3). For *in situ* technologies only SVE treats more soil as a total than bioremediation.

When surfactants are added to aqueous media, they form micelles above the CMC, with the hydrophobic tail of the surfactant within the micelle and the polar head in contact with the media. The PCB partitions in the hydrophobic core. MS/MS of cyclic peptides – protonated peptide as the base peak in the MS/MS spectrum from concurrent cleavage of the ester bond and the peptide bond in position two.

Surfactant enhances remediation in two ways. It causes a reduction in the PCB water interfacial tension when its molecules partition to PBC surfaces. This reduction decreases the capillary forces binding the globules in place allowing increasing the mobility. With extra mobility, hydraulic contact can be difficult to maintain. Sometimes it is better to achieve greater solubility than mobility. In such cases, surfactants that cause less reduction in interfacial tension can be chosen. Other system properties like ionic strength, and hydrophilic=lipophilic balance can be optimized. Aerobic microorganisms attack the less chlorinated PCBs and anaerobic microbes attack the highly chlorinated PCBs. They compliment each other (Cutter 2001). Fungi can possibly attack all the congeners.

Since the 1930s, Monsanto manufactured PCBs by bulk electrophilic substitution reactions, which in addition to steric effect, cannot control the where the chlorine atoms are placed, The chlorine content can be as little 21% (Aroclor 1221) or as high as 68% (Aroclor 1268) (Sather et al.al.2003).

For several years GC methods were dominant in PCBs separation and quantitation. More recently with the development of better stationary LC phases, HPLC methods are increasing used. Recently, several lipopeptide with antimicrobial and surface active properties have been produced from different *Bacillus subtilis* strains (Hagelin, 2005). Surfactin, fengycin, iturin, pumilacidin, bacillomycin, bacitracin and lichenysin are some examples. Most of the peptides are produced in mixtures and can be easily enriched from the cultures by HCl precipitation. Obtaining individual peptides is more time-consuming although separation is achievable by RP-HPLC.

2.7: Newer technologies for bioremediation

One novel technology involves supercritical oxidation which according to ProChemTech, Environment Australia, 1997) is the best way to treat organic contaminated wastewater. Under supercritical conditions (high temperature and pressure) with the addition of a suitable oxidant (e.g. oxygen, peroxide, nitrate), carbon is converted to carbon dioxide; hydrogen to water, chlorine atoms derived from chlorinated organics are converted to chloride ions; nitro-compounds to nitrates, sulfur to sulphates, and phosphorus to phosphates. The success of the technique stems from the unique properties of supercritical water. Gaseous oxygen and organic compounds are completely

soluble in supercritical water, whereas inorganic chemicals have much reduced solubility. The solubilized organic chemicals are completely and rapidly oxidized by oxygen at the high temperature and pressure conditions. Products of the process include, water, gas and solids. Gaseous effluents do not contain oxides of nitrogen or volatile acids such as hydrogen chloride or sulphur dioxide. No particulates are generated in the process and less than 10 ppm carbon monoxide has been measured.

Another technology, electrochemical oxidation, which was originally developed for the high efficiency conversion of a wide range of radioactive organic substances into environmentally acceptable waste streams. Experiments with chemical warfare agents were successful in destroying an organophosphorous nerve agent resulting in non-detectable levels after one hour of treatment while two hours of treatment were sufficient for an organochlorine agent called mustard gas (Environment Australia, 1997). The process uses an electrochemical cell to generate oxidizing species at the anode in an acid solution, usually nitric acid. The oxidizing agents then attack any organic compounds, converting them into carbon dioxide, water and inorganic ions at a low temperature (< 80 °C) and atmospheric pressure. The organic and water content of the waste can vary greatly enhanced / reduced without compromising the process. Aliphatic and aromatic hydrocarbons, phenols, organophosphorous, organosulphur compounds, chlorinated aliphatic and aromatic compounds have all been successfully destroyed by this process. (Environment Australia, 1997).

Solvated electron technology is a process whereby halogenated compounds are neutralized by the most powerful reducing agent known, solvated free electrons. The process involves dissolving a base metal such as sodium, calcium or lithium in anhydrous

liquid ammonia. As the metal dissolves, electrons are freed. Halogenated compounds having a strong affinity for the free electrons are instantaneously neutralized as they are introduced into the medium. Chlorine derived from PCBs is ionized in the process and reacts with the sodium to form sodium chloride, which is completely non-toxic. An added advantage of the technique when applied to contaminated soil is the enrichment of nitrogen in the cleaned soil resulting from the liquid ammonia.

Gas-phase chemical reduction with hydrogen at > 850°C can be used to reduce organic compounds to methane, hydrogen chloride, and minor amounts of low molecular weight hydrocarbons (benzene and ethylene). Steam is used in the process for heat transfer. The addition of caustic soda to the cooling reaction neutralizes the hydrochloric acid. The process quantitatively converts organic chemical including PCBs, polyaromatic hydrocarbons (PAHs), chlorophenols, dioxins, chlorobenzenes, pesticides, herbicides, and insecticides to methane. Because the process occurs in a reducing atmosphere devoid of oxygen, the formation furans and dioxins is eliminated (Environment Australia, 1997). The process has been used in tests at a commercial scale for the destruction of high-strength PCB oils and chlorobenzenes. Dioxins present in the PCB contaminated oil were destroyed with efficiencies ranging from 99.999 to 99.9999% (Schwinkendorf et al., 1995).

Halogenated contaminants can be dehalogenated by a chemical process that uses hydrogen or a reducing radical containing hydrogen donor. Two examples of the technique include the alkaline polyethylene glycolate process (APEG) and base-catalyzed decomposition These processes are applicable to halogenated aromatic compounds such as PCBs, PCDDs, ,chlorobenzenes, chlorinated phenols, organochlorine pesticides,

halogenated herbicides, and selected halogenated aliphatic including ethylene dibromide, carbon tetrachloride, chloroform, and dichloromethane.

Glycolate dehalogenation is a process that uses an alkali metal hydroxide and polyethylene glycol together to form a reagent called APEG (Tundo et al., 1985; EPA, 1990; EPA, 1992; EPA, 1995; EPA, 1996; EPA, 1997). Common alkali metal hydroxides include sodium and potassium hydroxide. The contaminated soil is mixed with the APEG reagent and heated. The alkali metal reacts with the chlorinated contaminant to form glycol ether and/or a hydroxylated compound and an alkali metal salt, all of which are water-soluble. The products of the reaction are non-toxic materials.

The APEG process consists of five steps: preparation, reaction, separation, washing, and dewatering. The preparation step involves excavation of contaminated soil and removal of large stones and logs. The reaction phase involves mixing of the contaminated soil with APEG reagent in a large blender (reactor) and heating for four hours. Vapors are collected and separated into water and gaseous contaminants by condensation; the water is recycled for use at a later step and the contaminants are captured with activated carbon filters.

The treated soil-APEG mixture is separated and the APEG reagent is recycled. The "detoxified" soil contains reagents and reaction products that are less toxic than the contaminants initially present. Treated soil is washed with the water collected from the previous condensation step, removing the last traces of residual APEG reagent, which is also recycled into the process. The washed soil is dewatered, and the collected water is treated to remove contaminants before release into an appropriate discharge area. Treated and washed soil is reanalyzed to determine if contaminant levels are below the desired

specification. Soil with unacceptable levels of contaminants can be recycled through the process or placed in an environmentally safe landfill. Clean soil can be returned to its site.

Base-catalyzed decomposition was developed in a joint effort between the EPA and the National Facilities Engineering services center (NFESC) to remediate liquids, soils, sludge and sediments contaminated with chlorinated organic compounds including PCBs, dioxins and furans. The process involves excavating contaminated soil with screening to remove large particles and debris followed by crushing and mixing with sodium bicarbonate. The mixture is heated to 200-400°C in a rotary reactor causing the volatilization of the halogenated compounds, which are captured and condensed for further treatment. Sodium bicarbonate was added to promote lower temperature desorption with partial decomposition of chlorinated organics. The technique uses an inert atmosphere above the heated soil preventing combustion and the formation of oxidized by products such as dioxins. The condensed gases are fed into a liquid phase reactor where they are mixed with sodium hydroxide (and other chemicals) resulting in the dechlorination reaction (National Research Council, 1993). The resulting dechlorinated mixture is then incinerated or treated by other technologies and recycled. The treated soil that now contains PCB concentrations less than 2 ppm can be safely returned to the site. The BCD process has the advantage of not requiring the removal of reactants from the treated soil.

Molten metal pyrolysis is a new technology for the conversions of hazardous wastes into useful materials. The process also stabilizes and reduces the volume of low level radioactive wastes to a fraction of the initial volume, reducing the costs of disposal.

The catalytic extraction process (CEP) involves adding industrial wastes to a sealed bath of molten metal (typically iron) heated to 1315-1650°C. Waste compounds are catalytically broken down to their elements by the molten metal. The reaction products are recovered as gasses that contain oxidized and decomposed contaminants, ceramics (consisting of silica, alumina and calcium chloride), alloys which are formed by adding appropriate materials such as oxygen and alumina, and liquid wastes may be obtained if wet scrubbers are used to control air emissions (Schwinkendorf et al., 1995). The gases may be used for the production of organic chemicals such as methanol. The ceramics may be used or buried in landfills, and the metal by-products (ferroalloy) can be used.

The catalytic hydrogenation of chlorinated wastes over noble metal catalysts has been known for several years (Environment Australia, 1997). Unfortunately, noble metal catalysts are susceptible to poising by a variety of components found in waste materials. The CSIRO Division of Coal and Energy Technology has developed a process for treating transformer fluids using metal sulphide hydrogenation catalysts. These catalysts are robust and can tolerate most catalytic poisons. The process has been demonstrated to destroy a wide variety of chlorinated hydrocarbons, producing hydrogen chloride and light hydrocarbons. Recent experiments conducted with POPs in hydrocarbon solvents resulted in the destruction of the contaminants to levels below the limits of detection (Table 2.6).

Advanced oxidative processes (AOPs) make use of O_2, H_2O_2, TiO_2, UV light, electrons, iron, and other oxidizing compounds for the detoxification of PCBs and volatile organic compounds (VOCs). Free radicals produced from the AOPs indiscriminately destroy organic compounds. Electrochemical peroxidation uses

electricity, steel electrodes and peroxide to degrade PCBs and VOCs. The process works by Fenton chemistry whereby free radicals are created that indiscriminately oxidize organic matter. Electrochemical peroxidation can be used for the low cost degradation of PCB present at low levels in soil, sediments, and dredged slurries. The process takes just minutes to degrade PCBs in sediment and water (Environmental Research Center, SUNY College, Oswego, NY 13126).

Table 2.6: Destruction of several persistent organic pollutants (POPs) by catalytic hydrogenation in a hydrocarbon solvent to below the limits of analysis (Duffy et al., 1997).

Compound	Feed concentration (mg/kg)	Product concentration (mg/kg)	Destruction efficiency, %
PCB	**40 000**	**<0.027**	**>99.99993**
DDT	40 000	<0.004	>99.99999
PCP	30 000	<0.003	>99.99999
HCB	1 340	<0.005	>99.99996
1,2,3,4-TCDD	46	<0.000004	>99.99999

Solvent extraction followed by chemical dehalogenation and radiolytic degradation can be used to decontaminate soil. The technique uses a solvent to extract specific contaminants from soil with subsequent treatment of the extraction mixture and recovery of the solvent to be recycled in the process. Soil or sediment cleaned by this process may be returned to the site of origin or placed in a landfill. US EPA guidelines allow the use of non-harmful solvents for the extraction of PCBs (USEPA, 1998). Although the method is cost effective in removing contaminants from soil, the extracted contaminants must be destroyed by some means. Chemical dehalogenation with immobilized reagents and gamma-ray irradiation have been successfully employed. PCB

concentration in transformer oil has reportedly been reduced from 700 ppm to non-detectable levels in less than five minutes using chemical dehalogenation. Radiolytic degradation of PCB with high doses of gamma ray radiation has also been reported (Nam et al., 1999).

Photochemical degradation using solar light has been used for the degradation of natural and synthetic organic compounds. The earth's atmosphere greatly attenuates the penetration of the UV light required for the direct and indirect photolytic degradation of pesticides and PCBs present in soil and surface waters. Given the attenuation by the earth's atmosphere, the effectiveness of the technology is strongly dependent on latitude, season and meteorological conditions (Plimmer, 1998). The main advantages of the technique are decreased fuel costs, improved thermal destruction of contaminants, reduced exhaust gas volumes including products of incomplete combustion. The processes can use either thermal energy or photochemical reactions. In order to make efficient use of the technique, solar radiation is concentrated by reflecting mirrors (heliostats) that direct the sunlight to a receiver that can reach temperatures of up to 2,300°K without any auxiliary fuel requirement. The technique has been shown to degrade organics, including pesticides, up to one hundred fold more efficiently than other thermal methods. High efficiencies have been obtained at a temperature of just 750°C, significantly lower than required for thermal incineration. The economic advantages of such a technique are particularly attractive given the current very high price of fossil fuels.

2.8: Surfactants

The primary use of surfactants is to lower the surface tension of a liquid and interfacial tension, increasing its ability to spread. Their properties also include wetting and penetrating actions. The word "surfactant" is a contraction of **surf**ace **act**ive **agent**. They are primarily organic amphipatic molecules (i.e. containing both hydrophilic and hydrophobic groups. For example, they can loosen oil that adheres to soil, keeping it dispersed in solution for eventual removal. These amphiphilic molecules prefer to reside between the hydrophilic and hydrophobic phases where they concentrate to form micelles.

Surfactants are used for a variety of applications including adhesives, flocculating, wetting, and foaming agents, deemulsifiers, and penetrants (Wilkinson et al, 1990). They are generally organic molecules that contain both hydrophilic and lipophilic portions (Rosen, 1975). They are classified as cationic, anionic, and nonionic. Octyl glucoside (Figure 2.8) is an example of a sugar-based non-ionic detergent. It is a member of the alkyl polyglucosides. An effective surfactant can significantly lower surface tension. For example, a good surfactant can lower the surface tension of water (air-water interface) from 72 mN/m to 35 mN/m and the interfacial tension (oil-water interface) for water against *n*-hexadecane from 40 nM/m to 1 nM/m (Rosen, 1975).

The surfactant concentrates at the interface (solid-liquid, liquid-liquid, or liquid-vapor). The surface tension correlates (decreases) with increased concentration of surface active compound until the critical micelle concentration (CMC) is reached. The CMC is defined as the minimum concentration of surfactant required to initiate micelle formation

(Cooper et al., 1980). Additional concentration of surfactant beyond the CMC does not significantly change the surface tension as the additional surfactant forms micelles in the

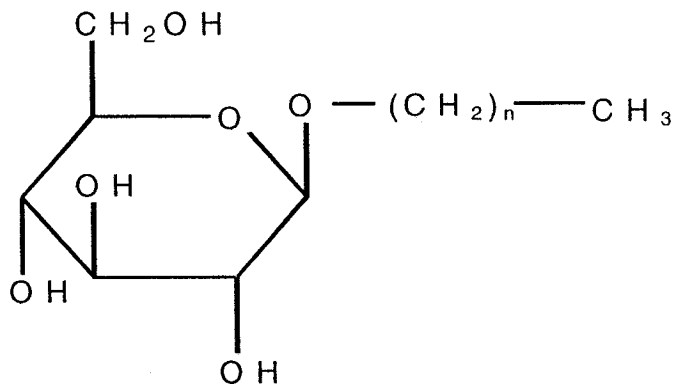


Figure 2.8: Octyl D-glucopyranoside (octyl glucoside)(n=7) (Wikipedia 2005)

bulk phase. Efficient surfactants have very low CMC (i.e. less surfactant is required to decrease the surface tension) (Cooper et al., 1979).

The main consumers are the petroleum industries. The ideal surfactant has good characteristics in terms of solubility, surface tension reduction, low critical micelle concentration, detergency power, wetting ability and foaming capacity (Myers, 1988). There are no surfactants that possess all of these characteristics. Rather, many surfactants exist each with their own strengths and weaknesses in terms of specific applications. As a result of their usefulness and shortage of large scale production, the demand for surfactants is high (Kosaric, 2001). Surfactants have been used in bioremediation of PAHs and in oil recovery operations (Hayes et al., 1986)). Its use in PCB biodegradation has not been fully exploited.

Microbial surface-active agents (biosurfactants) are metabolic by-products of bacteria, yeasts and fungi (Table 2.7). Several microbes can produce biosurfactants

(Table 2.8). They can be isolated from the extracellular medium or from the cell wall by several methods (Table 2.9). Biosurfactants are potentially as effective as the commonly used synthetic surfactants. They can also offer some distinct advantages (Cooper et al., 1986). Microbial surfactants show high specificity, making them well suited to new applications. These compounds have effective physicochemical properties (low interfacial tensions and CMC) and have good temperature stability characteristics. They also work at extreme pH and accept salinity. Several biosurfactants are of medium to high molecular weight containing a lipid moiety and can be produced in aerated bioreactors. Desired properties include biodegradability, reduced toxicity, biocompatibility and digestability (suitable for pharmaceutical and functional food applications), and a broad range of chemical structures (Kosaric, 2001).

The chemical and physical properties of biosurfactants can be modified, by genetic or chemical means, for specific applications. A selection of simple, potentially inexpensive substrates including *n*-alkanes, carbohydrates, vegetable oil, and wastes, can be used to produce biosurfactants. As an example, *Pseudomonas aeruginosa* can produce rhamnolipids using the following carbon sources C_{11} and C_{12} alkanes, succinate, pyruvate, citrate, fructose, glycerol, olive oil, glucose, and mannitol (Robert et al., 1989).

Biosurfactants are categorized as glycolipids, lipopeptides, phospholipids, fatty acids and neutral lipids (Biermann et al. 1987). With the exception of a few cationic biosurfactants (all of which have amine groups), most are anionic or neutral. Typically, the hydrophobic portion is based on long-chain fatty acids, hydroxyl fatty acids, or α-alkyl-β-hydroxy fatty acids. The hydrophilic portion can be a carbohydrate, amino acid,

cyclic peptide, phosphate, carboxylic acid, alcohol, etc. Numerous microorganisms produce these compounds (Kosaric, 2001). Most biosurfactants are produced from

Table 2.7: Examples of bacteria that are found in soil samples polluted with PCBs and PAHs. (Fritsche et al., 2002)

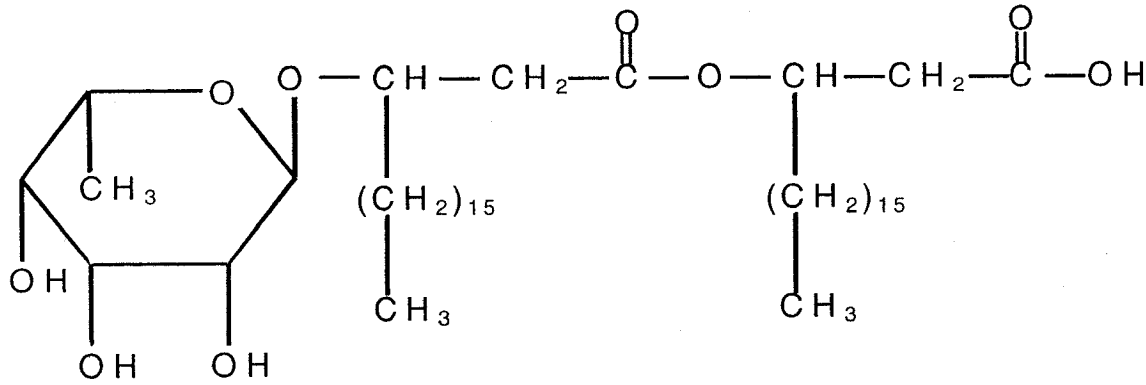
Gram-Negative Bacteria	Gram-Positive Bacteria
Pseudomonas spp.	***Bacillus spp.***
Acinetobacter spp.	*Mycobacterium spp.*
Alcaligenes sp.	*Corynebacterium spp.*
Flavobacterium	*Arthrobacter spp.*
Xanthomonas spp.	*Nocardia spp.*

Table 2.8: Selected microbially produced surfactants (Rosenberg et al., 1999)

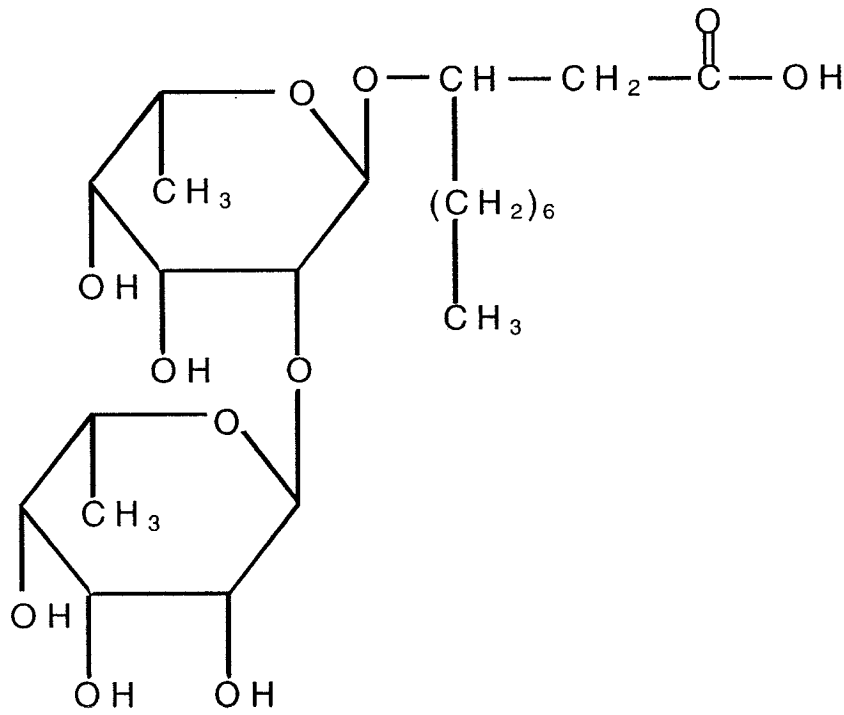
Surfactant class	Microorganism
Trehalose lipids	*Arthrobacter paraffineus, Corynebacterium* spp.
Surfactin	*Bacillus subtilis* ATCC 21332
Rhamnolipids	*Pseudomonas aeruginosa* 9027
Sophorose lipids	*Candida apicola, Candida bombicola*
Lipopolysaccharides	*Pseudomonas sp.,Acinetobacter calcoaceticus*

Table 2.9: Biosurfactant recovery processes (Biermann et al. 1987)

Process	Biosurfactants recovered by each method
Centrifugation	Glycolipids
Crystallization	Cellobiolipids, glycolipids
Precipitation by acetone	Bioemulsifiers, glycolipids
Solvent extraction	Sophorolipids, trehalose lipids
Ultrafiltration	Surfactin, glycolipids



R 1



R 4

Figure 2.9: Rhamnolipids produced by *P. aeruginosa* (Wagner et al.,1983)

Pilot studies for the production of the rhamnolipids have generated product at concentrations of 2.0 g/L with surface tensions of 29 mN/m (Reiling et al., 1986).

hydrocarbon substrates, with growth-associated production (Syldatk et al., 1985).

Biosurfactants produced from carbohydrates are usually secondary metabolites produced in the late logarithmic or stationary growth phases. The glycolipids produced by *P. aeruginosa* are well documented (Itoh et al., 1971). The rhamnolipids known as R1 and R4 can be produced from carbohydrates or hydrocarbons. R1 is a molecule consisting of two rhamnoses bound to β-hydroxydecanoic acid.

Surfactin (Figure 2.10) is an interesting lipopeptide produced by *Bacillus subtilis* (Cooper et al., 1981). It has a cyclic structure consisting of a 14 carbon fatty acid with a seven amino acid portion bound to the carboxy and hydroxyl groups of the fatty acid (Kakinuma et al., 1969). Growth of *Bacillus subtilis* in the presence of hexadecane totally inhibits surfactin production. Production of surfactin from glucose yields a very effective surfactant (27 mN/m at a concentration as low as 0.005%). Yields of 0.02 g/g glucose have been attained (deRoubin et al., 1989). Surfactin has been shown to improve the mechanical dewatering of peat by 50% at a concentration of only 0.0013g/g wet peat (Cooper et al., 1986).

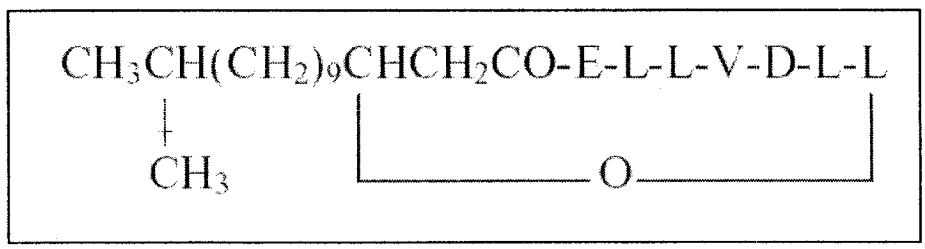


Figure 2.10: Surfactin produced from *B. subtilis* (amino acids are indicated with one letter codes E=glutamic acid, L=leucine, D=aspartic acid, V=valine)

2.9: Biotechnology and bioremediation

As a result of the strides made in biotechnology, bioremediation is one of the fastest growing areas in environmental restoration (Dua et al., 2002). Significant advances have been made in using microorganisms to detoxify pollutants of many different chemical classes. Successful application of bioremediation technologies depends on several factors. The biochemistry associated with the transformation of pollutants to less toxic chemicals is an important consideration. A given pollutant must be amenable to biochemical transformation. The microorganism must be able to access the pollutant and optimized conditions for biological activity will also impact the remediation efficiency. A study by Hamdy (1968) demonstrated evaluation of PCB degradation in waste contaminated with Aroclor 1248, shows that *P. aeruginosa* can biodegrade

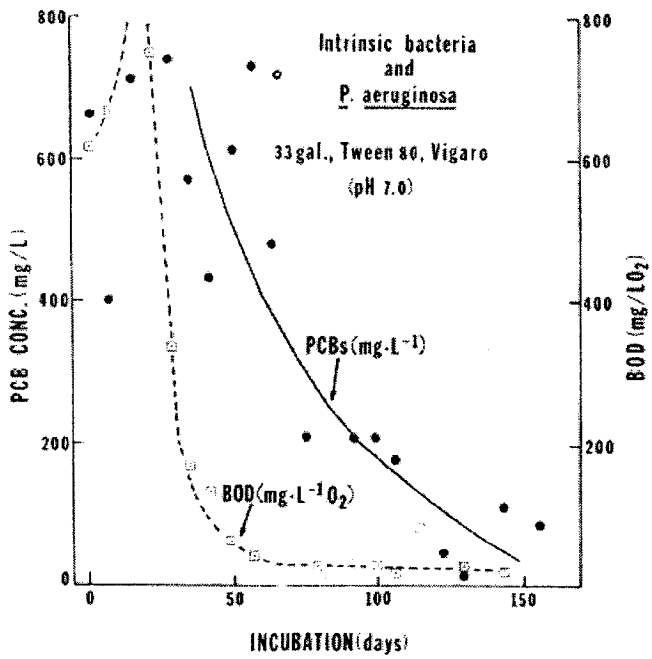


Figure 2.11: Biodegradation of PCB 1248 at 37°C in a reactor system as a function of time (Hamdy et al., 1986)

most of the Aroclor 1248 in the waste in 90-130 days of incubation (Figure 2.11). An interesting prospect for optimized bioremediation is the genetic engineering of organisms for enhanced biological activity. The microorganism must be able to access the pollutant and optimized conditions for biological activity will also impact the remediation efficiency.

Numerous synthetic organic chemicals are released into the environment intentionally on a large scale by industries for many applications including pesticides, fertilizers, herbicides, wood preservation agents, and insulators for electric transformers. Many of these chemicals are considered toxic to plants and animals. Some are readily degraded by microorganisms or decompose on their own while others can persist for years (Gibson and Parales, 2000). Moreover, pesticides that were once used in large amounts can have long half lives in soil. As an example, the commonly used insecticide, 4,4'-(2,2,2-trichloroethane-1,1-diyl)bis(chlorobenzene) (DDT) can persist for 3 to 10 years at a given site. Fundamental questions are being asked about how to dispose of the vast quantities of waste that are currently being produced and those that have accumulated in dump sites, soil and water systems (Dua et al., 2002).

The governments of developed countries are constantly raising the bar with regards to industrial practices that are respectful of the environment.. The situation in most developing countries is very different. These countries usually have few, if any, industrial environmental standards. In many instances, dumpsites are not set up with the proper barriers for ensuring that contaminants do not leak into the groundwater. A variety of solid and/or liquid pollutants may be found in the dumpsites, of known or unknown chemical composition. The cleanup of such a site may be difficult from a technology

standpoint, and costly. Alternatives to waste burial, the traditional method of waste management, are being pursued. Air stripping and incineration can be used for high concentration wastes in small dimensions. However, when contaminants are spread out over a large area, but still in significant concentration, such methods are no longer feasible. In such instances, microorganisms can be employed as a useful alternative (Dua et al., 2002).

2.10: Microorganisms

Billions of years of microbial evolution have resulted in many microorganisms adapting to using organic chemicals, both natural and synthetic, as sources of carbon and energy. The preexisting diversity of microbial enzymes is the foundation whereby the organisms can capitalize on new sources of carbon and energy derived from novel synthetic chemicals released into the environment by humans (Butler and Mason, 1997; Ellis, 2000).

Organisms capable of degrading a variety of chemicals such as benzene, phenol, naphthalene, atrazine, nitroaromatics, biphenyls, polychlorinated biphenyls (PCBs) and chlorobenzoates have been discovered and characterized (Sangodkar et al., 1989; Dickel at al., 1993; Faison 2001). Whereas unhalogenated aromatic compounds are readily degraded by several enzymatic pathways, halogenated analogs are more difficult to degrade. Microorganisms that can degrade aromatic halogenated chemicals usually can do so because they have evolved novel biochemical pathways (Chakrabarty, 1982; Engasser et al., 1990). The very properties that make industrial chemicals useful also

make them difficult to address from a detoxification standpoint. For example, substitution of chlorine on aromatic compounds renders the chemical resistant to degradation, both chemical and biological.

Much of the information gathered on biodegradation was obtained using aerobic conditions, since anaerobic conditions are more complex to control. Aerobic degradation is thought to be more efficient and generally applicable (Adriaens and Vogel, 1995). Aerobic degradation occurs by oxidation of the substrate at a carbon that is not substituted with a halogen. One or two oxygen atoms may be incorporated into the substrate, depending on which type of oxygenase is involved. Subsequent degradation steps my result in dehalogenation of the oxidized substrate. The aerobic oxygenases enzymes are not highly specific for their substrates.

In the modern era of molecular biology, it is now possible to genetically engineer microorganisms to perform specific biotransformations. To achieve this goal, genes from several different organisms encoding desirable enzymes may be cloned into a suitable host cell. The transformed organism can thus perform a variety of desirable biochemical reactions. In addition to the exchange of genes between organisms, site directed mutagenesis and gene shuffling based on three-dimensional protein structures can be used to produce enzymes with tailored characteristics (Singh 1999). An alternative approach would be to use random mutagenesis with a screening strategy to obtain mutants with desirable characteristics. Techniques used for this approach include DNA-shuffling, random priming and staggered extension processes (Stemmer 1994; Kuchner and Arnold 1997; Harayama 1998; Shao et al., 1998; Zhao et al., 1998). Interesting mutants obtained this way can potentially be refined by rational mutagenesis.

2.11: PCBs degradation by microorganisms

Given the requirement for a particular contaminant to enter a cell before it can be degraded, the solubility of the substrate may be a critical issue. As an example, PCBs have low solubility and bind tightly to soil; the combination of these factors greatly limits their degradation. Solubilization of PCBs with surfactants can result in an increased rate of degradation. Biosurfactants are better suited to bioremediation process due to their decreased toxicity and biodegradability relative to synthetic surfactants (Makkar and Rockne, 2003). A microbial strain that can produce its own surfactant would be ideal. In fact, microbial strains that produce surfactants are found ubiquitously in nature (Bodour et al., 2003). Such strains would be ideal for genetic transformation with genes encoding PCB degrading enzymes (Ohtsubo et al., 2004).

Although many microorganisms are capable of degrading chemical contaminants, no single organism is able to degrade all or even most contaminants in a given soil sample. The most efficient approach for biodegradation uses a community of microorganisms, which collectively provide a vast genetic library of enzymes that can act on the many organic compounds in the contaminated environment. The combination of genetic potential, temperature, pH, availability of nitrogen and phosphorus will determine the rate and extent of degradation. Among the various microorganisms, the aerobic gram-negative *Pseudomonads*, which never show fermentative activity, seem to have high a degradative potential, e.g. *Pseudomonas putida and P. fluorescens* (Fritsche and Hofrichter, 2002).

2.12 Biphenyl dioxygenase background and conditions of use

Presently eight major dechlorination pathways are known, each with its own congener specificity (Bedard,2003). The complete dichlorobiphenyl degradation pathway is depicted in Figure 2.12. The first stage of this cascade (Figure 2.13) utilizes an enzyme complex to convert the PCBs into more soluble intermediates. This reaction is one of the limiting factor in the pathway and its acceleration would enhance biodegradation.

Biphenyl dioxygenase (BPDO) is a four-component system consisting of a reductase, a ferredoxin and two oxygenases (Table 2.8). The oxygenases are comprised of alpha and beta subunits. The α subunit harbors two metal centers required for enzymatic activity; a catalytic mononuclear iron center and a [2Fe-2S] Rieske-type cluster required for electron transfer (Hurtubise et al., 1998). The enzyme uses molecular oxygen as the oxygen source and electrons derived from Nicotinamide adenine dinucleotide phosphate hydrogenase (NADPH) (Hurtubise et al., 1996). BPDO is expressed by a number of organisms. The oxidation of biphenyl substrates by *Comamonas testosteroni* B-356 and *Pseudomonas pseudoalcaligenes* KF707 occurs mostly as a *cis*-dihydroxylation at positions 2 and 3 (Gibson et al., 1993; Hurtubise et al., 1998). The BPDO of *Burkholderia cepacia* LB400 acts on many more biphenyl substrates, hydroxylates the 2,3 as well as the 3,4 carbons depending on the substrate and can also dechlorinate certain ortho-substituted congeners (Haddock et al., 1995). The broadened specificity of LB400 BPDO is attributed to the relaxed regiospecificity with regard to the position where molecular oxygen is added to the aromatic substrate (Arnett

et al., 2000). Thus, BPDO can transform several of the 209 polychlorobiphenyl (PCB) congeners into chlorobenzoates (Hurtubise et al., 1998).

PCB hydroxylation is the first step in the degradation of these persistent environmental contaminants. Once hydroxylated, additional ring cleaving enzymes can degrade the PCBs further (Haddock et al., 1995). Thus far BPDO capable of efficiently transforming highly chlorinated PCBs has not been discovered (Imbeault et al., 2000). Given the potential for bioremediation, the engineering of BPDO for broadened PCB selectivity and enhanced degradation is an attractive area of research. Alternatively, BPDO variants with desirable characteristics might be discovered by directed evolution of BPDO encoding organisms.

Comamonas testosteroni B-356 BPDO hydroxylates biphenyl and some polychlorinated biphenyls (PCBs) (Bergeron et al., 1994). The specificity of *Comamonas testosteroni* B-356 BPDO for various dichlorobiphenyl substrates is as follows; 3,3'->2,2'->4,4'- dichlorobiphenyl. The efficiency of BPDO catalysis depends to a large extent on the position of the dichloro substitutions on biphenyl. The k_{cat}/k_{mO2} for biphenyl, 2,2'- dichlorobiphenyl, and 3,3'- dichlorobiphenyl is 3.6±0.3, 0.06±0.02, and 0.4±0.07 x 10^5 $M^{-1}s^{-1}$ respectively (Imbeault et al., 2000). In addition, a considerable increase in the uncoupling of O_2 resulting in the formation of hydrogen peroxide was observed when biphenyl was utilized as a substrate in the presence of several dichlorobiphenyls. The ratio of biphenyl to O_2 consumed was 0.97, 0.44, 0.63, and 0.48 in the presence of biphenyl, 2,2'-, 3,3'-, 4,4'-dichlorobiphenyl, respectively (Haddock et al., 1995).

Studies with the BPDO of *Pseudomonas* sp. strain LB400 have addressed the oxidation of biphenyl and nine chlorinated biphenyls (Haddock et al., 1995). The enzyme converts biphenyl to 2,3-dihydrodiol. Dihydrodiols were produced from all the

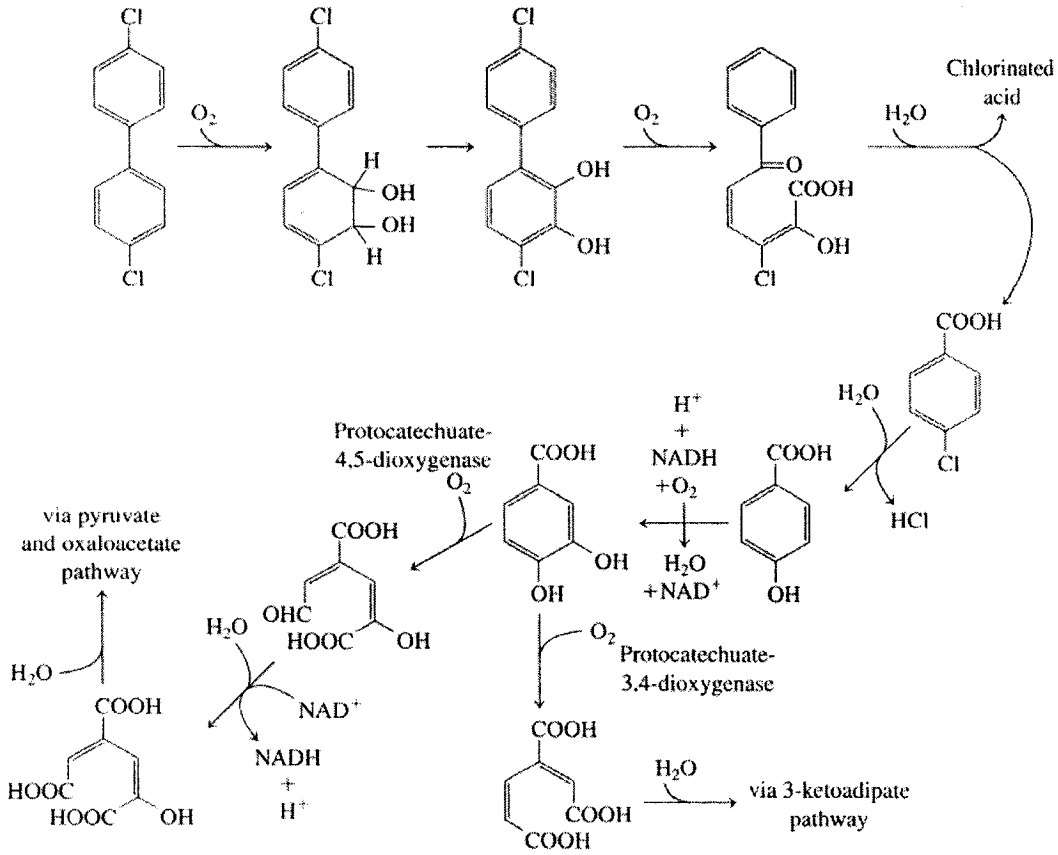
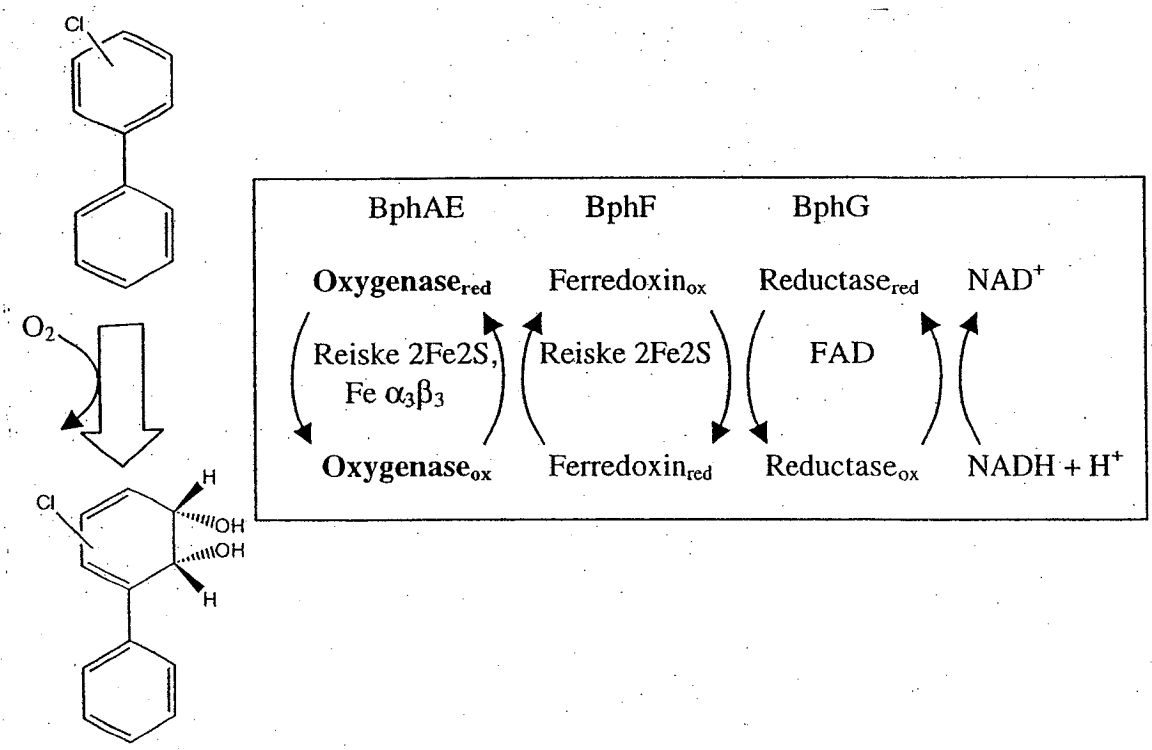


Figure 2.12 Complete dichlorobiphenyl degradation pathway (Sylvestre et al., 1993)

Table 2.10: Biphenyl dioxygenase enzyme system (SwissProt, 2005)

Protein	MW (kDa)	Number of amino acids
Biphenyl dioxygenase alpha subunit	51.69	457
Biphenyl dioxygenase beta subunit	21.55	186
Ferredoxin	11.91	109
Ferredoxin reductase	43.34	406

chlorobiphenyls with conversion of most substrates to more than one product. The dechlorination of a 2-chlorophenyl ring was observed resulting in the formation of a catechol. The oxidation of a 2,5-dichlorophenyl ring resulted in the formation of a 3,4-dihydrodiol. Thus, the LB400 BPDO has wide substrate specificity and the ability to oxidize at several ring positions.



2,3-dihydrodihydroxybiphenyl

Figure 2.13 Biphenyl dioxygenase multi-component system (Imbeault et al., 2000)

Highly purified, catalytically active BPDO has been crystallized for X-ray crystallographic structure determination (Imbeault et al., 2000). Structures for BPDO and BPDO complexed with a substrate (biphenyl) and the product (*cis*-(2R,3S)-dihydroxy-1-phenylcyclohexa-4,6-diene) have been obtained. The determined structures were useful

in directing experiments aimed at identifying specific residues involved in the reaction cycle of BPDO with biphenyl and chlorinated biphenyls.

Experiments with naphthalene dioxygenase have resulted in the proposal that asparagine 221 is a ligand to the active site mononuclear iron. The corresponding residue in BPDO is glutamine 226. From the crystal structures, its position is too far to be considered a ligand to the mononuclear iron in the substrate-free, substrate-bound or product-bound enzyme. To address the role of this residue in BPDO, several variants were engineered including BPDO_Q226A, BPDO_Q226E, BPDO_Q226N. Kinetic analysis of these variants indicates that glutamine 226 is not required for co-ordination to the mononuclear iron. However, the Q226E variant affected the electronic properties of the Fe-S cluster as observed by spectroscopic studies (Agar et al., 2005).

Two additional residues were modified (aspartate 230, methionine 231) in order to investigate their role in internal electron transfer and substrate specificity, respectively. The iron occupancy at the mononuclear active site of the BPDO_D230N variant was incomplete. Using biphenyl as a substrate, the mutant had very low activity, which clearly indicates the importance of the residue. Inspection of the BPDO crystal structure suggested that variants BPDO_M231A and BPDO_M231T might result in a preference for 2,2'-dichlorobiphenyl as a substrate. Steady state kinetic characterization of these variants revealed that they did have greater k_{cat} with decreased uncoupling when 2,2'-dichlorobiphenyl was used as a substrate (Agar et al., 2005).

The effect of 23 individual amino acid substitutions on BPDO of *Burkholderia* sp. strain LB400 has been investigated (Zielinski et al., 2003). The regiospecificity of substrate dioxygenation was used to monitor alterations to the steric and electronic

structure of the enzyme active site. Several residues predicted to interact with the substrate were found to strongly affect the site preference for dioxygenation. Several other residues, which do not contact the substrate, were also found to affect the site preference for dioxygenation.

The BPDO enzymes expressed by *Comamonas testosteroni* B-356 and *Pseudomonas* sp. strain LB400 are very closely related. Experiments have been performed where the α and β subunits of the two enzymes have been exchanged to generate two chimeras (Hurtubise et al., 1998). The purpose of the experiment was to examine the reactivity of the BPDO chimeras towards several chlorobiphenyls and their ability to catalyze meta-para dioxygenation. Both chimeras were prepared and found to be functional. The $\alpha_{B\text{-}356}\beta_{LB400}$, chimera was found to behave like LB400 BPDO in terms of its substrate regiospecificity. This result demonstrates the importance of the LB400 β subunit in determining substrate regiospecificity, even though the catalytic center is found in the α subunit. The $\alpha_{LB400}\beta_{B\text{-}356}$ chimera showed characteristics of both B-356 and LB400 BPDO as the enzyme was able to dioxygenate 2,2'-, and 3,3'-dichlorobiphenyl and catalyze the meta- para, oxygenation of 2,2',5,5'-tetrachlorobiphenyl. Collectively, these results showed that although the α subunit is the dominant determinant of enzyme specificity, the β subunit also contributes.

To completely remove PCBs from soil and sediments, a complete geochemical knowledge of the complex is necessary. At this time, the relationship between the physical and biological variables is not fully understood. When PCBs bind to soil, the desorption activation energies, the binding capacity and the type of soil, will determine the bioavailability, and hence the efficiency of the removal process. The low aqueous

solubilities and dissolution rates compound the problem (Opperhuizen et al., 1988). The key to successful bioremediation is the understanding of the environmental requirements of the microorganisms, and the sorption and transport mechanisms of the contaminants through pilot testing. Transformation of PCBs taken from the environment occurs readily, producing changes in the ratio of congeners. This makes the determination of the original mixture in an environmental sample extremely difficult.

Chapter 3

Materials and Methods

3.1: Chemical Supplies

Three monochlorobiphenyl isomers, seven dichlorobiphenyl congeners, seven trichlorobiphenyl and four tetrachlorobiphenyl PCBs were obtained from Sigma-Aldrich (St.Louis, MO). Polychlorobiphenyl kit #3 containing Aroclor, 1232, 1242 and 1254 were obtained from Supelco (Ph, PA). Two, 2, -2,3, -2,4, -3,3, -3,4, -4,4 dichlorobiphenyls were purchased from Accustandard Ltd. (New Haven CT). The purities were greater than 99% (certificate of analyses). The PCBs derivatives were synthesized by Bivan Consultants (Dorion, Que.). Cesium iodide (132.9054 amu), octapeptide ALILTLVS (829.5398 amu), the dipeptides (280.1059 ,131.0457 amu), rhamnose (164.0685 amu) , octyl glicoside (292.1186 amu) and reserpine (609.2821 amu) were obtained from Bachem (NY,US). Taurocholic acid was purchased from Calbiochem (San Diego, CA). All solvents and chemicals were HPLC grade and obtained from Fisher Scientific (Montreal, Quebec). The YM 10 membranes and ZipTip cartridges were obtained from Millipore (Bedford, MA). HPLC columns were obtained from Vydac (San Diego, CA) and Agilent (Montreal, Quebec). Uncontaminated garden topsoil, free of PCBs according to the manufacturer's specifications, and sandy loam soil which contains approximately 5% clay, 35% silt, 58% sand and 2% organic matter, were purchased from a local garden accessories supplier (Canadian Tire, LaSalle, Que.). A

contaminated soil sample containing 825 ppm Aroclor 1232, 1217 ppm Aroclor 1242 and 794 ppm Aroclor 1254 was obtained from a certified laboratory responsible for performing the analyses for the local municipality. The analyses were performed by an EPA-approved gas chromatographic method. The sample, originating from a contaminated site in Ville LaSalle, Quebec, Canada, and has been contaminated for approximately twenty-two years.

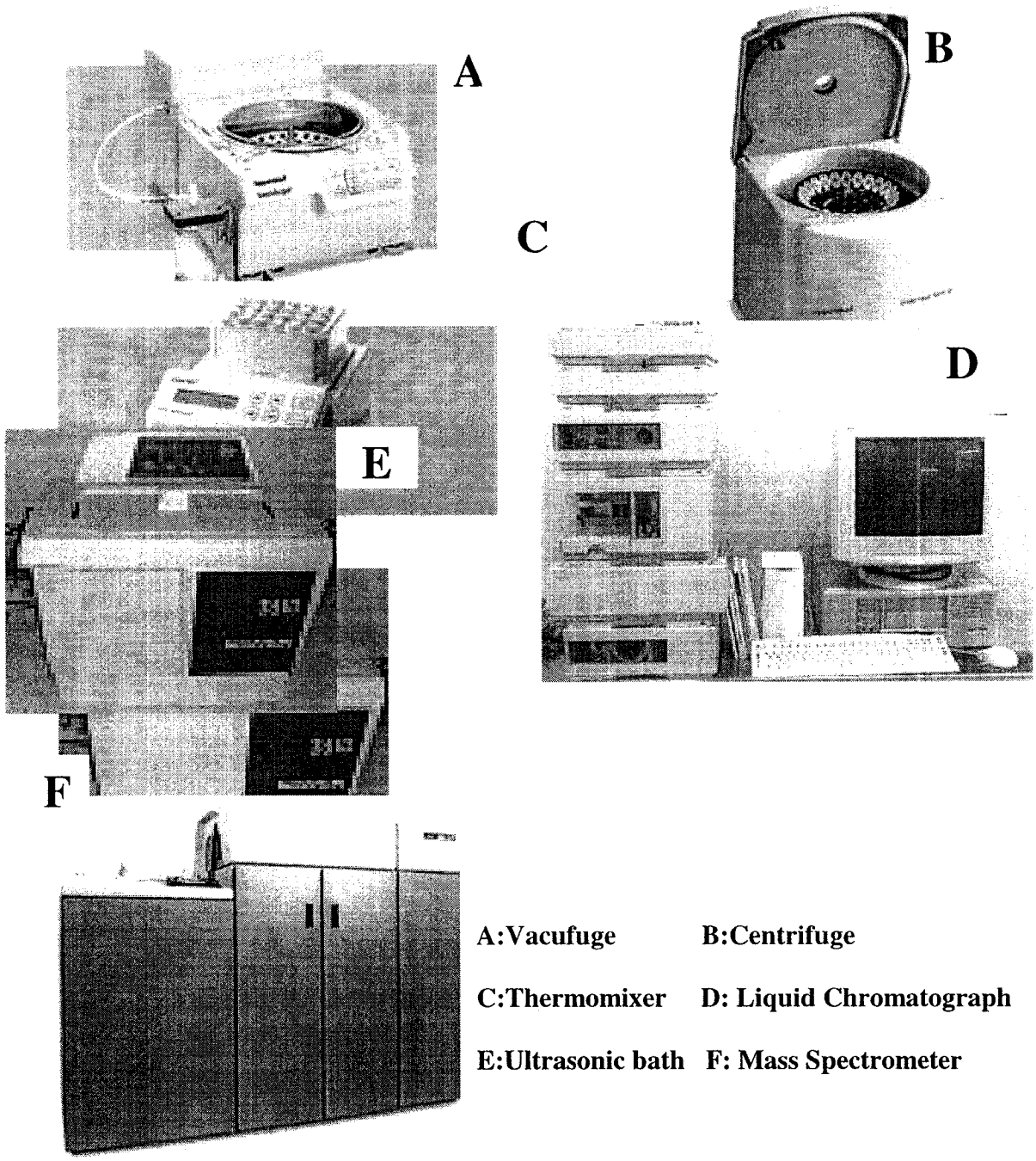
Most of the laboratory instruments used in this study are shown in Figure 3.1 and their manufacturers are shown in Table 3.1.

Table 3.1: Instrumentation used in the experiments

Manufacturer	Instrumentation
ABI Sciex	QSTAR mass spectrometer
Agilent	Capillary LC
Eppendorf	Vacufuge, Centrifuge, Thermomixer
Branson	Ultrasonic bath

3.2: Biosurfactant preparation.

Bacillus subtilis ATCC 21332 was maintained at 4°C on 4% glucose, mineral salt medium agar plates. *Pseudomonas aeruginosa* ATCC 9027 was maintained on agar P (Difco Cal, US). After 3 days growth in 100 cm^3 of 4% glucose and mineral salt medium supplemented with 3·2 x 10^{-4} mol dm^{-3} $FeSO_4$, 50 cm^3 of *B. subtilis* was transferred into 500 cm^3 (2 dm^3 flask). After 6h of growth, inoculum (0.5 dm^3 or 10.0 dm^{-3}) was added to a 20dm^3 Bioengineering fermentor. The following cultivation conditions were used:



A:Vacufuge **B:Centrifuge**

C:Thermomixer **D: Liquid Chromatograph**

E:Ultrasonic bath **F: Mass Spectrometer**

Figure 3.1: Photographs of some of the laboratory instruments used in the project

aeration at 20 dm^3 min^{-1}, pH control at 6·7, 100 rpm agitation and 37°C. Foam was collected and collapsed in a flask on the air exhaust line. *P. aeruginosa* was grown in a similar manner in proteose peptone medium. Cells were removed by centrifugation in a Beckman centrifuge at 12 000 *g* for 10 min (Mulligan and Gibbs, 1990).

Surfactin was isolated by adding concentrated hydrochloric acid to the collapsed foam after cell removal. Dichloromethane (1:1, v/v) was added to the suspension in a separatory funnel and shaken vigorously. The aqueous (bottom) layer was removed and extracted twice more as described above. The organic layer was pooled and evaporated. The residue was redissolved in water (pH 8·0) and filtered through Whatman No. 1 paper to remove undissolved impurities. Concentrated HCl was again added to the filtrate and extracted with dichloromethane (1:1, v/v) three times and evaporated as described (Mulligan and Gibbs, 1990).

Cell-free foam fractions from the surfactin isolation procedure were concentrated by an Amicon magnetically stirred ultrafiltration cell, containing a YM 10 membrane (Mol. Wt cutoff of 10,000) with a pressure of 172 kPa. A similar procedure was performed on surfaceactive rhamnolipids (R1 and R4) isolated from *P. aeruginosa.* The samples were lyophilized. Chemical analyses were performed on the retentate. Surface tension was determined by the de Nouy method with a Fisher Tensiomat Model 21. The CMC was determined by measuring the surface tension at various dilutions (Cooper et al. 1979). The logarithm of the dilution was plotted as a function of the surface tension. The CMC is the point at which the surface tension abruptly increases. The reciprocal of CMC is an indication of relative concentration.

Surfactin concentration was determined by amino acid analysis. A 10 μm^3 aliquot was dried and acid hydrolysed for 2-5 h at 105°C in a PICO-TAG amino acid analysis system. The residue was redissolved in 200 μm^3 of sodium buffer and injected on a Beckman System 6300 high performance analyser equipped with a Beckman Model 7000 data station. The concentration of surfactin was calculated by multiplying the lipopeptide concentration (mol dm^{-3}) by the molecular weight.

The purities of the isolated biosurfactants were checked by a LCMS on an Agilent Model 1100 Analytical LC equipped with a diode array detector, autosampler and ChemStation software. A Zorbax SB-C18 300 Å 4.6 x 250 mm column at a flow rate of 1ml/min was used, with a gradient from 5% to 75% acetonitrile in 30 min. The column was previously equilibrated with 5% acetonitrile and tested with the octapeptide standard to verify system performance (i.e. resolution, signal intensity, column bleeding, peak tailing, etc) before use. The eluent was monitored at 206 and 280 nm with a bandwidth of 2 nm and a reference wavelength of 550 nm.

To confirm the identity and integrity of surfactin, a product ion scan was performed The Rhamnolipids were quantitated using rhamnose (6-deoxy-L-mannose) as the internal standard. rhamnolipids R1 and R4 contain 34.09% and 24.50% of rhamnose respectively.

3.3: Enzyme preparation and assay

Briefly, the crude enzyme containing several proteins extract was loaded onto an anion exchanger. The eluent was concentrated with a YM 30 membrane column and

eluted in a linear sodium chloride concentration. The retentate was filtered and loaded onto a Phenyl Sepharose column, which was pre-equilibrated with a sodium chloride-saturated ammonium sulphate buffer. The oxygenase was eluted at with saturated ammonium sulphate. The eluant was concentrated and applied to a hydroxyapatite column with a linear sodium phosphate gradient.

Final purification of the dioxygenase enzymes was performed on a butyl Vydac analytical column on an Agilent Model 1100 LC system, to remove interfering adducts and low molecular-weight compounds. The gradient commenced from 20% acetonitrile to 80% in 30 minutes. The purified enzyme was lyophilized and stored at -20^0C until ready for use. The purity was confirmed by mass spectrometry.

The standard activity assay contained 70 μM $Fe(SO_4)_2(NH_4)_2$, 288 μM 2,3 dichlorobiphenyl,123 μM NADH, 1.2 μM $BphG_{B356}$, 2.8 μM $BphF_{LB400}$ and 0.36 μM oxygenase. The reaction was initiated by adding oxygenase after equilibrating the assay with all other components for 20 s. The assay was performed in a total volume of 1.3 ml of air-saturated 50 mM MES (I=0.05 M), pH 6.0, 25.0 ± 0.1°C. Activity determinations were corrected for Oxygen consumption observed in the presence of NADH, 2,3 PCB, reductase and ferredoxin only. One unit of enzyme activity is defined as the quantity of enzyme required to consume 1 μmol of O_2/min under standard assay conditions.

3.4: Mass Spectrometry (MS)

The mass spectrometer was calibrated in the positive mode with reserpine whose monoisotopic protonated mass is 609.12821 amu and major protonated fragment is

195.0657 amu. In the negative mode, the system was calibrated with taurocholic acid with exact mass MH^- 514.28385 amu, and its SO^{3-} fragment 79.95682 amu, at 20ng/μL concentration diluted to 500 μL with water / acetonitrile (50/50) containing 2mM ammoniun acetate. A 10 fmol/μL myoglobin in a solution of 0.1 % acetic acid dissolved in 50/50 acetonitrile/water was used for system verification in both modes.

Instrument tuning, data acquisition and processing were controlled by a Dell Precision 650 workstation with software provided by the instrument's manufacturer. The instrument was ready for sample injection when the drift was less than 3 ppm from the theoretical value. For quantitation of small molecules (<150amu) the synthetic dipeptide of molecular mass 131.0457amu was checked for mass accuracy and resolution before sample introduction. Briefly the samples and standards were dissolved in an acidified or basic aqueous organic mixture and infused through a stainless steel capillary (100 μM ID). A stream of air (pneumatic nebulization is introduced to assist in the formation of submicron droplets. These droplets are evaporated at the interface by nitrogen gas producing highly charged ions, which are detected and recorded by the analyzer. Approximate values for MS settings are shown in Table 3.2.

3.5: Liquid Chromatography Mass Spectrometry (LCMS)

In the final purification stage of crude biosurfactant,after after column

Table 3.2: Approximate mass spectrometric settings

Ion source	MS	MS/MS
Ion source Gas 1 (GS1)	20	
Ion source Gas 2 (GS2)	0	
Curtain Gas (CUR)	25	
IonSpray Voltage (IS)	4500.0	
Ion Release Decay (IRD)	6.0	
Ion Release Width (IRW)	5.0	
Ion Energy 1 (IE1)	1.0	
Grid (GR)	-40.0	
Einzel Lens Focus (TFO)	-10.0	
MCP (CEM)	2100.0	
Declustering Potential (DP)	60	50
Focusing Potential (FP)	200	200
Declustering Potential 2 (DP2)	20	20
Focusing Rod Offset (Q0)	20	30
Collision Gas (CAD)	2	3

purification, the eluent of the LC was connected directly to the MS. The flow from the column was optimised for efficient electrospray ionization. A similar approach was used for the final purification of alpha and beta dioxygenases.

3.6: Mass Spectrometry / Mass Spectrometry (MS/MS)

The octapeptide was fragmented with argon as the collision gas. All b and y ions were optimized with variable voltages throughout the instrument.

Parent ions of surfactin, rhamnolipids and octyl glucoside were also fragmented to generate product ion spectra for confirmation of molecular structures. The nature of the PCBs and their derivatives were also confirmed by product ion mass spectrometry.

3.7: Mass ionization response

The mass ionization response experiment was done to verify an adequate response can be obtained for the PCBs of interest and the selected internal standards. Five ng of the octapeptide and 0.5 ng cesium iodide (internal standards) were mixed and diluted to 500 μL with water / acetonitrile :50/50 containing 2mM ammonium acetate in an Eppendorf tube. The sample was mixed and divided into five equal aliquots each receiving 10, 20, 500, 800 and 1000mg/L of mono, di, tri and tetrachlorobiphenyl standards (Table 3.3). An aliquot was infused into the mass spectrometer. All experiments were performed in triplicate and statistically evaluated. Relative intensities were plotted versus concentration.

3.8: Degradation Activity

Activity determination experiments were also performed in the aqueous phase to determine the approximate conditions and quantities of sample required for process enhancement. Five ng octapeptide, 0.5ng cesium iodide, 1000 ppm each of mono, di, tri and tetrachlorobiphenyl standards were placed in an 1.5 ml Eppendorf tube, and taken to

Table 3.3: Concentration values used to check MS detector's response

PCBs conc(mg/L)	Internal standards per sample
10	0.1 ng cesium iodide
20	
500	1.0 ng octapeptide
800	
1000	

a total volume of 600 µL. The sample was mixed and divided into six equal aliquots and placed on a thermomixer (switch in off position). Ten µL of the reconstituted enzyme system were added to each aliquot, immediately followed by the addition of 1, 2, 50 and 100 mg/L of surfactin to five tubes, the sixth tube containing no surfactant was used as a negative control. Samples were incubated at various temperatures on the thermomixer at several agitation speeds (rpm) for eight hours. An aliquot was taken every two hours and the reaction arrested by lyophilization. The residues were resuspended in 20 µl of solution A and vortexed for five seconds. The samples were centrifuged at 10,000 rpm for 2 minutes. The supernatant was concentrated on a ZipTip cartridge before loading into a nanospray capillary and infusing into the mass spectrometer.

All experiments were performed in triplicate for statistical evaluation. Substrate and the products were monitored simultaneously. Experiments were also performed with the rhamnolipids and octyl gluocoside in aqueous solution to evaluate the potential

effective range required for enhancement. The concentration values chosen for the curve were based on the CMC obtained from the preparations.

3.9: PCBs-spiked soil preparation

Uncontaminated soil (10mg), a reference soil that did not contain PCBs according to the certificate of analysis, was spiked with 10, 20, 500, 800 and 1000 mg/L of PCB standards, 1ng octapepide and 0.1ng cesium iodide. Samples were sonicated for 10 minutes before incubation on the thermomixer at 22^0C for 12 hours at 400 rpm. Several extraction solvents (Table 3.4) were subsequent evaluated in this study and the best solvent(s) was selected for further experiments. Since the buffer used to control the pH can contribute to ion signal suppression, several buffers were evaluated for surfactant-mediated PCBs degradation (Table 3.4).

The scheme for the soil experiments is shown in Figure 3.2. Experiments were conducted at varying temperatures, degrees of agitation and incubation periods (Table 3.5). The samples were extracted three times with 20µL acetone/hexane. The extracts were pooled, lyophilized and redissolved in 20µL of buffer and infused in the mass spectrometer. All experiments were performed in triplicate and statistical evaluated.

3.10 :Aroclor –contaminated samples

The contaminated soil was sampled at approximately 25 mm to 30mm below the surface. The soil was moist and uniformly granular on receipt and stored in a closed

container for periodic testing. An analysis certificate was obtained on receipt.

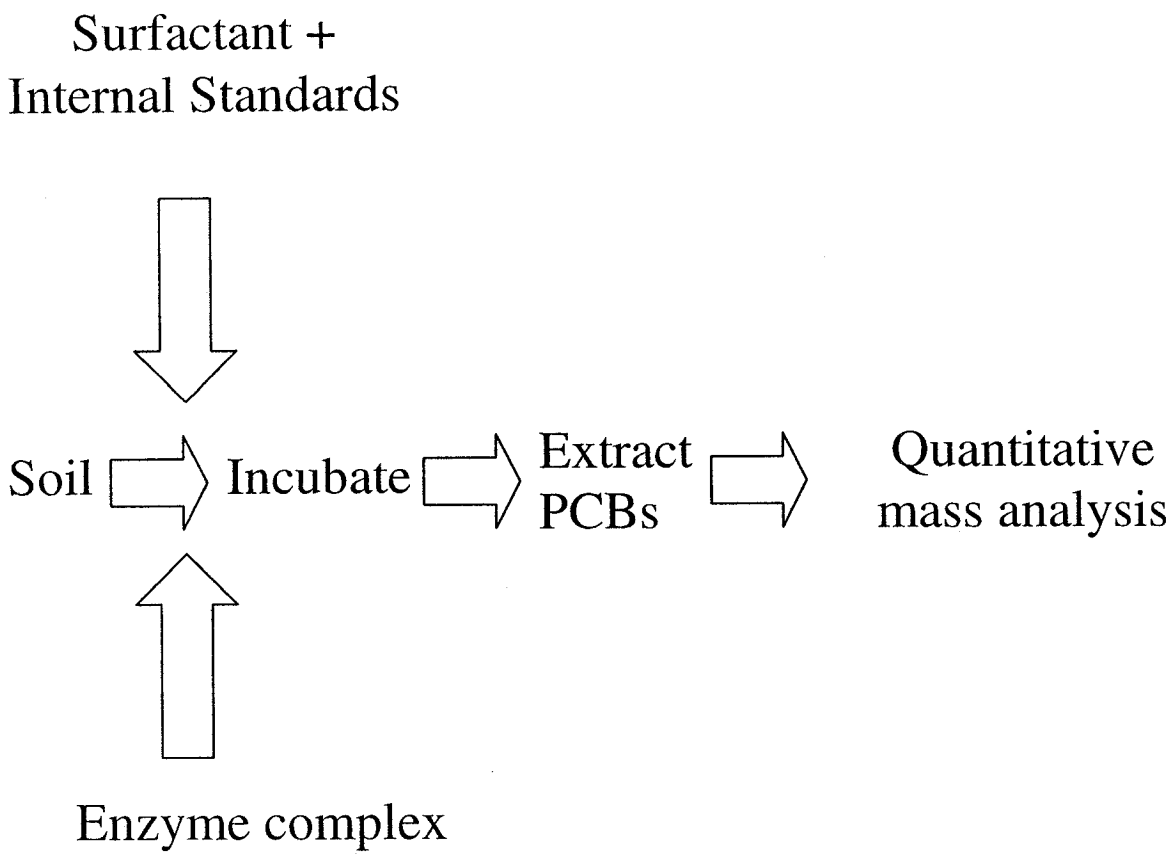


Figure 3.2: Experimental scheme for PCBs biodegradation and quantitation in soil

3.11:Preparation of calibration standards and quality control samples

For rigid quantitation, quality control samples were prepared in uncontaminated soil with the addition of PCBs and internal standards. Calibration standards and QC samples were used to assess precision, accuracy and determine the lower limit of quantitation (LLOQ). Five calibration standards ranging from 0-1000 mg/L were used to define the standard curve. QC samples at low (30mg/L), medium (200 mg/L and high

(700 mg/L) concentrations were prepared described above and analyzed in triplicate. These quality samples were estimated from the standard curve and statistically evaluated. Least square linear regression and statistical analysis were performed using an Excel spreadsheet .

Table 3.4: Solvents tested for efficient PCBs extraction

Extractants	pH adjusting solutions
Acetone	100 mM MES; pH 5.5
Hexane	1% acetic acid pH 5,5
Isooctane	0.1% formic acid pH 6
Methanol	0.1% TFA pH 6
Acetone/ hexane(50/50)	-
Acetone/ isooctane (50/50)	-

Table 3-5: Variables used in optimization experiments:

Temperature (^{0}C)	Degree of agitation (rpm)	Incubation period (h)
4	100	0.1
12	400	2
25	800	4
37	1000	8
45	1200	12

In this work, a purified biphenyl dioxygenase system, enzymes known to be involved in the first stage degradation of PCBs was utilized. Although the use of surfactants in PAHs degradation and oil recovery is well known, their role in PCB degradation is unclear. An enzyme solution was added to varying amounts of PCBs and internal standards. Varying concentrations of surfactin, a biosurfactant produced by *B. subtilis*, rhamnolipids produced by *P. aeruginosa* and octyl glucoside, a sugar-based non-ionic chemical detergent were added to the mixture. Samples were buffered and incubated over a period of time with different degrees of mixing. The reduction of the toxicants and appearance of the metabolites were monitored simultaneously. Product enhancement was achieved for the surfactant. Conditions were optimized before PCBs-spiked soil samples were analysed and quantitated. A contaminated sample was also investigated. The surfactants have not been previously reported as enhancers in PCB degradation.

Chapter 4

Results and Discussion

4.1: Introduction

In this chapter, the results of the experiments performed are discussed. As stated previously, experiments were done both in an aqueous environment, in normal soil spiked with PCB, and also in soil that has been contaminated for several years. Three surfactants were evaluated at several concentrations in a varying environment.

4.2: Purification and characterization of surfactants and enzymes

To ensure artifacts were not responsible for chemical or biochemical activities in the experiments performed, starting chemicals were purified and characterized. The LCMS of the surfactin is shown in Figure 4.1. Its measured monoisotopic protonated molecular mass was within 3 ppm of its theoretical molecular mass - 1036.6960 amu (atomic mass units). A product ion spectrum confirmed its structure (Figure 4.2) with the dominant y_6 ion fragment at 685.4579 amu, in addition to other characteristic ions known to be present in its standard product spectrum. Similarly LCMS of purified rhamnolipids is shown in Figure 4.3 with molecular masses in harmony with its chemical formulae. Their product ion spectra are also in harmony with known fragmentation pattern (Figure 4.4). The structure and molecular weight of octyl glucoside were confirmed by MS and tandem MS before use. The octyl glucoside purity was estimated to be greater 99%.

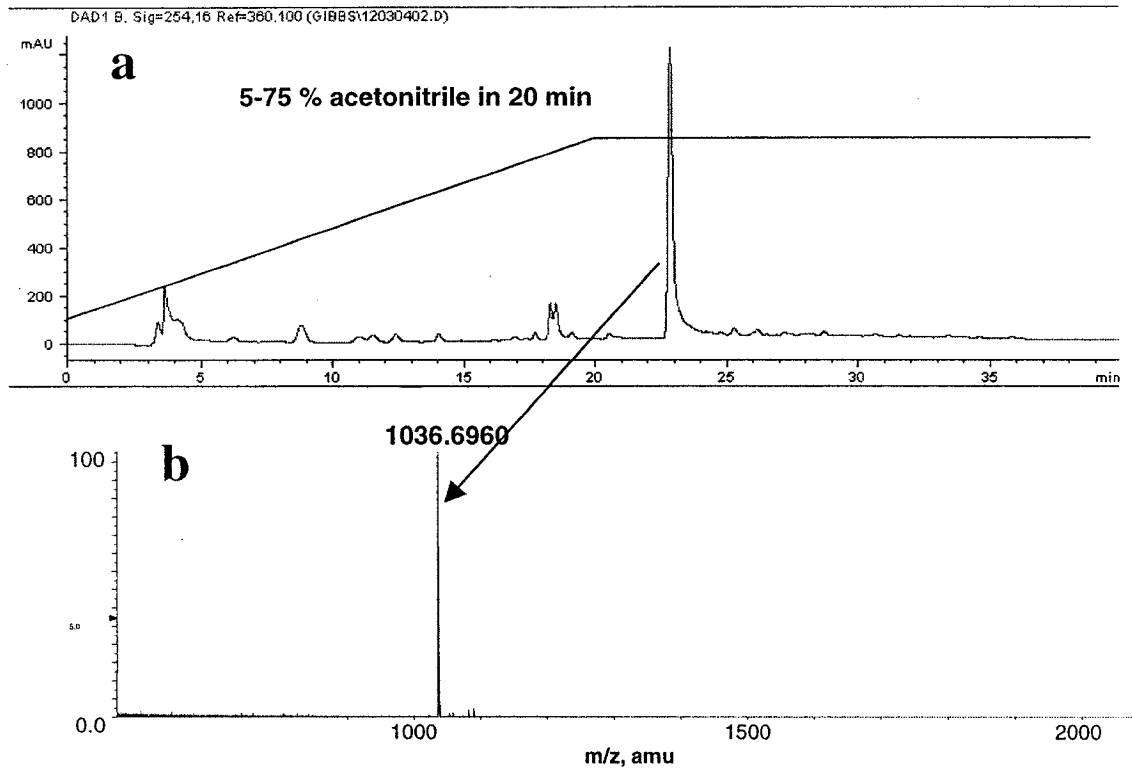


Figure 4.1: LCMS of surfactin (a) UV chromatogram (b) Mass spectrum of peak at 23.1 min.

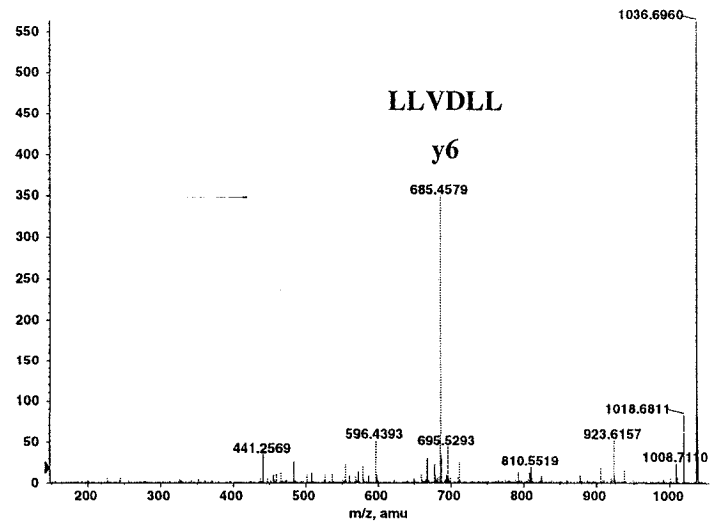


Figure 4.2: Product ion spectrum of surfactin.

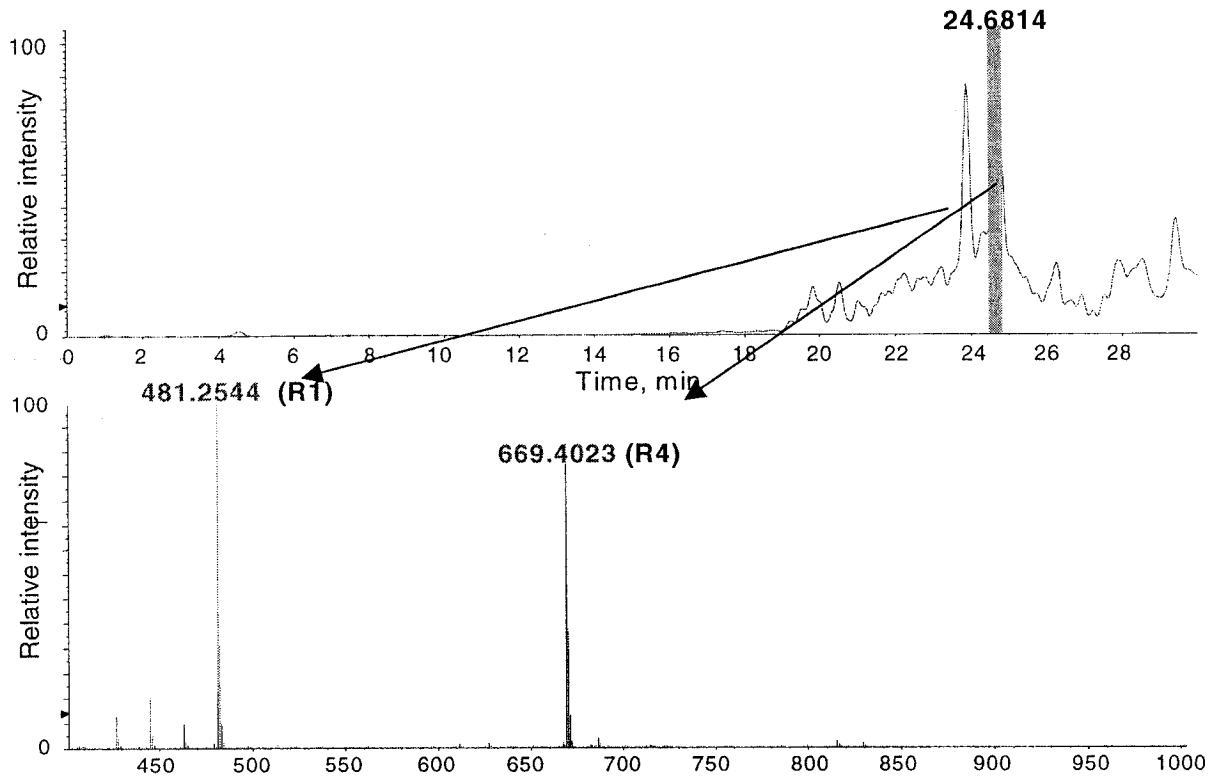


Figure 4.3: LCMS of rhamnolipids (a) Total ion chromatogram (b) Mass spectrum of R1 and R4

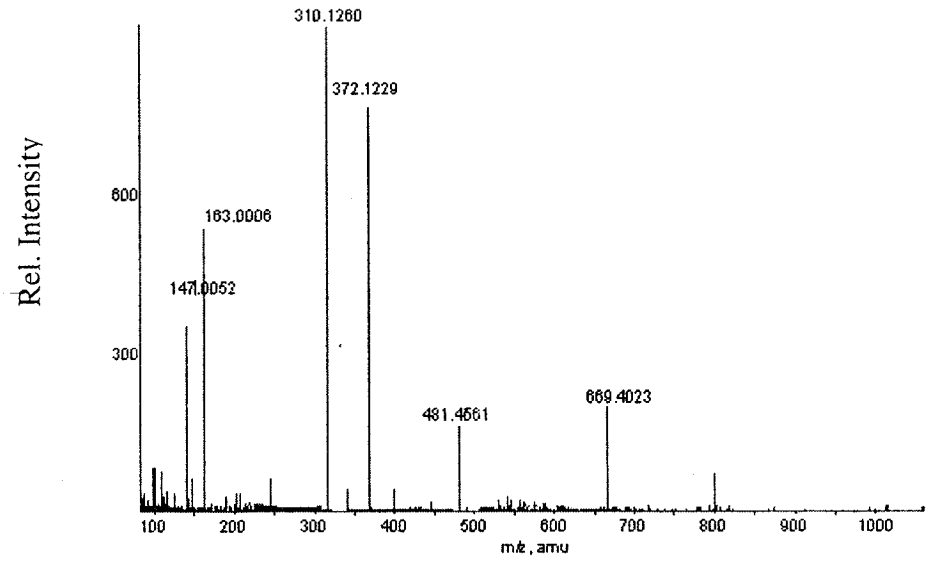


Figure 4.4: Product ion spectrum of rhamnolipids R1 and R4

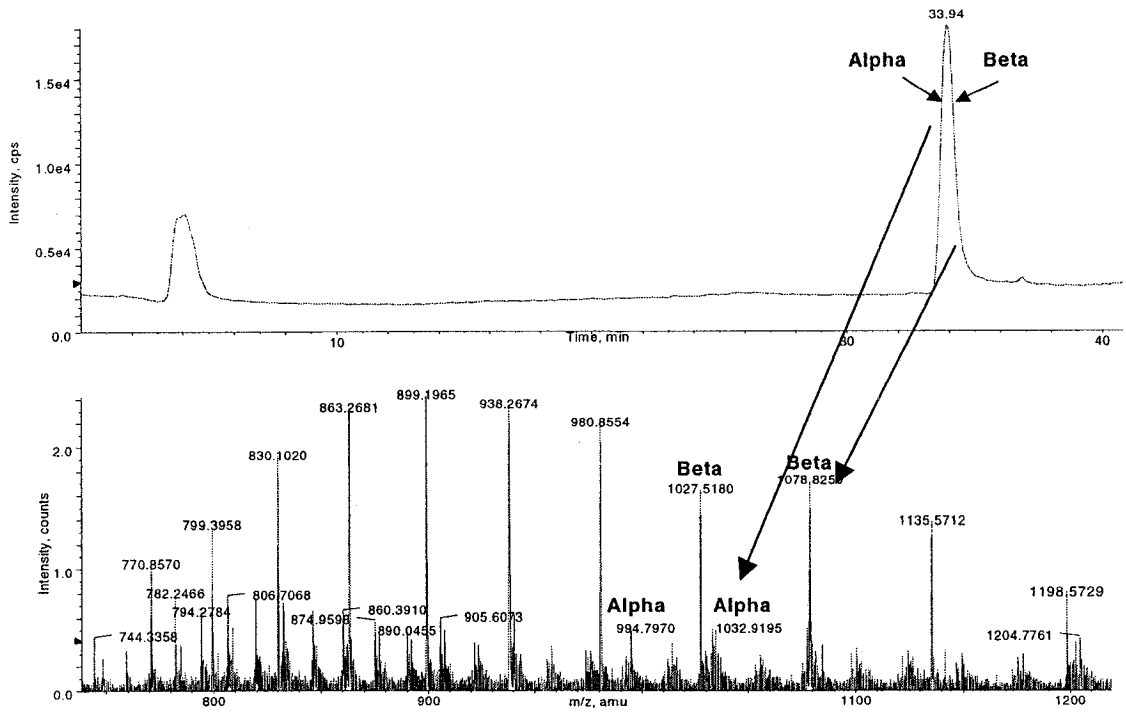


Figure 4.5: LCMS of biphenyl dioxygenase (alpha and beta subunits)

Table 4.1: Molecular weights of small PCBs

PCB type	Formula (Substrate)	MH^-	⇒	Formula Product	MH^-
Mono	$C_{12}ClH_9$	187.0315	⇒	$C_{12}ClH_{11}O_2$	221.0370
Di	$C_{12}Cl_2H_8$	220.9925	⇒	$C_{12}Cl_2H_{10}O_2$	254.9980
Tri	$C_{12}Cl_3H_7$	254.9536	⇒	$C_{12}Cl_3H_9O_2$	288.9591
Tetra	$C_{12}Cl_4H_6$	288.9145	⇒	$C_{12}Cl_4H_8O_2$	322.9200

The electrospray mass spectrum alpha / beta dioxygenase mixture is shown in Figure 4.5. Further separation was unnecessary since the mixture was found to be fully biologically active. Observed molecular weights of the two were within 0.01% of their theoretical values. The mass envelope of the beta subunit appears between 750 and 1027 amu, whereas the alpha subunit lies between 1056 and 1262 amu. Although used in diluted quantities, their mass location does not interfere with the analyses of substrates and products. After the activity was determined, the enzyme was aliquoted in 100µl portions in eppendorf tubes and frozen at -20^0 C. Samples were thawed prior to use.

4.3: Mass spectrometry

A high resolution mass spectrometer is required for this assignment since the mass differences between substrates and products are minimal. The molecular masses are shown in Table 4.1. For example, the monoisotopic molecular mass of a di-PCB is 220.9925 amu. But the dominant product formed from the biodegradation of a mono-PCB is 221.0370 amu, giving a difference of 201 ppm. Calibration must be precise and only a very small mass drift is acceptable. The approximate instrument settings are shown in Table 3.2. There is a tradeoff between sensitivity and resolution, appropriate voltages should be selected accordingly.

4.4: Mass ionization response

A mass spectrum of PCB standards and the internal standards is shown Figure 4.6. Internal standards were chosen based on their mass response, high purity, thermal stability and a molecular weight.of 500 to 1000 amu internal for a two-point calibration. The ratio of the response of the PCBs to the internal standards were plotted against a range of PCBs concentration. The plot was linear over the selected working range (Figure 4.7a and 7b). The concentration range of the PCBs curves was selected to reflect the expected values of samples to be analysed. The primary product of the degradation is shown in Figure 4.8 and th linearity and response was also verified for the PCB dihydrodihydroxy derivatives (Figure 4.9).

4.5: Activity determination.

In experiments where the enzymes were added to the mixture of PCB, internal standards and surfactants, new MS signals appeared after incubation, suggesting that degradation of PCBs occurred. Product ion spectra confirmed this speculation. The concentration of each surfactant used ranged from below to above their calculated CMC. PCB conversion to the dihydrohydroxy derivates was calculated when the reaction was observed.

Figure 4.10a shows an experiment with surfactin before the reaction commences, whilst Fig 4.10b displays the conversion after two hours of incubation with the appropriate concentration of surfactin. Figure 4.11a and Figure 4.11b show a similar reaction for rhamnolipids before and after degradation.

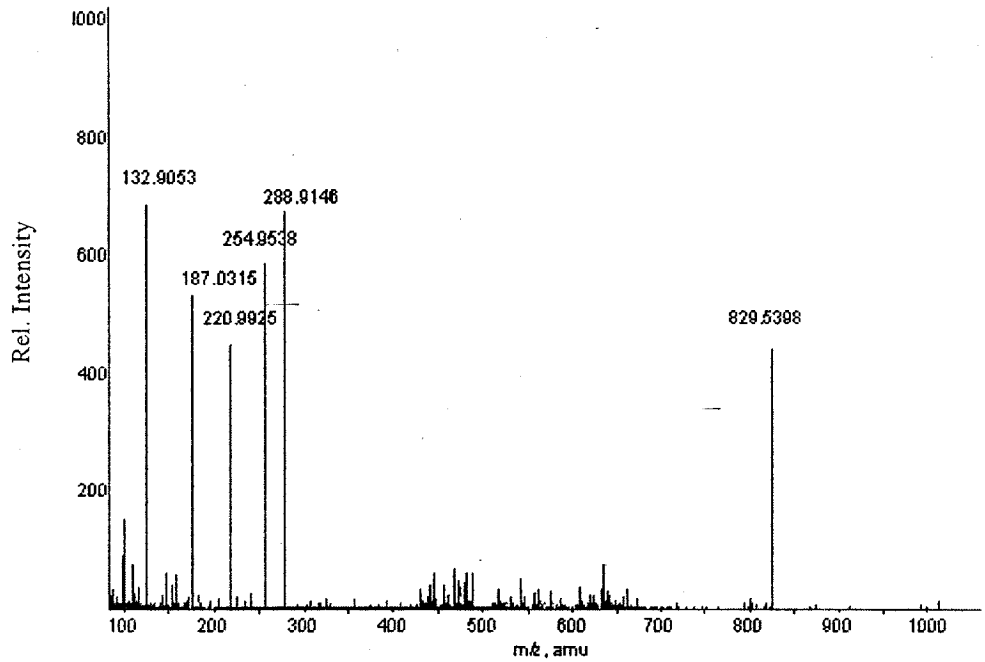


Figure 4.6: Mass spectrum of mono-, di-, tri- and tetra PCBs and internal standards

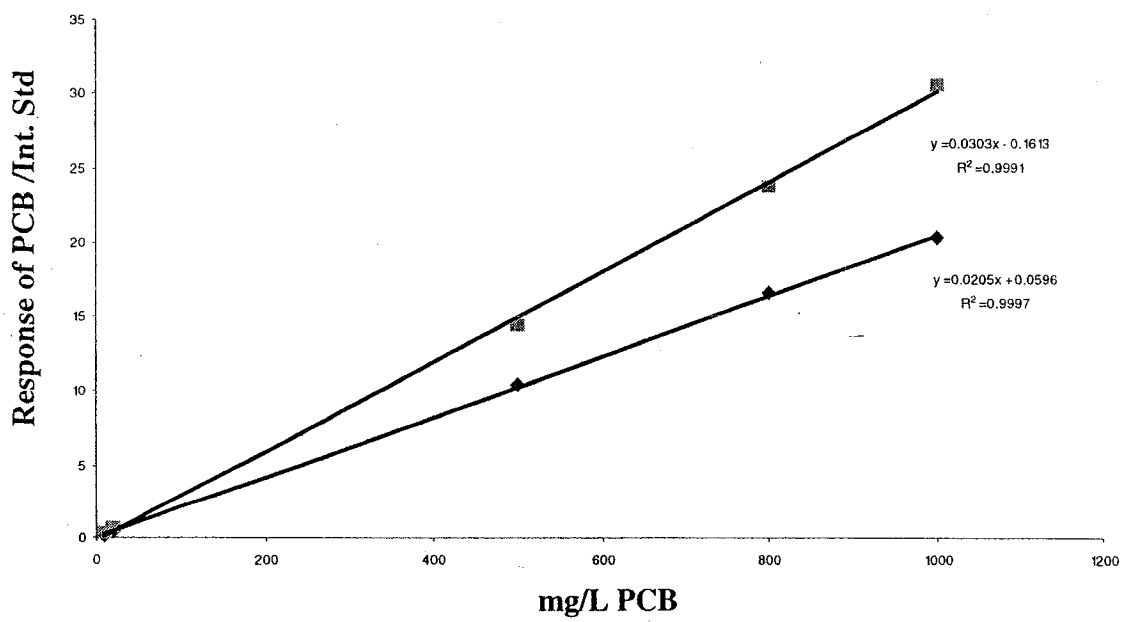


Figure 4.7a: Mass response of mono- and di-PCBs

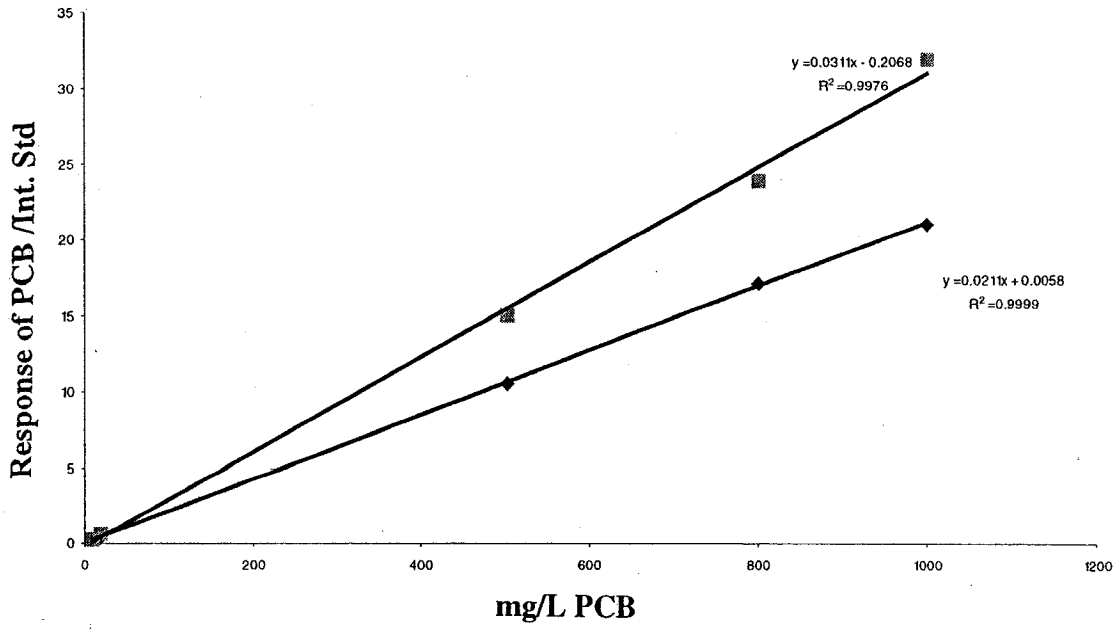


Figure 4.9a: Mass response of dihydrodihydroxy derivatives of mono- and di-PCBs

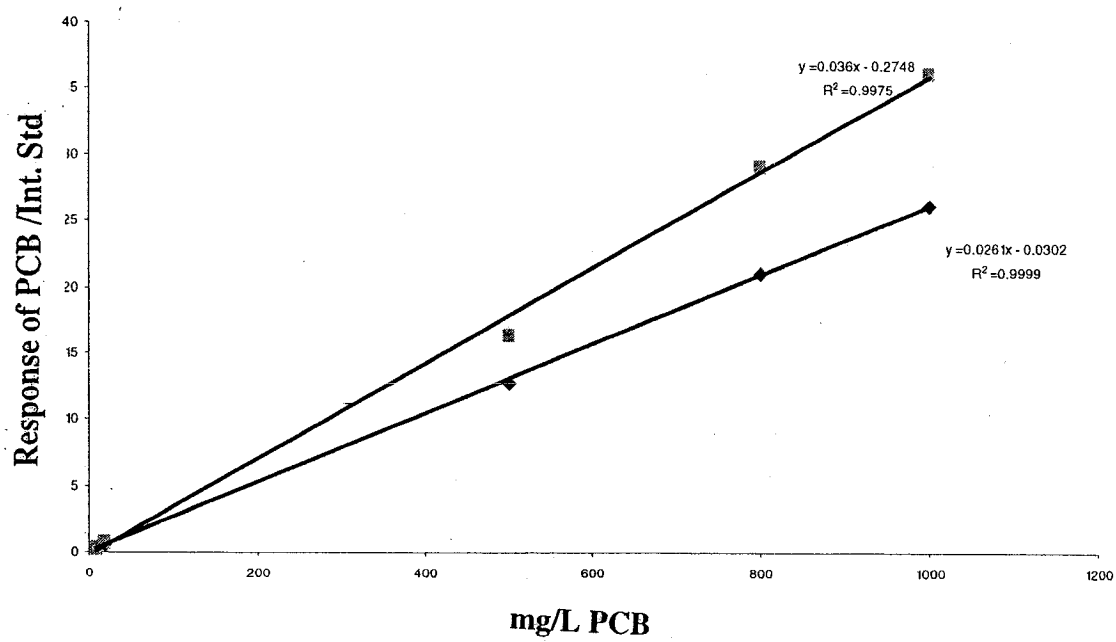


Figure 4.9b: Mass response of dihydrodihydroxy derivatives of tri- and tetra-PCBs

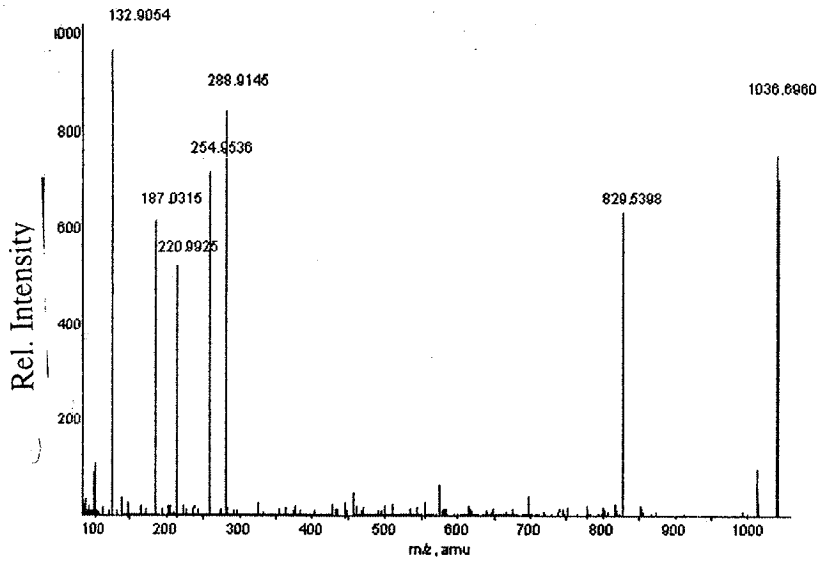


Figure 4.10a: Mass spectra of PCBs, internal standards and surfactin prior to the addition of enzyme complex

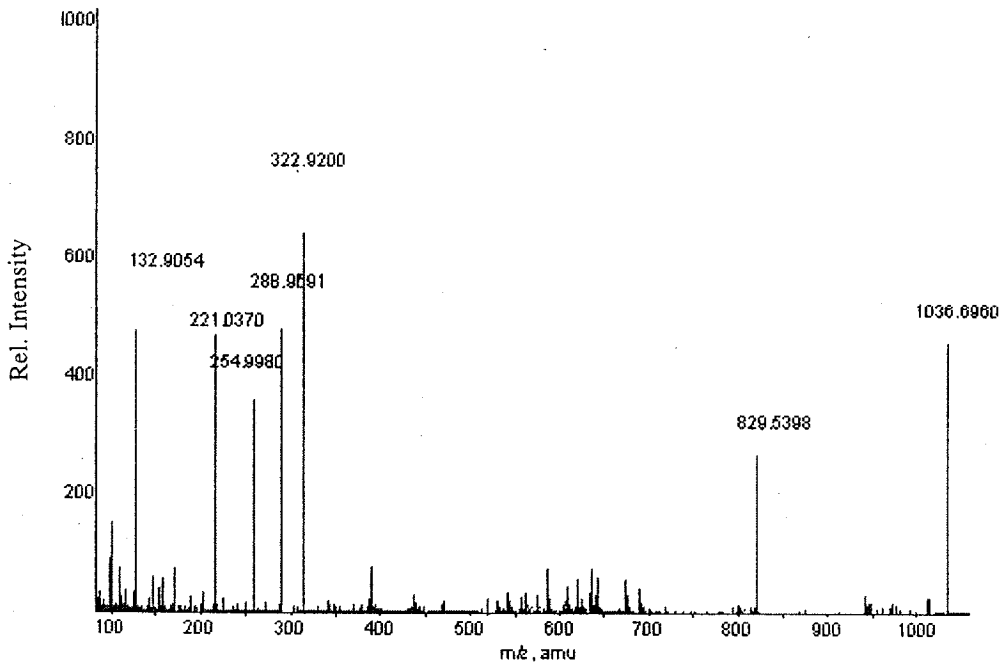


Figure 4.10b: Mass spectra of PCBs, internal standards and surfactin two hours after the addition of enzyme complex

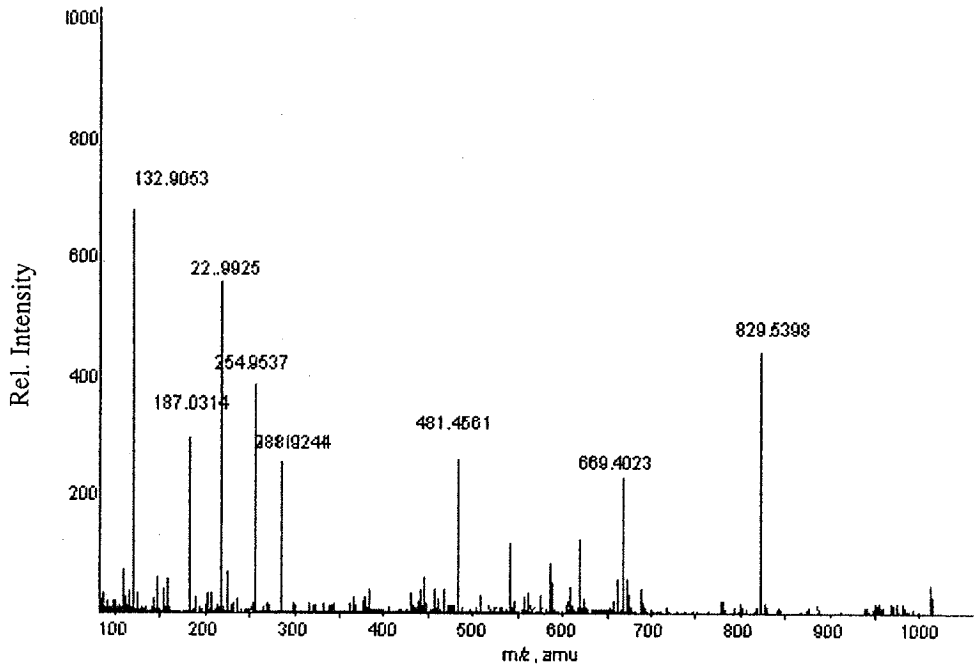


Figure 11a: Mass spectra of PCBs, internal standards and the rhamnolipids prior to the addition of the enzyme complex.

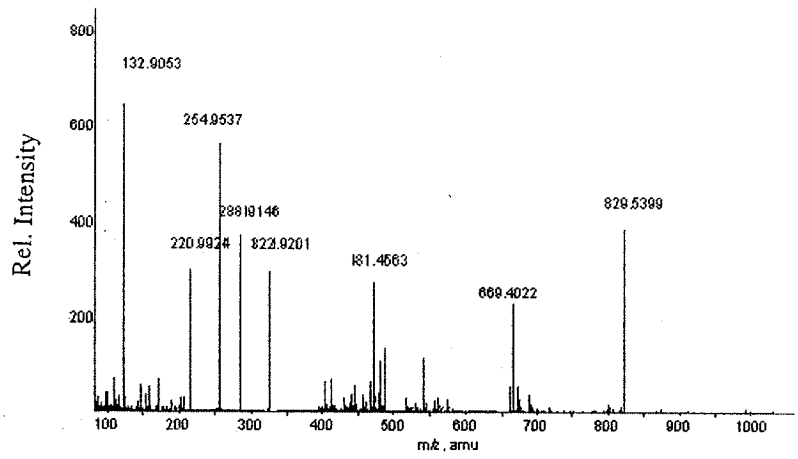


Figure 4.11b: Mass spectra of PCBs, internal standards and rhamnolipids two hours after the addition of the enzyme complex.

4.6: Spiked -soil extraction

The spiked soil samples were extracted with different solvents (Table 3.4) two hours after exposure to the toxicants. The solvent was added to the sample in a 100 μL eppendorf tube. It was briefly vortexed and upper phase removed. Two more extractions were made and the extracts pooled, evaporated and redissolved in starting buffer before infusion into the MS. The procedure was performed five times in triplicate and averaged. Results are shown in Table 4.2. All subsequent extractions were performed with acetone/ hexane.

Experiments were also performed with adjustment using different buffers (Table 4,3). As previously mentioned, the optimal enzyme activity and stability are observed in 100mM MES buffer at pH 5.5. Importantly, a useful mass spectrum could not be obtained in neat MES because of signal suppression. In ESI mass spectrometry, salts are avoided because of this phenomenon. In these experiments, the agitation rate was maintained at 400 rpm and incubation period was 2 hours. Subsequent reactions were performed in 100mM MES buffer pH 5.5 (maximum activity) but samples were thoroughly desalted on C_{18} ZipTip cartridges before infusion.

Experiments were also conducted at various temperatures. During that period, the agitation rate was maintained at 400 rpm and the incubation period was 2 hours. The optimal temperature was 25^0 C. Some activity was obtained at 12^0 C, but there were no activities at 37^0 C or 45^0 C. Subsequent reactions were conducted at 25^0 C. The degree of agitation was also varied to ensure that thorough mixing was achieved. The temperature

Table 4.2: Solvents used to extract PCBs.

Solvents	Percent recovery
acetone	68.0
n-hexane	70.3
isooctane	72.5
methanol	66.9
Acetone/hexane (50:50)	94.2
Acetone/isooctane (50:50)	88.3

Table 4.3: pH adjustment medium and recoveries

pH adjustment source	Percent recoveries
100mM MES	93
1% acetic acid	73
0.1% formic acid	76
0.05% trifluoroacetic acid	81

Table 4.4: Agitation rate and relative recoveries

Agitation Speed (rpm)	% Recovery
100	78.6
400	91.6
800	92.4
1000	89.2
1200	92.1

was held constant at 25^0 C. This could be of paramount importance to enable the surfactants to keep the PCBs in solution so they can interact with the enzymes. However as shown in Table 4.4, a rate of 400 rpm was ample to maintain the components in perfect contact, as recoveries were not improved at higher rates. In the time course experiments, most of the activity was completed to be within the first two hours of incubation as shown in figures.

4.7: Sample containing Aroclor

This sample was analysed in a similar fashion to the spiked sample. The optimal variables were used and the analyses were performed four times in triplicate and evaluated statistically.

Soils contaminated with PCBs for long periods of time are more difficult to treat. The longer the adhesion the tighter the binding. On the other hand, indigenous microorganisms in PCB- contaminated soils can sometimes utilize PCBs as substrates to initiate degradation. This process is referred to as reductive dechlorination (Barriault et al.1993).

4.8: Summary of results

This project was designed to evaluate the use of surfactants in conjunction with an enzyme complex for the degradation of PCBs in soil. In the first set of experiments, the

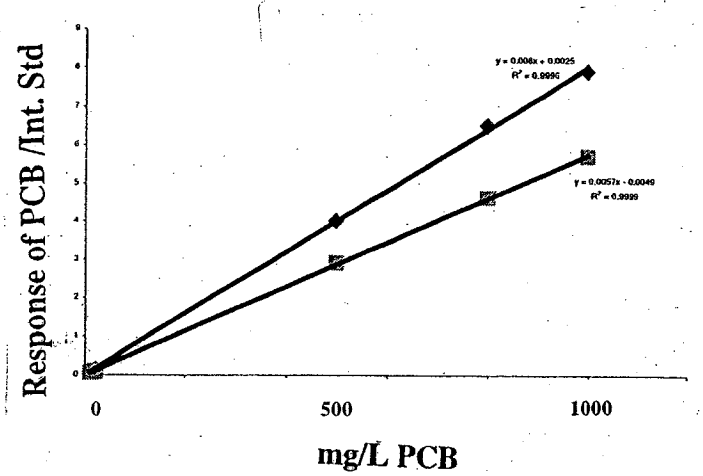


Figure 4.12: Tri- and tetra-PCB from soil extract

reactions were carried in an aqueous environment to optimize conditions whilst monitoring the degradation products. In other experiments calibration standards were prepared by serial dilutions of PCBs to internal standard ratios over a linear working range. Quality control samples were also prepared throughout the working range. Samples were analysed in a random fashion to eliminate biasness in the results. Several extractants were tested as the pH was adjusted with different chemicals with the aim of minimizing interferences which can occur in the final mass analysis. In later experiments, normal soil was spiked with varying amounts of PCBs and left in contact over a time course before extracting the pollutants. These xenobiotic compounds are very hydrophobic and adhere tightly to soil; factors that limit their degradation. Today, we still lack the knowledge on specifically how PCBs bind to different geochemical components of the sediment. This fundamental knowledge of PCBs association with soil is essential to comprehend the mechanism that determines their release from the sorbed state. It is

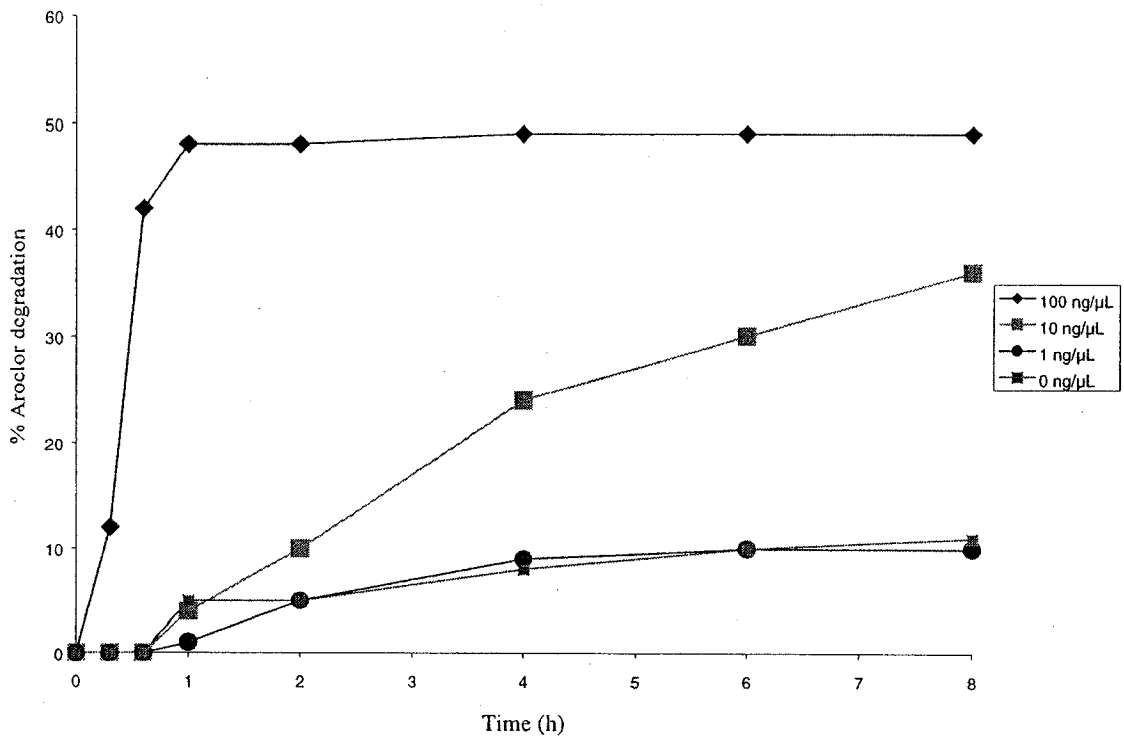


Figure 4.13:Aroclor degradation using different concentrations of surfactin

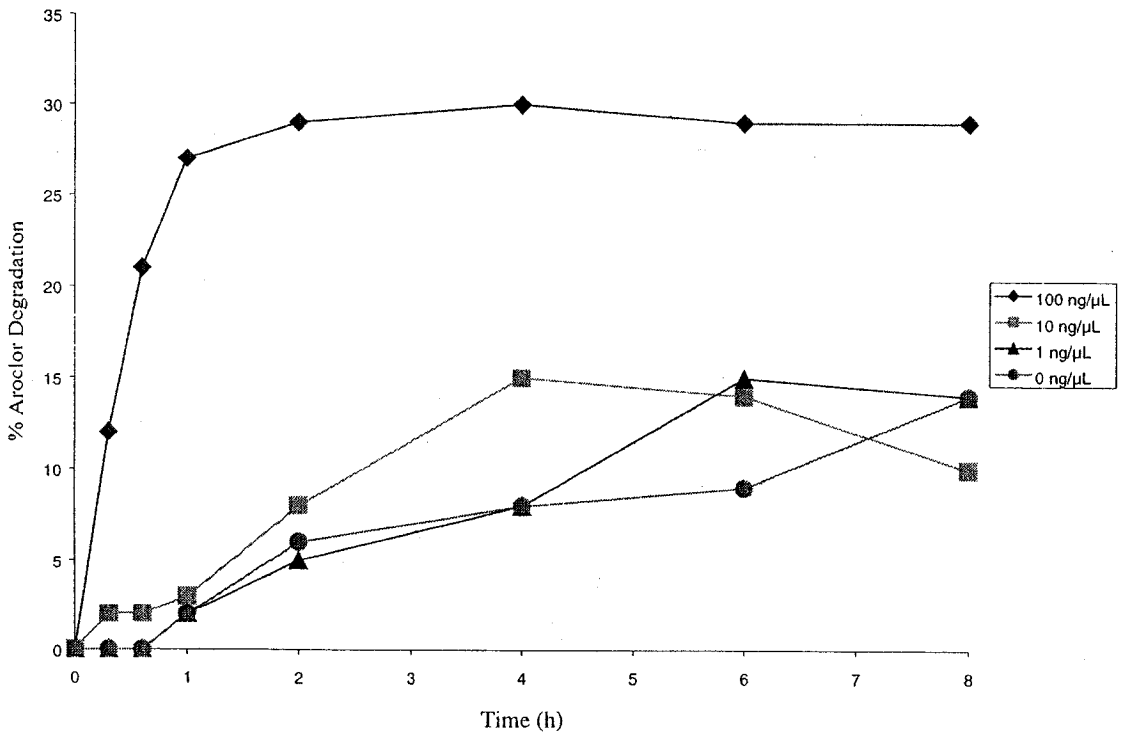


Figure 4.14: Arochlor degradation using different concentrations of Rhamnolipid

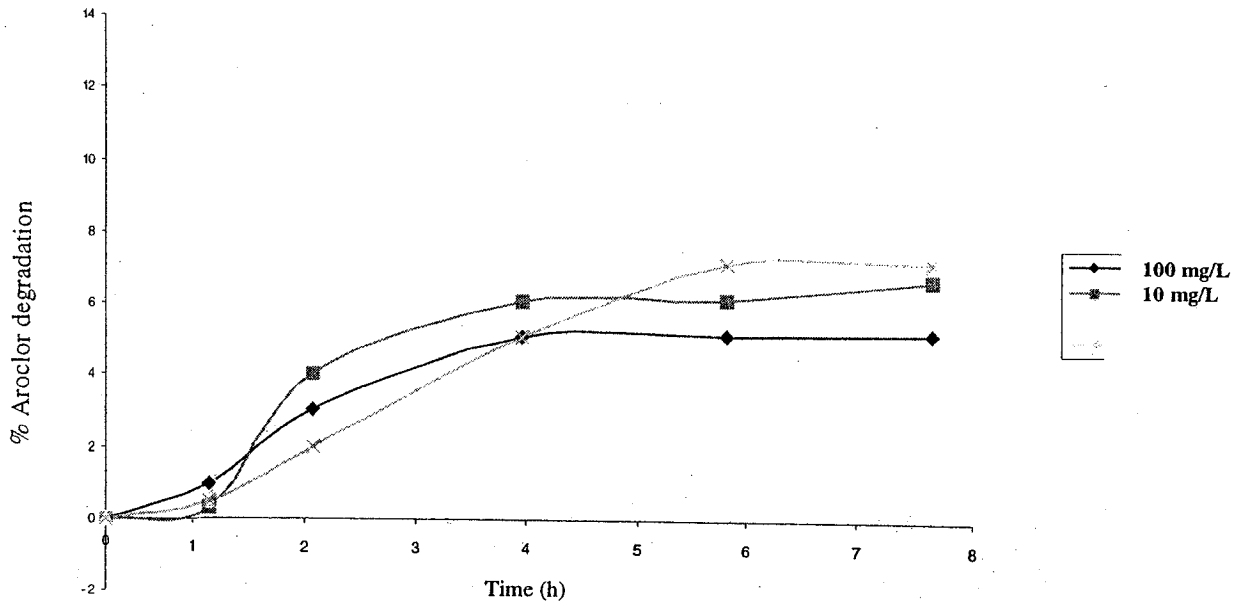


Figure 4.15: Octyl glucoside mediated Aroclor degradation

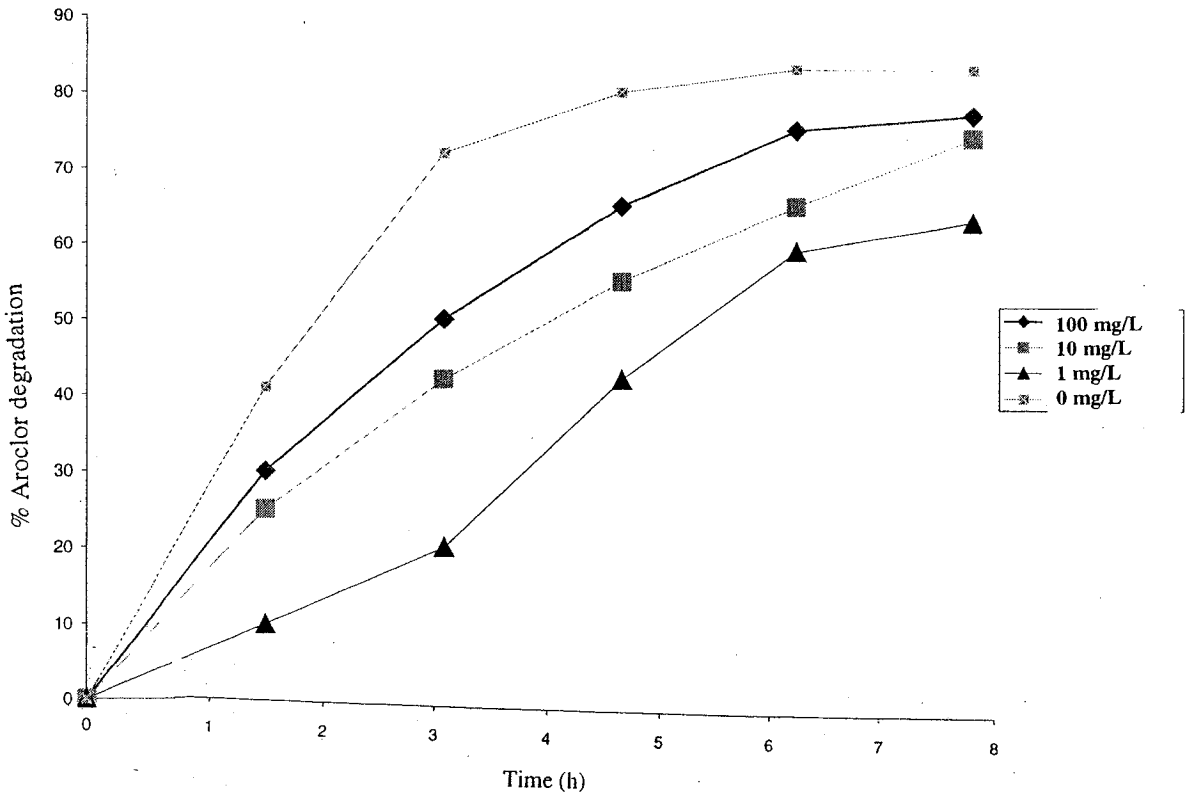


Figure 4.16: Aroclor degradation in soil sample

generally believed their slow release from the soil matrix to the aqueous phase is often the rate-limiting step in the process.

Figure 4.23 demonstrates the linearity of the working range when tri- and tetra-PCBs are extracted from soil. The PCBs extraction recoveries from the spiked samples two hours after exposure to the toxicants were 93% whereas average recovery from the samples extracted after 60 days of toxicant exposure was 67%. Within 2 hours of incubation, 47, 28 and 14% of PCBs were biodegraded from the contaminated sample by the addition of surfactin, rhamnolipids and octyl glucoside respectively, at above their critical micelle concentrations (Figures 4.13 to 4.15). In the aged PCB contaminated soil from a local site, 48% of the PCBs were biodegraded. The mass spectra of the non-incubated controls without surfactant revealed metabolites were not produced. The expected molecular masses and quantities of the parent compounds were observed. The incubated control without biosurfactant showed slight conversion to the dihydrodihydroxylated derivatives and other minor metabolites. The product ion spectra confirmed the nature of all compounds.

Table 4.5: Precision and accuracy data for mono- and di- PCBs calibration standards and QC samples

Calibration standards			QC samples			
Concentration (mg/L)	**Calculated concentration (mg/L)[a]**	**Accuracy (% bias)[b]**	**Concentration (mg/L)**	**Calculated concentration (mg/L)[a]**	**% CV**	**Accuracy (% bias)[b]**
Mono-PCB						
10	10.6	105.9	30	28.8	3.8	96.1
20	18.2	91.5		27.4		91.4
500	514.4	102.9		26.8		89.2
800	830.8	103.8	200	173.6	3.5	86.8
1000	969.1	96.9		172.4		86.2
				162.8		81.4
			700	637.8	2.4	91.1
				613.0		87.6
				641.0		91.6
di-PCB						
10	11.4	111.4	30	24.6	1.8	81.9
20	16.4	82.2		24.5		81.6
500	530.4	106.1		25.2		84.4
800	80.5	100.7	200	189.3	6.2	91.7
1000	980.4	98.0		207.2		103.7
				193.6		96.8
			700	681.6	2.8	97.4
				664.7		95.2
				704.2		100.6

[a] Concentration results were rounded to one decimal place

[b] % Bias is the calculated PCB concentration expressed as a percentage of its nominal concentration.

Table 4.6: Precision and accuracy data for tri- and tetra- PCBs calibration standards and QC samples

Calibration standards			QC samples			
Concentration (mg/L)	**Calculated concentration (mg/L)[a]**	**Accuracy (% bias)[b]**	**Concentration (mg/L)**	**Calculated concentration (mg/L)[a]**	**% CV**	**Accuracy (% bias)[b]**
Tri-PCB						
10	10.6	106.3	30		3.8	96.1
20	20.4	102.1				91.4
500	502.6	100.5				89.2
800	805.8	100.7	200		3.5	86.8
1000	1009.1	100.9				86.2
						81.4
			700		2.4	91.1
						87.6
						91.6
Tetra-PCB						
10	11.4	111.4	30	24.6	1.8	81.9
20	16.4	82.2		24.5		81.6
500	530.4	106.1		25.2		84.4
800	80.5	100.7	200	189.3	6.2	91.7
1000	980.4	98.0		207.2		103.7
				193.6		96.8
			700	681.6	2.8	97.4
				664.7		95.2
				704.2		100.6

[a] Concentration results were rounded to one decimal place

[b] % Bias is the calculated PCB concentration expressed as a percentage of its nominal concentration.

Chapter 5

Conclusions

5.1 Conclusions

The aim of this study was to determine the optimum conditions for the bioremediation of PCBs, a persistent toxicant in the environment, which are potentially carcinogenic. From the analysis of analytical data produced, the following conclusions were drawn.:

- Surfactants can enhance degradation of PCBs.
- Microbial surfactants are more effective than the chemical analog, at equal concentrations.
- These catalysts are much more effective at concentration above their critical micelle concentrations,
- It appears that the biosurfactants increase the solubility of the PCBs at those concentrations making them more available for biodegradation
- Surfactin was found to be more effective than rhamnolipids followed by octyl glucoside.
- When PCBs adhere to soil for long periods, the resident time for release of the toxins is much longer.

- The results demonstrated that the contact time is very crucial in the binding mechanism between soil and PCBs.
- Since the chemical composition of soil is quite variable, this phenomenon will also play a significant role in bioremediation.
- The BPDO enzyme complex system initiates the ring opening on its own but that is the rate-limiting step of the entire pathway.

5.2 Suggestion for future studies

- Although this project was undertaken in a laboratory, the results from the experiments suggest that if the soil composition and mechanism for removal at a contaminated site are fully understood, *in situ* bioremediation can be exploited.
- The use of other surfactants that can be more economically feasible to produce in crude mixtures can also tested.
- Several mutants of BPDO have been produced in our Chemistry / Biochemistry Department. These can also be analysed for enhancement.
- Utilizing other enzymes in the dechlorination pathway should also be examined for even further enhancement.

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