MICROBIAL DEGRADATION OF TOXAPHENE

Martha L. Lacayo Romero

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Abstract

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Abstract

This works deals with the degradation of a group of recalcitrant compounds known as toxaphene. Toxaphene is a complex mixture of chlorinated compounds including hexa- and octachlorocamphenes, hepta-, nona- and decachlorobornanes. Some components of toxaphene are very persistent while others are degraded rapidly by both abiotic and biotic processes. Not all components of toxaphene have been identified. The main purpose of this research was to develop inexpensive and robust technology for degradation of toxaphene using microorganisms. To achieve the degradation two approaches have been used: anaerobic dechlorination with a subsequence metabolism of the carbon skeleton, and use of strong oxidizing reagents from fungal peroxidase/laccase to at least initiate degradation.

A sequential anaerobic-aerobic bioreactor configuration was used for the purpose of toxaphene degradation. Aerobic processes permit the attainment of higher organic matter removal rates and are often less sensitive to pollutant toxicity. In this study, COD removal efficiencies of up to 90% were attained in the anaerobic step, confirming the potential of this process for the initial attack of toxaphene. No significant enhancement in the removal of COD was recorded in the aerobic step. The anaerobic-aerobic system implemented to degrade toxaphene in water was effective and after 42 days, the total toxaphene concentration was reduced by 87%. Final degradation, achieved after 269 days, was very similar to the results obtained already from the anaerobic reactor (98%).The anaerobic step was responsible for high removal efficiency and small improvements were obtained in the aerobic step. Toxaphene isomers having more chlorine substitutions were degraded relatively fast, resulting in an initial rise in concentration of isomers with less chlorine substitutions.

White-rot fungi often need the presence of easily biodegradable co-substrate to trigger the production of extracellular enzymes. Wheat husk, wood chips and molasses were compared for their ability to support toxaphene degradation by a *Bjerkandera* sp. Toxaphene biodegradation was intrinsically linked with the production of lignin peroxidase (LiP). Approximately 85% of toxaphene was removed when wheat husk was used, while lower removal efficiencies were recorded when using wood chips and molasses, respectively. There are several evidences that ligninolytic enzymes produced by *Bjerkandera* sp. are the oxidizing agents of recalcitrant compounds; however, the capability of toxaphene removal has not been studied before. Future work should focus on the development of continuous fungal-based processes and further comparison with bacterial based processes.

Toxaphene biodegradation in bio slurry reactors is often limited by pollutant bioavailability. Removal efficiencies of toxaphene isomers of up to 96% were achieved in 79 days of operation. In our study, no improvement in the biodegradation of less chlorinated isomers was observed when adding the surfactant Triton X-114 or lactic acid. The removal of heavily chlorinated compounds was enhanced by the addition of the compounds above mentioned.

From toxaphene-contaminated soil it was possible to identify one species that was capable of degrading toxaphene on its own. *Enterobacter* sp. Strain D1 is a facultative anaerobic, Gram negative heterotrophic bacterium. Based on 16S rDNA analysis, the strain D1 was clustered closely with the species *Enterobacter cloacae* subsp. *dissolvens* and *E. cloacae*. The ability of this microorganism to utilize and transform the different chlorinated camphenes present in toxaphene was investigated under anaerobic conditions. Levels of hexachlorocamphenes and heptachlorobornanes were initially reduced, but the levels increased again during the last 20 days of cultivation.

Key words
Toxaphene, biodegradation, anaerobic, aerobic, aged contaminated soil, *Enterobacter* sp, *Bjerkandera* sp.

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List of Publications

This thesis is based on the following papers, which will be referred to by their roman numerals in the text.

I Lacayo-Romero, M., van Bavel, B., Mattiasson, B. Degradation of toxaphene in water during anaerobic and aerobic conditions. *Environ. Pollut*. 2004, **130**, 437-443.

II Lacayo-Romero, M., Quillaguamán, J., van Bavel, B., Mattiasson, B. A toxaphene degrading bacterium related to *Enterobacter cloacae*, Strain D1, isolated from aged contaminated soil in Picacho, Chinandega, Nicaragua. *Syst. Appl. Microb*. (Accepted)

III Lacayo-Romero, M., van Bavel, B., Mattiasson, B. Degradation of toxaphene in aged and freshly contaminated soil. *Chemosphere*. (submitted for publication)

IV Lacayo-Romero, Terrazas, E., van Bavel, B., Mattiasson, B. Degradation of toxaphene by *Bjerkandera* sp, using waste biomass as a cosubstrate. *Appl. Microbiol. Biot.* (submitted for publication)

My Contribution to the papers

Paper I I designed the experimental work, analyzed the results and wrote the first draft of the manuscript which was then revised by the other authors.

Paper II I planned the experiments and did most of the experimental work except the characterization and identification of the strain which was carried out by Jorge Quillaguamán. I wrote the first draft of the manuscript which was then revised by the other authors.

Paper III I did practically all the work except the isolation and characterization of the fungal strain which was carried out by Enrique Terrazas. I wrote the first draft of the manuscript which was then revised by the other authors.

Paper IV I planned the experiments and did most of the experimental work. I wrote the first draft of the manuscript which was then revised by the other authors.

Chapter 1: Introduction

Persistent Organic Pollutants (POPs) is the name for the most problematic group of chemicals. POPs are toxic chemicals which pervade and contaminate air, soil and water over extended periods because they break down only slowly in the environment. They may also accumulate in the adipose tissue of humans and animals. Since the boom of the chemical industry, enormous amounts of these chemicals have been, and continue to be, produced globally (Tanabe, 1991).

The story of POPs begins with the growth of the organic chemical industry in the early 20th century. DDT was first synthesized in 1874, but its insecticidal properties remained unknown until reported in 1939 by the Swiss chemist Paul Hermann Muller. A skin rash called chloracne was reported by Karl Herxheimer to be afflicting German workers in chemical industry producing organic chemicals. The causal agent, dioxin, remained elusive for many decades (EPA, 1999).

PCBs were first produced commercially in 1929. Their production peaked in 1970 and they were finally banned from production in the United States. Dieldrin and aldrin were first synthesized as pesticides in the United States in the late 1940s. With World War II came a broad public awareness of the potential marvels of chemicals such as DDT for disease vector control. Faith in DDTs potential was exemplified by international efforts seeking to eradicate diseases such as malaria. At the same time, newly developed organochlorine pesticides and herbicides were rapidly filling the needs of the growing agrochemical industry (Wania and Mackay, 1996).

1.1 World Environmental Pollution

The environmental contamination of POPs on a global scale has been thoroughly documented (Marcus and Renfrow, 1990; Sericano et al., 1990; Calero et al, 1993; Readman et al., 1992; Rugama et al., 1993; Daskalakis and O'Connor, 1995; Long et al., 1995; Muir et al., 1995; Cruz et al., 1997; Castillo et al., 1999; Carvalho et al., 1999; Castillo et al., 2000; Lacayo et al., 2000; Dorea et al., 2001).

POPs reach the environment in a variety of ways. They may be introduced to the environment through chemical plant discharge and agricultural inputs, but also through losses from many consumer products such as computers, paints and household products, (in which they may be used as additives). POPs can be transported by air and sea currents and may travel great distances. As a result, they do not only contaminate the immediate surroundings of chemical plants, but may also reach pristine and remote areas. Contamination from POPs is a global problem. Although, some dangerous chemicals (POPs) have already been banned in certain European countries, progress on the most problematic chemicals is relatively limited in developing countries. As POPs do not acknowledge national boundaries, continued production and use in one country can lead to increased widespread contamination (Wania and Mackay, 1996).

1.2 Toxaphene Contamination

Toxaphene, "also known as camphechlor, chlorocamphene, polychlorocamphene and chlorinated camphene" is a pesticide with a range of applications. After the usage of DDT was forbidden in the early 1970's, toxaphene was its major replacement as an agricultural insecticide (Saleh, 1991).

Toxaphene, is one of the most heavily used pesticides. The global use of toxaphene between 1950 to 1993 was estimated to be 1 330 000 tonnes. However, lack of data or records from some countries makes these figures a bit uncertain. During this windows of time the United States, former Soviet Union, Central America and Brazil were using the largest amounts of the pesticide (Voldner and Li, 1995).

Due to its chemical stability, environmental persistence, and molecular properties, toxaphene has been accumulated and bioconcentrated in the food chain. Thus, enrichment has taken place in fat-rich tissues of plants and animals (Terrierre et al., 1966; Muir et al., 1992; Bidleman et al., 1993; Zhu and Norstrom, 1993; Kidd et al., 1995).

During 1980's the first signals of toxaphene as a global pollutant came from Canada and Scandinavia. In these regions, located thousands of kilometers from the main application sites, toxaphene was detected especially in lipid rich polar fish and mammals (Muir et al., 1992; Gregor, 1993). Toxaphene constitutes a global threat similar to DDT, polychlorinated biphenyls (PCB), and other organochlorines (Saleh, 1991).

In Nicaragua toxaphene was produced in a factory near Managua starting in 1974. National production was 9,500 tones in 1974 and

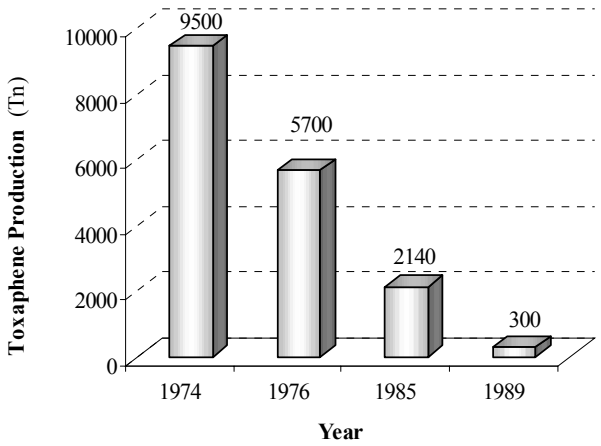
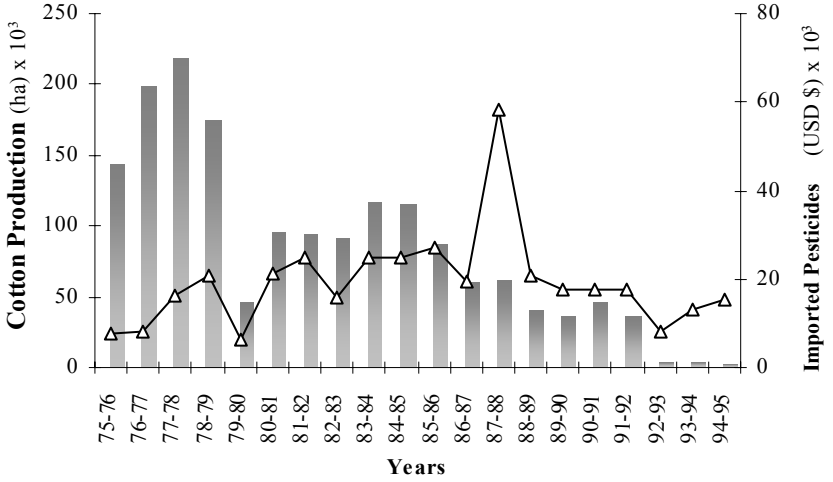


Fig. 1.2.1. Toxaphene production in Nicaragua

decreased to 290 tones by 1989 (Fig. 1.2.1). In 1990 the production of toxaphene finally ceased. Confirmed by statistics of formulation plants, large stocks of the chemical were used to prepare mixtures of toxaphene with imported DDT, parathion and methyl-parathion for several years (Appel, 1991). The total production of toxaphene in the period 1974-1990 was estimated to be about 79,000 tones and most of it was used within the country (Rodezno, 1997).

The use of toxaphene in Nicaragua was always closely correlated with cotton production and, still today, the cotton farmers consider toxaphene as the most effective of all pesticides used in cotton production. In 1985, the rates of application of toxaphene were as high as 31 kg ha^{-1}. However, the reduction of cotton prices in the international markets in the early 80s, together with the introduction of other pesticides, significantly decreased the use of toxaphene (Appel, 1991).

Chinandega, in northwestern Nicaragua, is a fertile lowland agricultural district with a hot climate and an annual rainfall of 1500-1700 mm (between May and October). Chinandega has the nation's best soil quality and therefore has a large agricultural impact. It is the main region for growing cotton and toxaphene was used to a wide extent in this area (Fig. 1.2.2). An investigation carried out by Aquatic Research Investigation Center (CIRA) in 1995/96 indicated concentrations of toxaphene in agricultural soils as high as 40 $\mu g.g^{-1}$ soil. In the water courses, the concentration of



Font : Economic Indicators (Nicaraguan Central Bank, 1996)

Fig. 1.2.2. Cotton Production (bars) and imported pesticides (triangles) in Nicaragua.

toxaphene was found to be higher in deeper sediment layers due to heavier use in the past.

The decreasing price of cotton on the international market led to a decrease in cotton production in Nicaragua. This was compensated by an increase in production of crops like corn, coffee and vegetables. For these types of crops, organophosphorous pesticides are preferable, so the overall demand for organochlorine pesticides consequently diminished. The persistent toxaphene introduced into the soil from Nicaragua's cotton production will continue to contaminate rivers, lagoons and groundwater for many years, severely affecting the drinking water quality and the sensitive mangrove ecosystems (Carvalho, et al., 2003). The Chinandega district on Nicaragua's Pacific coast is a region of particular concern.

A screening of contaminants in the Pacific coast of Nicaragua carried out in 1995, indicated the presence of relatively high levels of toxaphene in the lagoons of the Chinandega district (Carvalho et al., 1999). More studies in different compartments of the ecosystem show the environmental contamination by toxaphene (Calero et al, 1993; Rugama et al., 1993; Cruz et al., 1997; Lacayo et al., 2000; Castillo et al., 1999; Carvalho et al., 1999; Dorea et al., 2001).

Stockpiles of toxaphene continue to threaten the environment in some areas. In Nicaragua, 230 tones of toxaphene were recently reported to be stockpiled in a zone of high risk for earthquakes near Lake Managua, a unique ecosystem and home to many rare species of wildlife (EARTH, 2000).

1.3 The aim and outline of the thesis

The main purpose of the thesis work was to find a biotechnological method to degrade the persistent organic pollutant called toxaphene. For the treatment of this compound, it is essential to develop an inexpensive technique in order to make it affordable for impoverished regions afflicted with toxaphene-related problems.

The first two chapters of the thesis provide a background of persistent organic pollutants (POPs) including its environmental impacts and fate if left alone. The properties of toxaphene, and other important information are included.

The chapters 3 and 4 summarize the results presented in paper I-IV. In chapter 5 innovative biological methods for treatment of POPs are presented.

Finally, chapter 6 gives some concluding remarks.

Chapter 2: Persistent Organic Pollutants (POPs)

POPs are highly stable, molecules that are essentially non-reactive under common conditions. POPs are used as herbicides, pesticides, fungicides and as raw materials for chemical production. Twelve POPs identified for priority action by the United Nations Environment Programme **(**UNEP) are listed in Table 1. Nine of these are pesticides while the other three include polychlorinated biphenyls (PCBs), polychlorinated dibenzo-p-dioxins (PCDD) and polychlorinated dibenzofurans (PCDFs) (Fisher, 1999). The last two have never been produced intentionally. They are produced by incineration processes and, unintentionally, as by-products during chemical reactions (Wittich, 1998).

There are many thousands of POPs, often coming from certain series or families of chemicals (e.g. there are theoretically 209 different polychlorinated biphenyls (PCBs) congeners, 419 congeners of polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and polychlorinated diphenyl ethers (PCDEs), 75

congeners of polychlorinated naphthalenes (PCNs), etc.) (Bünz and Schmidt, 1997; Falandysz, 1998).

Table 1. List of 12 priority POPs identified by the UNEP

POPs	Applications
Aldrin	Insecticide for crops like corn and cotton
Hexachlorobenzene	Solvent for a pesticide, intermediate, fungicide for treatment of crops
Chlordane	Broad spectrum contact insecticide, additive in glues
Mirex	Insecticide against leaf cutters, ants and termites.
Dieldrin	Insecticide for crops like corn and cotton
Toxaphene	Insecticide mostly used on cotton crop
DDT	Production of dicofol, insecticide
Dioxins	Not intentionally produced.
Endrin	Insecticide used mainly on field crops, rodenticide to control mice.
Furans	Not intentionally produced
Heptachlor	Insecticide primarily used against soil insects and termites
PCBs	Heat transfer medium, paints, transformers oil, dielectric

2.1 Physicochemical Properties

POPs are hydrophobic and lipophilic. These substances possess physical and chemical properties which determine their transport pathways and distribution in the environment. The physical properties of greatest importance are water solubility, vapor pressure (P), octanol water partition coefficient (Kow). Persistence in the environment is the other important characteristic of POPs since transport can extend the range of exposure far beyond the immediate area of use and or release (Scholtz and Voldner, 1992).

The volatility of POPs gives rise to a certain mobility that allows relatively great amounts of the substance(s) to enter the atmosphere and be transported over long distances (Barrie et al., 1992). It is common for these substances to volatilize in hot regions and condense and tend to remain in colder regions. This phenomenon is called global distillation. It is supposed that much of the pollution of polar regions by POPs can be ascribed to this phenomenon (Fig. 2.1.1). Volatilization may occur from plant and soil surfaces following application of POPs used as pesticides. (Scholtz et al., 1993, Benjey, 1993).

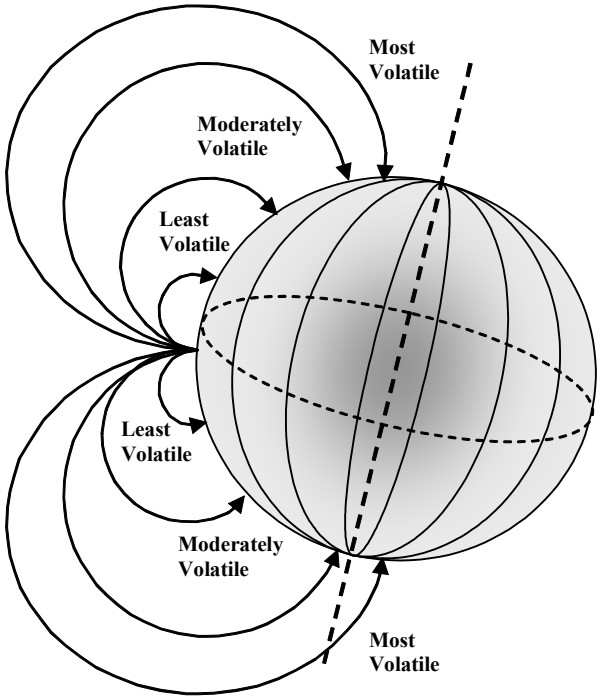


Fig. 2.1.1. Global distillation of POPs in the world.

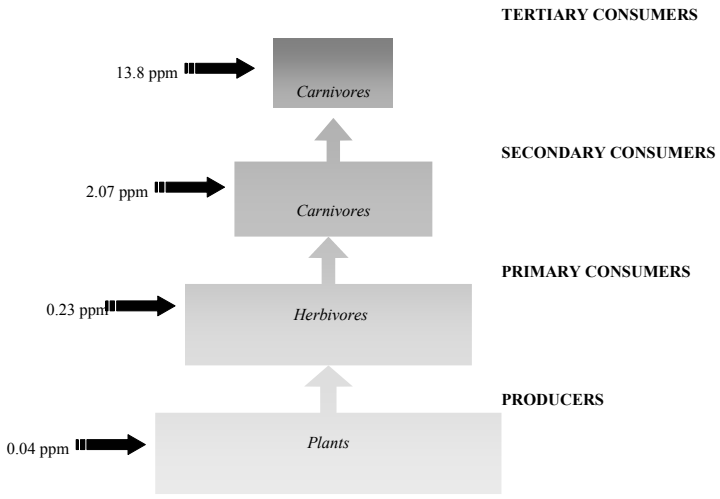


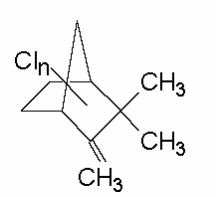
Fig.2.1.2. DDT Biomagnification through the food chain

Due to their high lipophilicity, POPs tend to dissolve preferentially into lipids and fat rather than in water. This lipophilicity (combined with their resistance to natural biological degradation) results in biomagnification throughout the food chain. This results in much greater concentrations of POPs in organisms at the top of the

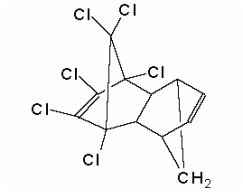
food chain (Scholtz and Voldner, 1992) (Fig. 2.1.2).

2.2 Structures

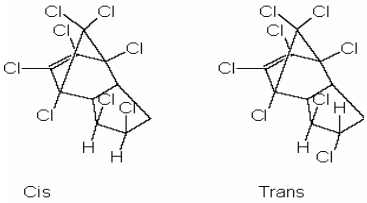
The chemical and physical properties are determined by the structure of the molecule and the nature of the atoms present in the molecule. Fig. 2.2.1 shows the chemical structure of the 12 priority POPs.



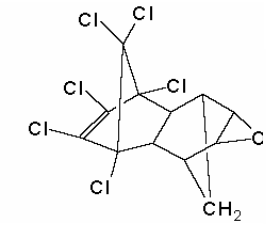
Toxaphene



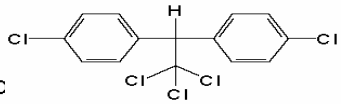
Aldrin



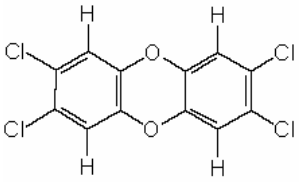
Clordane



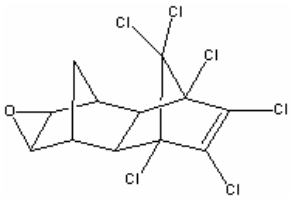
Dieldrin



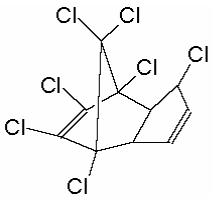
DDT



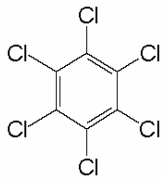
Dioxins



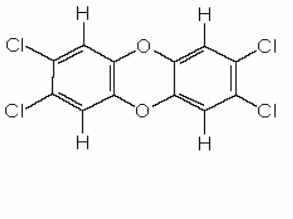
Endrin



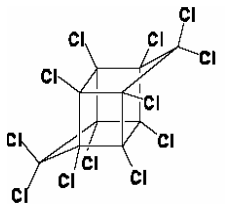
Heptachlor



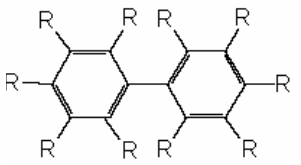
Hexachlorobenzene



Furans (PCDFs)



Mirex



Polychlorinated biphenyls (PCBs)

Fig. 2.2.1. The 12 priority POPs by the UNEP.

2.3 Sources

The sources of POPs are from agriculture, industry, manufacturing discharge and municipal sewage disposal practices. As mentioned above sources of organochlorine pesticides (OCPs) are mainly derived from agricultural applications. Polychlorinated biphenyls (PCBs) had a wide range of industrial applications as dielectric and hydraulic fluids, prior to their ban in many countries. However, PCBs continue to be released by a wide range of industrial activities, particularly via the disposal of electrical waste. (OCPs) are widely used for pest vector control. Polybrominated diphenyl ethers (PBDEs) continue to be used as flame retardants in a range of materials and electrical components (Kannan et al., 1997).

By the late 1970s, nine POPs had been either banned or subjected to severe use restrictions in many countries. Current information indicates that some of these POPs are still in use in parts of the world where they are considered as essential for ensuring public health (UNEP, 2002). In an effort to further reduce their use in these countries, it is important to understand which countries are using POPs, and how they are applied. It was found that there is considerable information that describes the aggregated volume of POPs produced and used in the world; however, there is very little reliable data about the specific uses in each country (UNEP, 2002).

2.4 The mechanisms of soil binding

For many pesticides, the binding is a consequence of transformation of the parent product into phenolic and amino derivatives. Some recalcitrant pollutants like highly energetic chemicals (2,4,6-trinitrotoluene - TNT, hexahydro- 1,3,5-trinitro-1,3,5-triazine - RDX and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine - HMX) (Hawari et al., 2000), and polyaromatic hydrocarbons, PAHs (Ressler et al., 1999), have been reported to disappear after *ex-situ* bioremediation treatment. Despite the disappearance of the POPs in almost all reported studies, little or no mineralization is produced; and the fate of the compounds and their bio-transformed products has not been determined. Consequently, several authors have recently addressed the importance of understanding the mechanisms of interactions between the POPs and their degradation products with various matrixes (soil, organic matter, and biomass) (Achtnich et al., 1999, Knicker et al., 1999).

POPs can exist bound in soil in many different ways. The term easily extractable relates to the fraction which is set free rapidly (< 1 h of contact) at room temperature (Vanneck, 1996). Easily extractable forms are those that are adsorbed to surfaces, present in liquid films on the soil particles or as particulate pollutant in the soil matrix. Poorly extractable forms are adsorbed in soil, and/or entrapped in the water phase of micro or nanopores. Non extractable forms are chemically bound (Volkering, 1996). The term poorly extractable relates to pollutants which are hardly or not at all extractable by normal solvents (e.g. methanol or hexane). However, by extensive refluxing with a solvent (8 to 24 h) at higher temperatures (e.g. 40 °C for dichloromethane, 34 °C for diethylether) in Soxhlet apparatus these poorly extractable pollutants can be set free. Alexander (1995) stated that some organic solvents, often under vigorous conditions, can extract from aged polluted sites.

The term ageing does not include reactions that alter the structure of the molecule like polymerization or covalent binding to humic substances (Hatzinger and Alexander, 1995). Hence, aged compounds are considered to belong to the category of poor extractable. The term non extractable relates to altered structures which can only set free upon chemical modifications of the molecules concerned. The term bound residues is

proposed to relate to all residues retained in a physical or chemical way in the soil, reversible or irreversible (Verstraete and Devliegher, 1996).

2.4.1 Sorption

The term adsorption relates to molecules which are adsorbed onto the soil matrix without formation of covalent bonds. Surface sorption is considered as the rapid and reversible partition process of the chemical between the water and solid phase. Sorption is strongly dependent on the hydrophobic/hydrophilic properties of a chemical as well as on its concentration in the water phase. Adsorption is partly due to minerals, especially the clay fraction, and partly to the organic matter. Whereas clay minerals are responsible for adsorption of polar and hydrophilic compounds, organic matter consists of hydrophilic and hydrophobic regions, which results in an adsorption capability for either polar or charged and apolar or lipophilic chemicals. Surface sorption on soil, is most important since both its cation exchange capacity and surface area is higher than any other soil component (Khan, 1982).

Residues adsorbed to soil organic matter are subject to microbial degradation. The adsorbed parent compound or metabolite is in equilibrium in trace amounts with the pore water solution and thus available for degradation. Moreover, it has been argued that microorganisms might be capable to directly act on surface sorbed compounds. Microbial uptake of surface bound substrates stimulates their desorption by maximizing the concentration gradient. They exist over very short distances between the particle surfaces and cell walls. Desorption can even be more pronounced if microbial cells are attached to the surface of the soil particles (Harms and Zehnder, 1995).

2.4.2 Chemical binding

Chemical binding is the result of a covalent bond that is formed between a molecule and the humic matter of the soil. The stable chemical linkage that is formed is known as an oxidative coupling. These formed complexes are highly resistant to acid or base hydrolysis, thermal treatment and microbial degradation. During humus formation, as a result of the gradual removal of reactive substituents, organic matter in the soil becomes a quasi inert polycondensate. This process is the consequence of the minimization of free energy and results in an end product which is not harmful for the environment. On the contrary, the humus in the soil provides stability for water holding capacity and aggregate formation. The humus heteropolymer is slowly degraded (a few percent per year), not by specific enzymes, but through overall physical and chemical erosion. (Verstraete and Top, 1999).

Nonpolar hydrophobic POPs adsorb tightly to the organic fraction of soil and sediments. Sorption to the mineral fraction of soil is usually much weaker and is important for bioavailability, mainly by intraparticle diffusion. Within the fraction of organic soil compounds, humic substances (humic acids, fulvic acids and humus) play an important role for binding nonpolar POPs or their metabolites (Ressler et al., 1999). Humic substances are considered to be a sink for intermediates of aromatic and non-aromatic compounds.

The formation of humic acid-like substances during microbial degradation of hazardous organic pollutants like PAHs and other POPs has been an important matter of concern in the field of bioremediation of contaminated soil in recent years.

2.5 Toxicity

The toxicity of a substance can be reported in a variety of ways. Substances may be classified and measured according to as acute or chronic, acute (short-term) or chronic (long-term) effects, lethal or effective dose levels (LD50 or ED50, the dose that will kill

or affect 50% of test animals), or tissue levels associated with an adverse effect. Even though certain toxic effects and levels may be easily detected and quantified in laboratory settings, measurement of these compounds in the natural environment is considerably more difficult (UNEP, 2002).

Some studies have demonstrated that continuous exposure to toxaphene can cause acute toxicity to aquatic and terrestrial wildlife and pose carcinogenic and mutagenic risks to humans (Hooper et al., 1979; Reuber, 1979; Calciu et al., 1997). However, up to now, little data is available concerning the pharmacokinetics of toxaphene as such studies are plagued by the complex chemical mixture of toxaphene congeners and the technical difficulty in accurately quantifying their environmental concentrations. Some limited kinetic studies indicate that toxaphene has a relatively long retention time and a generally high accumulation rate in the animal lipid tissues (Mohammed et al., 1983).

Studies using ^{36}Cl- and ^{14}C-labeled technical toxaphene demonstrated that in rats more than 50% of the orally administered dose was eliminated in urine and faeces in about 2 weeks (Crowder and Dindal, 1974; Ohsawa et al., 1975). The major metabolite of ^{36}Cl-toxaphene is chloride ion (Crowder and Dindal, 1974; Ohsawa et al., 1975). However, these previous studies have principally been concerned with the time-course distribution of toxaphene. Little research has been conducted on the pharmacokinetics of accumulation, elimination, and tissue burden of this compound.

2.6 Impacts and Fate

The behaviour and fate of persistent organic pollutants in the environment is determined by their chemical and physical properties and by the nature of their environment. Effects include carcinogenicity, toxicity to the reproductive, nervous and immune systems and adverse effects on development (Silberhorn et al. 1990, Allsopp et al. 1997, Longnecker et al. 1997).

Organochlorines, such as DDE, may have weak estrogenic properties and while some authors have suggested a possible role in estrogen receptor positive breast cancer (Wolff et al., 1993), others have been unable to demonstrate such a role for DDT or its metabolites (Krieger et al., 1994). Halogenated aromatic hydrocarbons are also known to affect endocrine function and reproductive systems (Peterson et al., 1992; Gray, 1992; Thomas and Colborn, 1992). Vonier et al., 1996, examined the ability of chemicals to bind to the estrogen receptor and progesterone receptor in a protein extract. Unlike some DDT metabolites, toxaphene did not interact with the estrogen receptor.

POPs may affect one or more steroid hormone receptors in the body. They might affect receptors for the male sex hormone testosterone, the female sex hormones estradiol progesterone, and various hormones produced by the pituitary and adrenal glands. These hormones receptors are common in fish, reptiles, birds and mammals including humans. They are essential for regulation of many body functions especially during the formative stages of growth and development. It has been hypothesized that endocrine-disrupting chemicals can interfere with the levels of circulating hormones in the body and may elicit a wide range of adverse effects (Colborn et al. 1993, EPA 1999).

Other mechanisms by which POPs could cause effects on health include those mediated via enzyme systems. Cytochrome p450 enzymes located in the liver are involved in both the regulation of steroid hormones and in the detoxification of chemicals, including steroid hormones. This enzyme system is common to all vertebrate animals. Some POPs, notably persistent organochlorines, are known to stimulate the production of certain cytochrome p450 enzymes. Various biological effects, including reproductive and immunological effects, may result from induction of these enzymes by POPs (Tanabe et al. 1994).

In humans, the exposure to POPs is mainly from food intake. However, today some of these compounds are commonly found in ground water and this poses a serious threat to all who use it for consumption purposes. Toxaphene has been observed in groundwater in Nicaragua. Since many POPs are fat soluble, the highest levels are

present in fish meat and dairy products. Dioxins, PCBs, several organochlorine pesticides and brominated flame retardants are detectable in human breast milk, adipose tissue and blood worldwide. This reflects widespread exposure to these chemicals. (Hileman, 1993; Gilman, 1991).

Organochlorines such as dioxins have been linked to immunotoxic effects including suppression of antibody and humoral immune responses in laboratory animals. Studies in exposed human populations and non human primates have shown that halogenated aromatic hydrocarbons have been associated with measurable alterations in immune function (Holsapple et al., 1991).

2.7 Toxaphene Analysis

Toxaphene analysis is difficult and further complicated by interferences from other chlorinated hydrocarbons such as PCBs, DDT, chlordane, etc. Analytical methods for measurement of toxaphene have been developed and improved over the last 15 years. Electron capture detection (ECD) and negative chemical ionization (NCI) mass spectrometry (MS) are most frequently used for the quantification of total and congeners of toxaphene (Vetter and Oehme, 1999). The NCI can be combined with low resolution (LRMS) or high resolution (HRMS) techniques. HRMS monitor the exact mass of the most abundant electron ionization fragment of all toxaphene compounds (Wade et al., 1995), and **(Papers I, II, III, IV)**. However, it does not allow determining the degree of chlorination.

ECD requires the complete removal of interfering classes of compounds such as PCB. However, co-elution with chlordane and other substances of the same polarity is still possible since they elute in the same fraction as toxaphene for any column clean-up. The ECD response factors vary much less (about a factor of 2) between isomers than those for NCI±MS (at least one order of magnitude). ECD is more suitable for the quantification of congeners which are not available as reference compounds.

In our study total toxaphene was analyzed by GC-ECD and specific congeners of toxaphene were measured by GC/MS-NCI (Fig. 2.7.1).

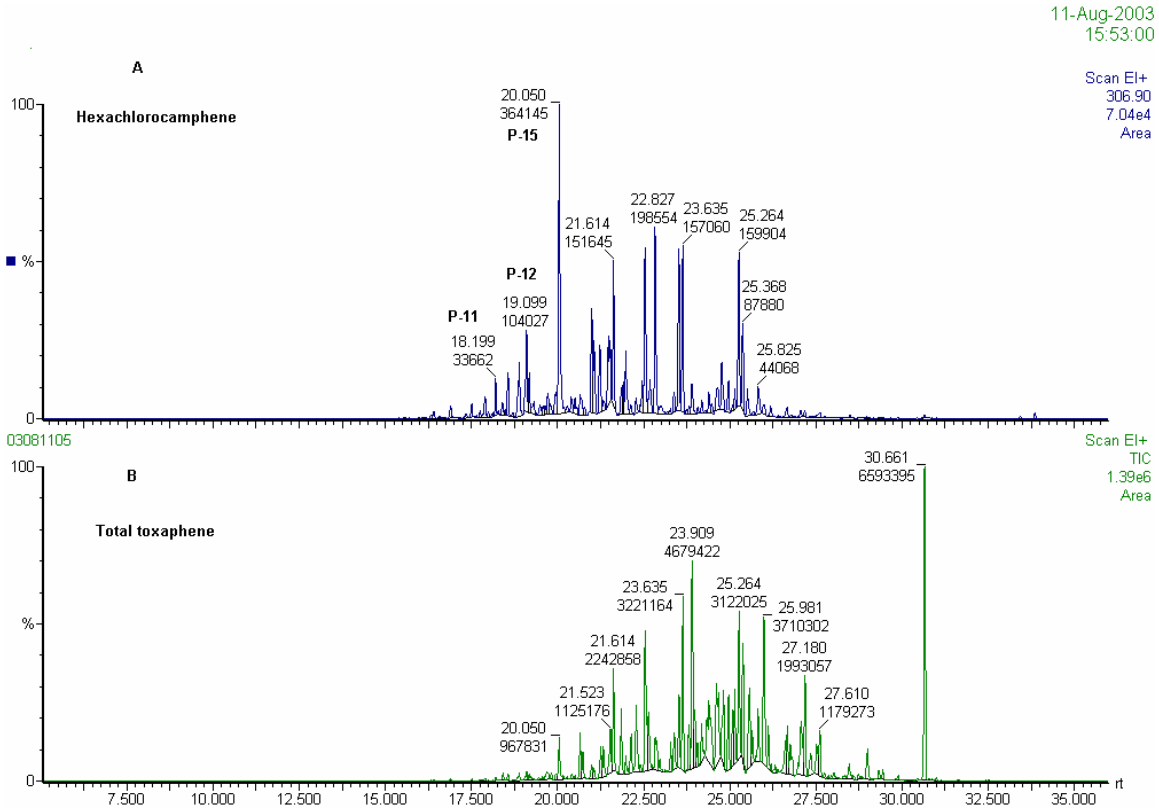


Fig. 2.7.1. Chromatograms for hexachlorocamphenes detected with a specific mass of 306.90 (A) and for total toxaphene by GC/MS- NCI (B).

Chapter 3: Degradation Methods of Toxaphene

With over 4 million chemicals pervading our biosphere and more than 1250 new xenobiotic compounds being added annually to the list of more than 850 000 synthetic molecules, it is not surprising that we are confronted by a growing problem of soil, sediment, groundwater and atmosphere pollution (Sarokin, 1988). Therefore, cost-effective technologies for removal of theses hazardous toxic pollutants are urgently needed. Physical/chemical methods have been traditionally used, however they are often logistically difficult, expensive and environmentally unfriendly. By comparison, biological methods are cheaper, safer and more efficient, especially when the pollutants are present at low concentrations and the volumes to treat are large.

A number of important papers are addressing how and whether the enhanced biodegradation successfully implemented in the lab can be achieved in field conditions.

Other papers focus on techniques to promote ageing or to stimulate immobilization of the compounds *in situ,* in order to make them less bioavailable (Hawari et al., 2000).

Recalcitrance of a synthetic compound may be attributed to the concentration or the inclusion of chemical bonds rarely found in nature; the same bonds which convey the important property of persistence in chemicals such as pesticides (McBain et al., 1996; Alexander, 1987; Zaidi et al., 1988). Other important factors include physical properties, particularly chemical binding (incorporation), bioavailability, adsorption (Roane et al., 2001, Verstraete and Top, 1999, Rieger et al., 2002). The presence/absence of an appropriate gene pool for transport and/or catabolism (Fewson, 1988), and interacting environmental variables, some of which are responsible for abiotic transformations.

Bioavailability is a phenomenon of great importance both when evaluating ecological effects on pollutants and their potential biodegradability. The limited bioavailability is one of the most important obstacles in application of bioremediation methods **(Paper III).** It has been shown that bioavailability can decline during compound ageing and weathering. This is extremely important as the enhancement of compound solubilization and bioavailability is required for a successful biological treatment (Hatzinger and Alexander, 1995, Cornelissen et al., 1997, Luthy et al., 1997). The bioavailability can be increased by the addition of surfactants to the soil. Adding surfactants increase the solubility of the hydrophobic contaminant and also increases the surface area of the contaminant available for microbial attack, making biodegradation of the pollutant easier.

3.1 Physicochemical treatments

Toxaphene is commonly found at very low concentrations in large volumes of contaminated soil and ground water. This significantly increases the size of the treatment facility and therefore construction and operation costs. In this regard, a preliminary concentration step of toxaphene will reduce the reactor volume and consequently the treatment costs. Physical adsorption by Amberlite in contaminated ground water and

toxaphene extraction from contaminated soil by Supercritical fluid extraction constitute the most common approaches for pollutant concentration.

3.1.1 Amberlite^{TM} XAD-4 Adsorption

Amberlite is a non-ionic crossliked polymeric adsorbent made of divinylbenzene. It has both continuous polymer and pore phases, which gives a high chemical and thermal stability (Fig. 3.1.1.1). This adsorbent can be used for removal of hydrophobic organic pollutants such as pesticides, phenols or aromatic compounds from ground water or wastewater. There are different kinds of adsorbents for compounds of different molecular weights or structures.

The pollutant can be eluted with a solvent that dissolves the compound well. For hydrophobic molecules, water miscible organic solvents like methanol, ethanol or acetone are preferred. Through elution the Amberlite is regenerated and can be used many times.

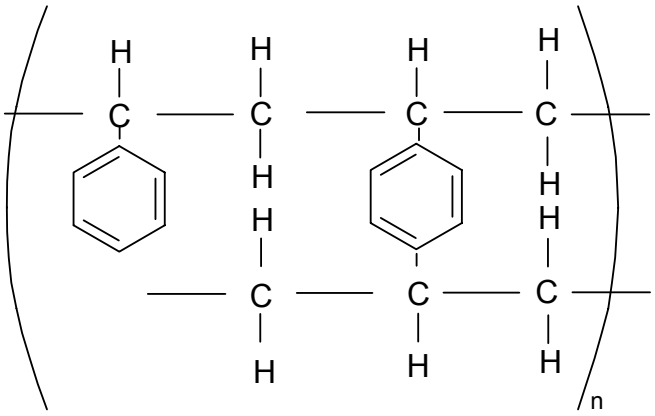


Fig 3.1.1.1. Physical structure of Amberlite XAD-4

To investigate the adsorbing efficiency and the optimal adsorbing time, the following experiment was carried out in the lab. Ten grams of Amberlite XAD-4 (Rohm and Haas Company) were washed in distilled water and put in a tube made of muslin fabric, which was placed in a bottle of water with a magnetic stirrer and a toxaphene concentration of 0.5 mg/l. Water samples of 5 ml were taken every 4 h. Since the results indicated the adsorbing efficiency much faster than expected, the experiment was done again, this time with samples taken every 30 min for 6 h. To improve the efficiency, approximately 35 g of Amberlite was put in a 50 ml column. Water samples taken from wells in Picacho (Chinandega-Nicaragua) were pumped through this filter with a peristaltic pump at flow rates in the range of 3-6 ml/min.

Several different methods were tried for elution of the insecticide and, at the same time, regeneration of the adsorbent. Ethanol was used for elution of toxaphene from the Amberlite column. The flow was 1-2 ml/min as recommended by the manufacturer. To develop an easy, economically and environmentally beneficial method to elute toxaphene, water steam pressure was used. Steam was pumped through the column with an air pump from a sealed beaker of boiling water.

The column was working efficiently at all flow rates between 3 and 6 ml/min. The absence of toxaphene in the samples taken at the end of the column indicates that almost all of it was adsorbed in the Amberlite filter (Fig 3.1.1.2). The efficiency was calculated to be approximately 97 % in the beginning of the experiment, but it seemed to deteriorate after extended use. Approximately 70 % of the toxaphene was eluted with ethanol in the beginning. This yield decreased with time to 37 % and later on to 14 % The losses may be the result of evaporating the eluting solvent and filtering of the eluate. At the same time, the adsorbing efficiency decreased considerably. Solid residues and crystallisation during elution were problems for quantification. These were more common when using methanol. Water steam pressure did not elute the pesticide at the pressures used. The optimal adsorption time turned out to be approximately 3 hours.

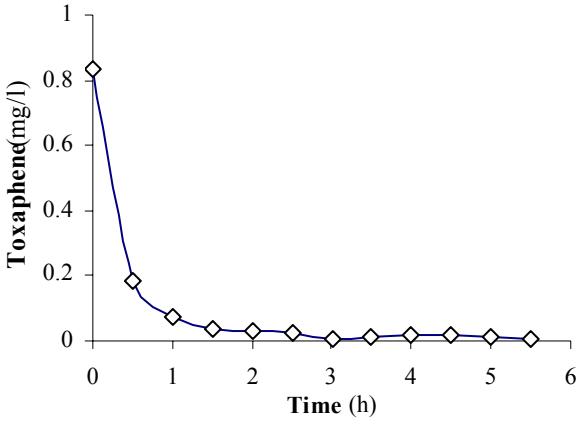


Fig. 3.1.1.2. Optimisation of adsorption time for Amberlite XAD-4.

The Amberlite XAD-4 was shown to be an efficient adsorbent of toxaphene; almost all of the pesticide was removed from the water. However, further research is needed concerning regeneration of the filter to maintain the adsorption qualities of the material.

Amberlite XAD-4 adsorbent can be used in contaminated ground water wells. Amberlite will trap toxaphene and therefore purify the drinking water before the local population can consume it. The eluted toxaphene from the adsorbent will then be transferred to a bioreactor for destruction.

3.1.2 Supercritical fluid (SCF) extraction

Supercritical fluid extraction (SCF) of organic hazardous waste from contaminated soils is a promising new technique for hazardous waste site cleanup. The ability of SCFs to solubilize heavy molecular weight organics is well documented (Groves et al., 1985).

Supercritical carbon dioxide (SC-CO_2) is used to extract PCBs, DDT, and toxaphene from contaminated top soils and sub soils. An attractive feature of this process is that the CO_2, being virtually inert, leaves no solvent residue on the processed soil (Paulaitis et al., 1982).

The separation of the extracted solute from SC-CO_2 results in the creation of a small volume of concentrated waste. This improves the efficiency of subsequent treatment processes such as combustion. Typically, in SCF, CO_2 is contacted with a solid or liquid phase at high pressure and moderate temperature. Slight changes in the temperature or pressure of the system can cause large changes in the density of the solvent and consequently change its ability to solubilize heavy nonvolatile waste compounds from the solid or liquid phase (Groves et al., 1985).

Although supercritical fluid densities are comparable to liquid densities, their viscosities and diffusivities range between those of liquids and gases. Thus, SCFs exhibit better mass transfer characteristics than typical liquids. As a result, separation efficiencies for SCF extractions can be much higher than for liquid solvent extractions (Paulalitis et al, 1982).

3.1.3 Reductive dechlorination of Fe(II)/Fe(III) redox couple

Williams and Bidleman, (1978) investigated the degradation of toxaphene in four systems using Fe(II)/Fe(III) redox coupled. They used sterilized sediment, unsterilized sediment, sterilized sand containing $Fe_3(OH)_8$, and the Fe(II)/Fe(III) redox coupled. Controls were performed at pH 5.6, and included a redox potential of -250 mV vs. saturated calomel electrode (SCE), sterilized sand and distilled water. Toxaphene was added to all four systems at 5 $\mu g.g^{-1}$ of wet sand or sediment, and the systems were kept in glass stoppered flasks at room temperature. Toxaphene was quickly degraded in both the sterile and unsterile sediments and in the sand containing the Fe(II)/Fe(III) redox couple (Khalifa, et al, 1976).

Results showed that the normal toxaphene fingerprint was greatly altered within a few days by the formation of compounds having gas chromatographic retention times shorter than those of standard toxaphene components. This suggested the breakdown of toxaphene in sterile as well as unsterile samples and was confirmed by the similar GC pattern of products isolated from the Fe(II)/Fe(III) sand system. These changes in the sediment system were likely caused by chemical rather than biological processes No breakdown of toxaphene in the sand control system was noticed. The breakdown of toxaphene in salt marsh sediments and in the Fe(II)/Fe(III) sand system probably occurred by a reductive dechlorination mechanism (Khalifa, et al, 1976).

3.2 Bacterial Degradation

The chemical and microbiological processes involved in dechlorination of many persistent organic pollutants are well understood. Technological applications of these processes to environmental remediation are relatively new, particularly in pilot or field scale. It is well established, however, that there are several mechanisms which result in dehalogenation of some classes of organic contaminants, often rendering less toxic compounds. These include: stimulation of metabolic sequences through introduction of electron donor and acceptor combinations; addition of nutrients to meet the needs of

dehalogenating microorganisms; possible use of engineered micro-organisms; and use of enzyme systems (Lovley, 2003).

Organisms have detoxification abilities (i.e. mineralization, transformation and/or immobilization of pollutants). Microorganisms, particularly bacteria, play a crucial role in biogeochemical cycles contributing to the maintenance of ecological balance. This ecological service has existed since the dawn of time and is now severely under significant threat. Bacteria have developed strategies for obtaining energy from virtually every compound under aerobic or anoxic conditions (using alternative final electron acceptors such as nitrate, sulphate, and ferric ions); (Hutzinger and Verkamp, 1981) and therefore almost every compound may be degraded by microbiological systems.

Microorganisms have evolved an enormous catabolic potential for numerous natural and synthetic compounds that were considered recalcitrant. In addition, their potential and genetic flexibility should allow the generation of new catabolic pathways for xenobiotic compounds. Under real world conditions these evolutionary processes, however, may require long periods of time to develop. Certain structural elements such as halo or nitrosubstituents are rare in natural compounds (Timmis and Pieper, 1999) and they therefore constitute a challenge to the metabolic machinery.

Large scale production of synthetic halogenated organic compounds, which are often resistant to both biotic and abiotic degradation, has occurred only in the last few decades (Hutzinger and Verkamp 1981). However, naturally occurring halogenated organic compounds have existed in marine systems for perhaps millions of years. These compounds, including aliphatic and aromatic compounds containing chlorine, bromine, or iodine, are produced by macroalgae and invertebrates. The presence of these natural compounds, at potentially high concentrations, may have resulted in populations of bacteria that are effective dehalogenators (King 1988). Microorganisms exposed to halogenated compounds in soil **(Paper II)** and ground water may also have developed enzymatic capabilities similar to those in marine environments. Enzyme systems that

have evolved to degrade non-chlorinated compounds may also be specific enough to degrade those that are chlorinated. (Reijnders and Stevens 1987).

Organic compounds generally represent reduced forms of carbon, making degradation by oxidation energetically favorable. However, halogenated organic compounds are relatively oxidized by the presence of halogen substituents, which are highly electronegative and thus more susceptible to reduction. A compound with more halogen substituents is therefore more oxidized and more susceptible to reduction **(Paper I)**. Thus, with increased halogenation, reduction becomes more likely than oxidation (Vogel et al. 1987).

An organic compound is considered to be reduced if a reaction leads to an increase in its hydrogen content or a decrease in its oxygen content; however, many reduction reactions (e.g., the vicinal reduction process) do not involve changes in the hydrogen or oxygen content of a compound. Vicinal reduction occurs when two halogens are released while two electrons are incorporated into the compound. Oxidation and reduction reactions are more precisely defined in terms of electron transfers (Vogel et al., 1987).

Generally, organic compounds present at a contaminated site represent potential electron donors to support microbial metabolism. However, halogenated compounds can act as electron acceptors, and thus become reduced in the reductive dehalogenation process. Specifically, dehalogenation by reduction is the replacement of a halogen such as chloride, bromide, fluoride, or iodide on an organic molecule by a hydrogen atom. Reductive dehalogenation may require the induction of enzymes responsible for dehalogenation (Linkfield et al., 1989).

3.2.1 Anaerobic

Research findings indicate that anaerobic processes that remove halogens from POPs produce dehalogenated compounds that are generally less toxic and more susceptible to further microbial attack. Both aromatic and non-aromatic organic compounds are subject to these dehalogenation processes. Some investigations also have shown that anaerobic dehalogenation reactions specifically involving reductive processes can effectively degrade a wide variety of halogenated contaminants in soil **(Paper III)**, and ground water (Vogel et al. 1987, Kuhn and Suflita 1989a).

In such environments, biodegradation is carried out by either strict anaerobes or facultative microorganisms using alternative electron acceptors, such as nitrate (denitrifying organisms), sulphate (sulphate reducers), Fe(III) (ferric-ion reducers), CO_2 (methanogens), or other acceptors like chlorate, Manganese(IV) and Chromium(VI) (Gibson and Harwood, 2002; Lovley, 2003; Widdel and Rabus, 2001). When oxygen is not present (anoxic conditions), microorganisms can use organic chemicals or inorganic anions **(Paper I)** as alternative electron acceptors under metabolic conditions referred to as fermentative, denitrifying, sulfate-reducing or methanogenic conditions.

Some anaerobes release Fe (III) chelators, which solubilize Fe (III) from Fe (III) oxides, and electron-shuttling compounds, which accept electrons from the cell surface and then reduce Fe(III) oxides. It has been shown that enhancing the availability of some electron acceptors, such as Fe (III) by adding suitable ligands, can greatly stimulate anaerobic degradation of contaminants in subsurface environments (Lovley, 2003).

Decontamination of toxaphene has been achieved by biological anaerobic degradation in water, sewage sludge and on-site processes **(Paper I;** Buser et al., 2000 and Mirsatari, et al.1987). However, studies aiming at toxaphene degradation, employing only one microorganism, have been few. *Dehalospirillum multivorans*, a strictly anaerobic bacterium, was the only example of a prokaryotic organism reported to degrade this chlorinated pesticide when operating in pure culture, Ruppe, et al., (2002).

Enterobacter sp. is a facultative anaerobic bacterium. It was able to use toxaphene as the sole carbon and energy source **(Paper II)**.

The potential for anaerobic biological processes to reductively dehalogenate organic compounds may be important in the bioremediation of soils and aquifers contaminated with these compounds. (Fig. 3.2.1). These environments often become anaerobic due to depletion of oxygen by the microbial degradation of more easily degradable organic matter. When compounds can be degraded under anaerobic conditions, it is not necessary to create aerobic conditions. The costs associated with the maintenance of an aerobic environment by providing air, ozone, or hydrogen peroxide would thus be avoided (Suflita et al. 1988).

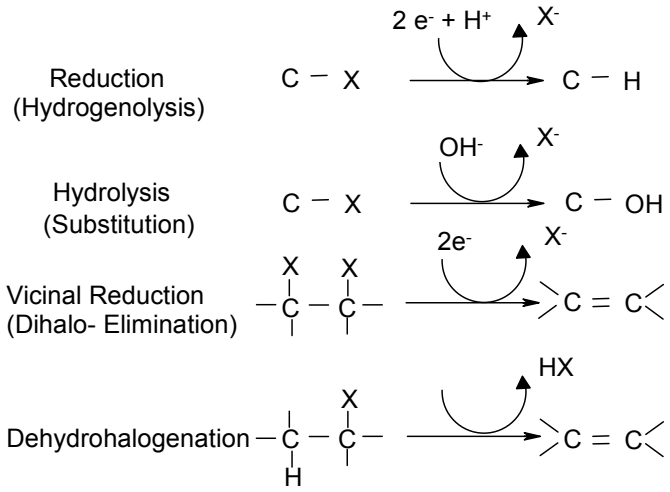


Fig. 3.2.1. Anaerobic dehalogenation mechanisms of some non-aromatic pesticides

3.2.2 Aerobic

Oxygen is the final electron acceptor for microbial respiration in aerobic processes (Wacket, 2003). In chemotrophic reactions, a portion of the substrate is oxidized to obtain energy or to be assimilated in biomass. A wide phylogenetic diversity of microorganisms such as *Pseudomonas* species is capable to perform the aerobic degradation of POPs. These microorganisms have been extensively studied because of their ability to degrade oxic organic contaminants (Wackett, 2003). However, many polluted environments such as aquifers, aquatic sediments and submerged soils are often anoxic. This poses a serious disadvantage since the anaerobic degradation of toxaphene in the above mentioned environment should be followed by an aerobic stage. For instance, Clark and Matsumura (1979) reported that toxaphene was first dechlorinated under anaerobic conditions and

then followed by oxidative actions on the less chlorinated products in an aerobic environment. In **paper I** we found that an anaerobic-aerobic system was successful for the degradation of toxaphene in contaminated wastewater. Thus, after 42 days of the total toxaphene concentration was reduced by 87%. However, most of this degradation was due to the anaerobic step.

3.3 Fungal Degradation

Lignin is a complex molecule of conjugated aromatic structures. An organism capable to of degrading such a molecule has enzymes that will catalyze the degradation of many environmental pollutants. Processes of natural bioremediation of lignin involve a wide range of organisms, but predominantly fungi. Laboratory studies on the degradation of lignin-containing substrates such as wood, straw and cereal grains **(Paper IV)** have focused mainly on a few fungal species (Hammel, 1989).

The only organisms reported to degrade lignin efficiently are the white rot fungi that, under natural conditions, mostly colonize dead or living wood. White rot fungi that degrade lignin rather than cellulose are called selective degraders and are especially interesting for use in biotechnological applications within paper and pulp processing. This interest lies in that they remove lignin and leave the valuable cellulose fibers intact (Eriksson, 1997).

The ability of the fungi to degrade a wide variety of compounds has been attributed, at least in part, to the action of *ligninolytic enzymes* (Aust and Barr, 1994). The lignin degrading enzymes (*lignin peroxidases*, *manganese peroxidases* and *laccases*) are essential for the fungal survival (Hatakka, 1994; Pelaez et al., 1995).

There are several reasons to use white rot fungi in decontamination of polluted sites. First, they are capable of mineralizing a wide variety of toxic xenobiotics. Second, they occur ubiquitously in natural environments. Third, they have the potential to oxidize substrates that have low solubility because the key enzymes involved in the oxidation of

several pollutants are extracellular. Fourth, the constitutive nature of the key enzymes involved in lignin degradation obviates the need (in most cases) for these organisms to be adapted to the chemical being degraded. Fifth, the preferred substrates for the growth of white-rot fungi, such as corn cobs, straw, peanut shells, and saw dust, are inexpensive and easily added as nutrients to the contaminated site. Sixth, the lignin degrading system (LDS) of *P. chrysosporium* is expressed under nutrient-deficient conditions, which are prevalent in many soils. And finally, as filamentous fungal grow by hyphal extension and extend through the soil with growth, they can reach pollutants in the soil in ways that bacteria cannot (Morgan et al., 1991).

White rot fungi are capable of producing activated forms of oxygen which are involved in the reactions with aromatic structures. Since peroxidases and laccases are extracellular enzymes, the reaction can take place outside the fungal organism. Furthermore, when the activated oxygen is formed there is no more influence by enzymes on specificity of the oxidizing reactions, thereby expanding the range of substrates that can be processed **(Paper IV)**.

Chapter 4: Characterization of microorganisms in soil

Soil bacteria and fungi play important roles in various biogeochemical cycles (Molin and Molin, 1997; Trevors, 1998b; Wall and Virginia, 1999) and are responsible for the cycling of organic compounds. Torsvik et al., (1990a,b) estimated that in 1 g of soil there are 4000 different bacterial genomic units based on DNA–DNA reassociation. It has also been estimated that about 5000 bacterial species have been described (Pace, 1997). Approximately 1% of the soil bacterial population can be cultured by standard laboratory practices. It is not known if this 1% is representative of the bacterial population (Torsvik et al., 1998). An estimated 1,500,000 species of fungi exist in the world (Giller et al., 1997). But unlike bacteria, many fungi cannot be cultured by current standard laboratory methods (Thorn, 1997; Van Elsas et al., 1995).

Methods to measure microbial diversity in soil can be categorized into two groups: biochemical-based techniques and molecular-based techniques. Typically,

diversity studies include the relative diversities of communities across a gradient of stress, disturbance or other biotic or abiotic difference (Hughes et al., 2001).

4.1 Enrichment and Acclimation of microorganisms

The classical approach to cultivating microorganisms is to prepare a solid or liquid growth medium containing an appropriate carbon source, energy source and electron acceptor depending on the organism being isolated. The medium is then inoculated with a suitable source of microorganisms and left to incubate at a desired growth temperature until organisms multiply to the point at which we become aware of their presence by colony formation or increased turbidity (Asano, 1998).

Enrichment culture is a technique to isolate microorganisms having special growth characteristics. Some microorganisms grow faster than others in media with limited nutrients, high temperature, extreme pH values, toxic chemicals, etc. (Asano, 1998). Microorganisms that grow faster than other species become dominant after several transfers of the culture. When using biofilm reactors that are exposed to a selective medium with increasing selection pressure, during extended period of time there will be just a few organisms surviving. Sometimes they are dependent upon each other, in other cases the organisms are growing individually under the conditions applied. Such selections are observed in **Paper I and II**.

An acclimation technique is applied when a toxic or unnatural compound is used as a substrate **(Paper II).** An adaptation to a synthetic medium containing a target compound often results in the isolation of microorganisms having a new enzyme (Asano, 1998).

4.2 Isolation and Characterization

Characterization and identification of pure cultures of microorganisms is based upon description of morphological, cultivation and physiological characteristics. The

determination of the growth and nutrition of the cells must be studied (e.g. whether they are aerobic or anaerobic or facultative, what sugars they ferment, **(Paper II)** whether they are motile, the antigenic properties of cell wall proteins, etc.).

Several methods such as sample dilution, filtration, micromanipulation etc. have been used successfully to isolate microorganisms. Sample dilution may work when the target organism is numerically dominant in a microbial community. The sample is simply diluted until only the target organism remains. Sample filtration separates cells according to size, so if the target group is particularly large or small, this might be useful for initial sorting away from the primary inoculum. Micromanipulators and optical tweezers are instruments for physically moving single cells or tight clusters of cells from a mixture of cells to fresh growth medium, where the cell(s) can grow in isolation. These methods are most suitable for isolation of large, morphologically conspicuous microorganisms, such as filaments (Liesack et al., 1997).

Density-gradient centrifugation separates cells according the density and may be useful for initial sorting of communities to enrich for the target organisms. Cell sorting by flow cytometry is a high-throughput method for quickly isolating target cells from a mixed culture; it is most suitable for singly-occurring cells because cell aggregates can interfere with the hydrodynamic focusing in the apparatus. The isolation procedure can not be directly monitored by Fluorescence *in situ* hybridization (FISH) when cells have been previously isolated by micromanipulators or optical tweezers. Indeed, FISH monitoring involves the inactivation of cells (due to fixation with paraformaldehyde) and therefore cells will not be viable for subculture. Instead, monitoring by filtering the cells or density-gradient centrifugation can be used (Zinder and Salyer, 2001).

PCR amplification of 16S rRNA genes (16S rDNA) using consensus bacterial primers **(Paper II)** and separation of the resultant PCR amplicons either by cloning, by denaturing gradient gel electrophoresis (DGGE) or temperature gradient gel electrophoresis (TGGE) constitutes the most popular molecular ecology technique used to describe soil bacterial ecology to date. Clones or bands on gradient gels can be

sequenced and the resultant information is used to infer something about the diversity of the original sample (Muyzer and Smalla, 1998).

Sequences can be retrieved and submitted to the European Molecular Biology Laboratory (EMBL). They are analyzed and compared to others in GenBank and RDP databases. In **paper II** Strain D1 was found to bear closest phylogenetic relationship to *Enterobacter cloacae* subsp. *dissolvens* and *Enterobacter cloacae*, with 99.8% and 99.4 of sequence similarity, respectively. The phylogenetic tree shows a heterogeneous distribution of the *Enterobacter* species.

Many other techniques have been developed to assess microbial community diversity. In these methods, DNA is extracted from the environmental sample and purified **(Paper II)**. Target DNA (16S, 18S or ITS) is amplified using universal or specific primers and the resulting products are separated in different ways (Tiedje et al., 1999).

Since the majority of microorganisms still need to be characterized and described, it is important when developing degradation processes to focus only on those organisms that have some unique properties to offer. This means that one shall let the selection process sort out one or a few organisms that are essential for the process before starting to isolate and characterize them.

Chapter 5: Innovative biological methods for treatment of POPs

Contaminated places with recalcitrant compounds are a global problem, contributing to loss of habitat, poisoning of wildlife, and threats to human health. Biological treatment methods play an increasing role in cleanup procedures of POPs. Degradation of complicated structures occurs in several steps. First, halogenated compounds loose the halogen molecules at an early stage in the process. This takes place more rapidly under anaerobic conditions. After dechlorination, degradation under aerobic conditions is favoured. The stereochemical structure is of great importance for predicting the time required for degradation. In general, molecules with substituents in ortho position will be degraded much faster than a similar molecule with meta positioned substituents. This is referred to as the meta- effect (Saleh, M., 1991).

It has not been elucidated yet whether dehydrochlorination or dechlorination is involved in the degradation pathway of toxaphene (**Paper I**). Reductive dechlorination removes one chlorine substituent from the carbons with two functional groups attached. Every degradation step releases one chlorine ion involving the transfer of two electrons and is replaced by an hydrogen (Fingerling, et al., 1996). For instance, nonachlorobornanes are dechlorinated more rapidly than compounds with less chlorine. This was confirmed in degradation process when most of the heavily chlorinated compounds decreased concomitantly with an increase in the concentration of less chlorinated compounds **(Paper I)**.

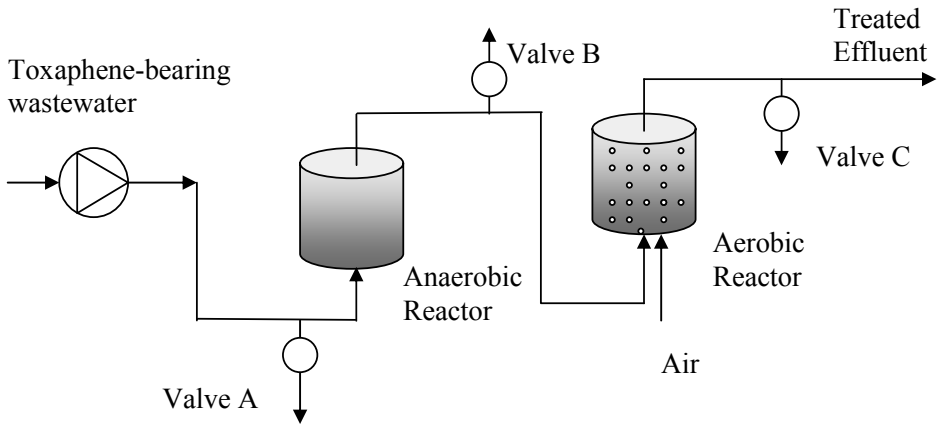


Fig. 5.1. Schematic representation of the sequential bioreactor set-up.

In paper I, a sequential anaerobic-aerobic bioreactor configuration was investigated for the toxaphene degradation purposes (Fig 5.1). Anaerobic bioreactors are cheap since no oxygen is required but not all compounds are dechlorinated in this process. A packed bed configuration with the biomass immobilized on Poraver® beads (foam glass particles) was used to implement the anaerobic step. The use of biofilm prevents from biomass wash-out and allows attaining higher biomass densities. In addition, biofilm systems offer the microbial cells a higher protection towards pollutant toxicity. This is advantageous in the case of toxic organic pollutants such as toxaphene. By combining with an aerobic step, further dechlorination and degradation can be expected. Aerobic processes attain faster organic matter removals and are often less sensitive to pollutant toxicity. However, the high operational cost due to the need for

external oxygen supply and the production of large amounts of biomass limit their application.

In our study, COD removal efficiencies up to 90 % were attained in the anaerobic, confirming the potential of this process for the initial attack of toxaphene. There was no significant enhancement in the removal of COD recorded in the aerobic step (Fig. 5.2). The low microbial activity in the aerobic bioreactor could likely be due to high recalcitrance of the metabolites produced during the anaerobic degradation of toxaphene. In fact, new peaks were observed in the chromatographic analysis of samples taken from the effluent of the anaerobic reactor (**Paper I**).

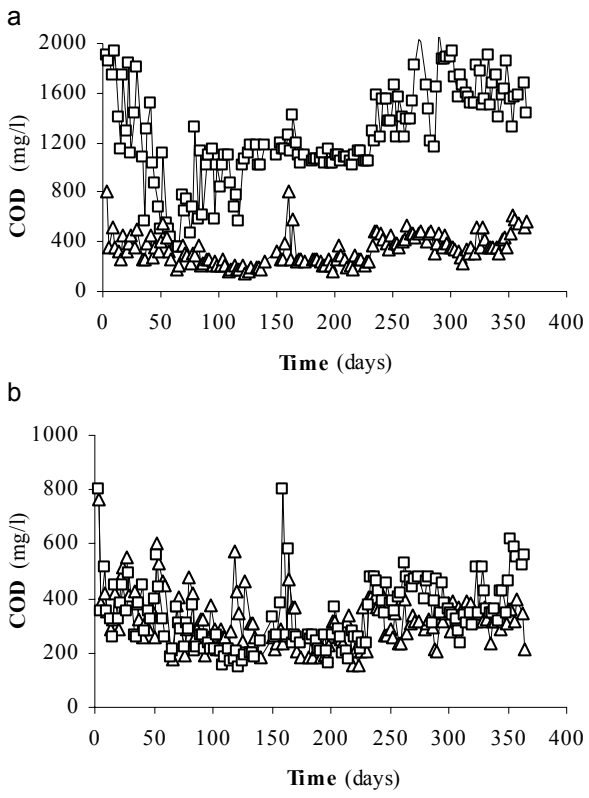


Fig. 5.2. Time course of influent (squares) and effluent (triangles) COD in the anaerobic reactor (a) and in the aerobic reactor (b).

White-rot fungi also offer an alternative to the bacterial degradation of toxaphene. Fungal-based bioreactors are advantageous as they permit to carry out toxaphene degradation in a single stage process, with subsequent in construction and operational costs. The constitutive enzymes involved in the biodegradation of lignin (lignin peroxidases, manganese peroxidase and laccases) can also degrade toxaphene (Hatakka, 1994; Pelaez et al., 1995) (**Paper IV**). White rot fungi often need the presence of easily biodegradable co-substrate in order to trigger the production of extracellular enzymes. In our study, molasses, wheat husk and wood chips were compared for their ability to support toxaphene degradation by a *Bjerkandera* sp. It was found that toxaphene biodegradation was intrinsically linked with the production of lignin peroxidase. Approximately 85% of toxaphene was removed when wheat husk was the

main substrate, while 52 and 49 % removal efficiency were recorded when using wood chips and molasses, respectively. Future work should focus on the development of continuous fungal-based process and further comparison with bacterial based processes.

Toxaphene biodegradation in bio slurry reactors is often limited by pollutant bioavailability. Thus, due its high hydrophobicity, toxaphene is strongly bound into the organic fraction of the soil, which reduces the amount of contaminant available for the microbial cells. In this regard, pollutant bioavailability decreases as a result of soil ageing. Indeed, it has been reported that pollutant binding to soil becomes stronger with time and therefore aged contaminated soils are more difficult to treat. Toxaphene bioavailability was a key issue in order to improve the remediation efficiency (**Paper III**). In our study, a bio slurry reactor for the treatment of toxaphene contaminated soil was investigated (Fig. 5.3). Removal efficiencies of toxaphene congeners up to 96 % were achieved in 79 days of operation. An enhancement in the pollutant bioavailability can be achieved via surfactant addition to the soil. In our study, no improvement in the biodegradation of less chlorinated congeners was observed when adding the surfactant Titron X-114 or an additional carbon source as lactic acid (**Paper III**). However, the removal of heavily chlorinated compounds was enhanced by the addition of the above-mentioned chemicals.

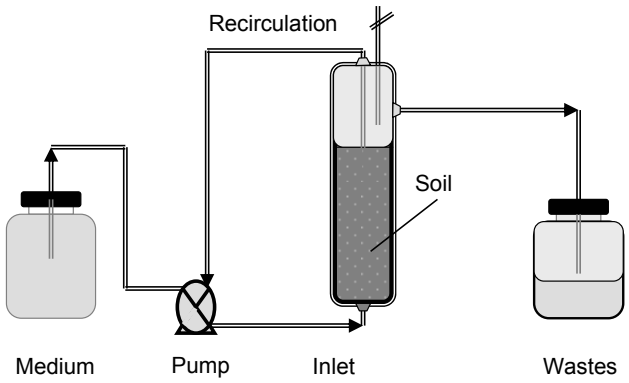


Fig. 5.3. Schematic representation of the sequential bioreactor set-up.

Environmental pollutants can either be present in relatively well defined polluted sites which can be remediated, or they can be spread into ground water and air and then be present at very low concentrations. The latter makes it more difficult to address, since it

is usually in large volumes and the concentrations are so low that it can not be expected that cells capable of degrading the compounds will get enough energy to their maintenance metabolisms. Therefore, such microbial processes will be less efficient. In these cases it may be advantageous to first concentrate the pollutant before degrading it.

Toxaphene in the ground water in Nicaragua is a serious problem in many regions where cotton farming was intense. Work was done within the project to develop an efficient adsorbent to treat ground water, and then to regenerate the adsorbent in an efficient and inexpensive mode (see 3.1.1, this thesis). The released toxaphene could then be transferred to a biological treatment system (Fig. 5.4). The first and the last step seems to function well, while the desorption step needs to be developed further before it can be introduced as a realistic tool to cope with the problem of toxaphene in drinking water.

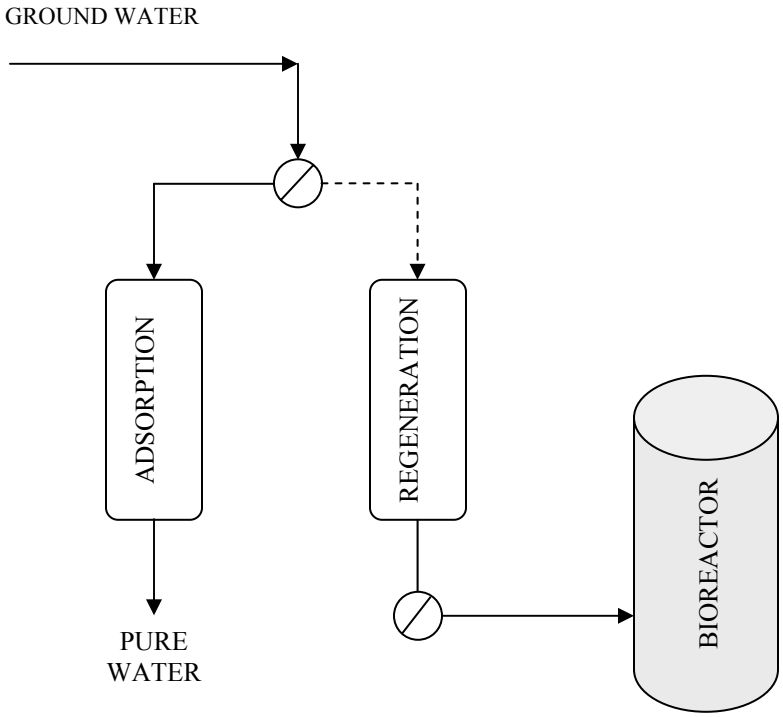


Fig.5.4. Schematic illustration of a process configuration devised to remove toxaphene in an adsorption column packed with Amberlite followed by the biological regeneration of the adsorbent.

Chapter 6: Concluding remarks

Toxaphene is a complex mixture of chlorinated compounds that are difficult to degrade. The present investigation has been focusing on two approaches to achieve degradation: anaerobic dechlorination with a subsequence metabolism of the carbon skeleton, or use of strong oxidizing reagents from fungal peroxidase/laccase to at least initiate the degradation.

The anaerobic system, when operated as a biofilm growing on a solid support and with a history of being exposed to increasing concentrations of the toxic compounds, turned out to be very efficient in attacking the heavily substituted compounds. This occurred while the concentrations of the less chlorinated compounds increased during a transition phase. It is thus likely that upon dechlorination of the heavily substituted compounds, less substituted compounds are formed. The organisms can not degrade toxaphene as sole carbon source, there has to be a co-substrate present as well. In the

anaerobic system, lactate turned out to be a very good co-substrate. This was true in comparison to a number of other potential co-substrates

Use of lignin degrading fungi is a natural choice when a very recalcitrant compound is to be degraded. White rot fungi have developed very powerful oxidative enzyme systems which produce a range of activated species of oxygen. These highly reactive compounds will efficiently contribute to the degradation of toxaphene. A great advantage from an engineering point of view is that the selectivity in the enzymatically catalyzed process is in the formation of the activated oxygen species, while the subsequent reaction with the recalcitrant compounds was controlled by other factors. Thus, selectivity is much lower than what is normally expected when enzymes are involved.

Toxaphene can be degraded aerobically or anaerobically. The latter was shown to be efficient in releasing the chlorine from the toxaphene in the form of chloride ions. After the dechlorination, the carbon skeleton was degraded. However, in the aerobic steps catalyzed by white rot fungi, it was obvious from the analyses that a range of new substances appeared. Since no chloride ions were released upon dechlorination, it is realistic to assume that a range of intermediates have been formed containing chlorine, or that the chlorine has been transferred to other organic molecules. These observations clearly illustrate the need for proper analytical back-up when performing biodegradation of toxic chemicals. Furthermore, tests on toxicity must be included before such treatments are being implemented.

The degradation studies have clearly pointed towards the need for a highly sensitive analytical back up, since it will otherwise be difficult to properly monitor the progress. The analytical challenge is quite substantial since toxaphene as such is a poorly defined substance; it is rather a mixture of many similar structures.

The use of biofilm reactors is advantageous since it helps to create a high cell density and thus a high catalytic density in the reactor. Furthermore, when starting such a

reactor, the population is very diverse. However, as time goes on with increasing concentrations of the recalcitrant compound, the biodiversity is reduced until eventually only very few species that can stand the selection pressure will be present.

Among these selected organisms it was possible to identify one species that was capable to degrade toxaphene on its own.

The needs for efficient and inexpensive methods for combating the toxaphene pollution in Nicaragua are not completely fulfilled with the work done in this thesis. However, there is now knowledge to be applied when addressing the pollution with recalcitrant compounds. Since the problem is widespread on the countryside and that one even finds toxaphene in the local wells, there is a need to purify the drinking water before the local population drinks it. Work along that line has been initiated during this thesis work. A good adsorbent has been identified and even tried under realistic conditions. The limitation today is that there is also a need for an efficient and inexpensive mode to regenerate such an adsorbent. With regards to the adsorbent, the idea is that the pollutants should be enriched on the adsorbent and then be released in an inexpensive mode so that microorganisms could degrade it into carbon dioxide, water and some hydrochloric acid.

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