

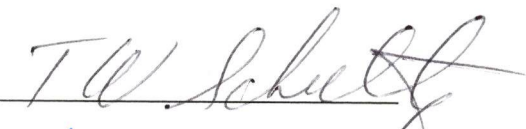
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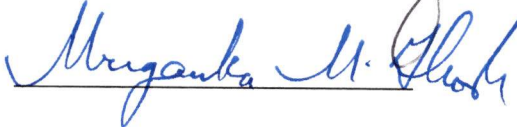
I am submitting herewith a thesis written by Maurizia Muccini entitled "Toxicity Evaluation of PCB-Contaminated Soils". I have examined the final copy of this thesis for form and content and recommend that it be accept in partial fulfillment of the requirements for the degree of Master of Science, with a major in Ecology.



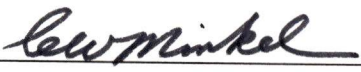
Gary S. Sayler, Major Professor

We have read this thesis
and recommend its acceptance:





Accepted for the Council:



Associate Vice Chancellor
and Dean of The Graduate School

**TOXICITY REDUCTION EVALUATION OF
PCB-CONTAMINATED SOILS**

**A Thesis Presented for the
Master of Science Degree
The University of Tennessee, Knoxville**

MAURIZIA MUCCINI

August, 1997

DEDICATION

This thesis is dedicated to my husband

Alexandre Rivas

and my son

Gabriel Muccini Rivas

whose love and patience were fundamental to this achievement

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ABSTRACT

The ultimate goal of bioremediation is to completely biodegrade environmental toxic substances. However, pollutants can undergo partial biodegradation or biotransformation producing more toxic byproducts than the parent compound. In this manuscript, two aquatic ecotoxicological analyses, *Tetrahymena pyriformis* and MicrotoxTM, were used to assess the toxicity of a bioremediation process that uses a surfactant solution to extract PCBs from soil and two genetically engineered microorganisms to degrade the PCBs. Approximately 50% of PCBs and 90% of surfactants were degraded in the bioremediation process and there was no increase in the toxicity. The relative toxicity of the major metabolites formed by aerobic PCB degradation (chlorobenzoic acids) was studied using both an acute and chronic *Tetrahymena* assay. Log K_{ow} and pK_a were used as descriptors for the development of QSARs. Results showed that neutralized chlorobenzoic acids are less toxic than non-neutralized and chlorine substituents at *meta* and *para* position render benzoic acids more toxic than benzoic acids with chlorines at *ortho* position.

TABLE OF CONTENTS

	PAGE
PART I	
INTRODUCTION.....	1
LITERATURE CITED.....	12
PART II	
ACUTE TOXICITY ASSESSMENT OF AN IN SITU SOIL WASHING/ BIOREMEDIATION PROCESS FOR PCBs DEGRADATION	18
INTRODUCTION.....	19
MATERIALS AND METHODS.....	24
Chemical and Soil Selection.....	24
Soil Washing Phase	25
Bioremediation Phase	25
Using Soil Washing Solution	25
Using Aroclor 1242	26
Comparison of Uncontaminated and Contaminated Soils.....	27
Methods for Chemical Analysis	28
PCB Analysis from Contaminated Soil.....	28
PCB Analysis from Contaminated Soil Washing and Aroclor Solutions	29
Surfactant Analysis.....	29
Methods for Toxicological Analysis	30
16-Hour <i>Tetrahymena</i> Mortality Assay.....	30
Microtox Test.....	31

RESULTS	32
DISCUSSION	41
LITERATURE CITED	46

PART III

QUANTITATIVE STRUCTURE-ACTIVITY RELATIONSHIPS FOR CHLOROBENZOIC ACIDS (CBAs)	51
INTRODUCTION.....	52
MATERIALS AND METHODS	54
Chemical Selection and Physicochemical Parameters Determinations	54
16-Hour <i>Tetrahymena</i> Mortality Assay	55
40-Hour <i>Tetrahymena</i> Growth Impairment Assay	55
Statistical Procedures.....	56
Aliphatic Monocarboxylic Acid Model	57
Excess Toxicity.....	57
RESULTS	57
Acute Toxicity	58
Chronic Toxicity	61
DISCUSSION	68
LITERATURE REVIEW.....	71

PART IV

SUMMARY AND CONCLUSIONS.....	76
VITA	78

LIST OF TABLES

	PAGE
Table 1 - PCB groups with high potential environmental significance.....	8
Table 2 - Composition of Aroclor 1242 and TVA soil	33
Table 3 - Degradation of PCBs	34
Table 4 - Degradation of Surfactant	37
Table 5 - Recovery of PCBs in the soil washing solution experiment.....	38
Table 6 - Recovery of PCBs in the Aroclor 1242 (low conc.) experiment	38
Table 7 - Recovery of PCBs in the Aroclor 1242 (high conc.) experiment	38
Table 8 - Toxicity evaluation of uncontaminated and contaminated TVA soil	39
Table 9 - Toxicity evaluation of Aroclor 1242 (low concentration) degradation	40
Table 10 - Toxicity evaluation of Aroclor 1242 (high concentration) degradation	41
Table 11 - Toxicity evaluation of the TVA soil bioremediation	41
Table 12 - Baseline models for acute and chronic <i>Tetrahymena pyriformis</i>	58
Table 13 - Physicochemical parameters and acute toxicities for <i>Tetrahymena pyriformis</i>	59
Table 14 - Excess acute toxicity of non-neutralized and neutralized benzoic acids	60
Table 15 - Linear regression models for acute toxicity	61
Table 16 - Multiple regression models for acute toxicity.....	62
Table 17 - Physicochemical parameters and chronic toxicities for <i>Tetrahymena</i> <i>pyriformis</i>	63

Table 18 - Excess chronic toxicity of non-neutralized and neutralized benzoic acids.....	64
Table 19 - Linear regression models for chronic toxicity	66
Table 20 - Multiple regression models for chronic toxicity	66
Table 21 - Expected metabolites resulting from aerobic metabolism of aroclor 1242	67
Table 22 - Expected metabolites resulting from aerobic metabolism of TVA soil.....	68

LIST OF FIGURES

	PAGE
Figure 1 - Structure and numbering for biphenyl.....	3
Figure 2 - <i>o</i> , <i>m</i> , <i>p</i> denotes <i>ortho</i> , <i>meta</i> and <i>para</i> respectively (a); Structure of 2,3,7,8-TCDD (b); Structure of PCB congeners IUPAC numbers 77 (c), 169 (d), 126 (e) and 81 (f).	7
Figure 3 - Degradation of chlorobiphenyl by the 2,3 dioxygenase pathway. Gene designation: bphA = biphenyl 2,3 dioxygenase; bphB = dihydrodiol dehydrogenase; bphC = 2,3 dihydroxybiphenyl dioxygenase; bphD = 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (meta cleavage product) hydrase.....	10
Figure 4- <i>In Situ</i> Soil Washing/Bioremediation Process.....	19
Figure 5 - Bench scale for <i>in situ</i> soil washing system	20
Figure 6 - Schematic diagram of a surface active molecule.....	21
Figure 7 - Degradation of polyoxyethylene 10 lauryl ether by IPL5::TnPCB and B30P4::TnPCB.....	22
Figure 8 - Degradation of Aroclor 1242 by IPL5::TnPCB and B30P4::TnPCB	36
Figure 9 - A scatter plot of toxicity (LC ₅₀) versus hydrophobicity (log K _{ow}) for non-neutralized benzoic acids	59

LIST OF ABBREVIATIONS

BA	- Benzoic acid
CAS	- Chemical abstract service
CBA	- Chlorobenzoic acid
EC ₅₀	- Effective median concentration
f	- Fisher statistic
GC	- Gas-chromatograph
IGC ₅₀	- 50% Inhibitory growth concentration
K _{OW}	- 1-Octanol/water partition coefficient
LC ₅₀	- 50% Lethal concentration
mg/l	- Milligrams per liter
mM/l	- Millimoles per liter
MW	- Molecular weight
PA	- Phosphate buffer medium concentrate
PAS	- Phosphate buffer minimal salts medium
pKa	- Ionization constant
PCB	- Polychlorinated biphenyl
POL	- Polyethylene (10) Lauryl Ether
Pr > f	- Level of significance
QSAR	- Quantitative Structure-Activity Relationship
r ²	- Coefficient of determination
s	- Square root of the mean square for error
SAS	- Statistical Analysis System
T.U.	- Toxic units
T _e	- Excess toxicity

PART I

INTRODUCTION

Many naturally occurring compounds are degraded in the environment by microorganisms after their synthesis. In contrast, xenobiotics (chemically synthesized compounds that do not exist naturally) may be refractory to degradation. The main reason is that they cannot be recognized by naturally present organisms and therefore do not enter common metabolic pathways. Some compounds may be transformed only in the presence of another compound which appears as a carbon and energy source. Very often compounds are degraded sequentially through the activity of a series of different organisms (Rozgaj, 1994). Due to rapid rates of multiplication and great metabolic potential, microorganisms may be able to adapt to new substrates.

Polychlorinated biphenyls (PCBs) are members of the halogenated aromatic group of environmental pollutants that have been identified worldwide in diverse environments (Safe, 1994). Improper disposal practices resulted in discharge of large quantities of PCBs into soils, river and lakes sediments, and landfills. Concerns over their toxicity and bioaccumulation potential have emphasized the need to bioremediate these contaminated sites.

PCBs consist of a biphenyl ring having multiple chlorines attached to its nucleus. Chlorines can be placed at any or all of the 10 available sites (Figure 1), with 209 different compounds theoretically possible, varying in the number and position of the attached chlorines. The individual isomers and homologs are generally referred to as congeners.

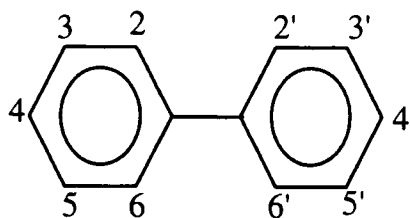


Figure 1 - Structure and numbering for biphenyl.

PCBs were manufactured and sold as complex mixtures differing in their average chlorination level. The crude mixtures resulting from the chlorination were fractionally distilled to produce commercial mixtures with the desired properties (Abramowics, 1990). The lower chlorinated mixtures are mobile, colorless oils, whereas the higher chlorinated are an immobile, viscous liquids or amorphous solid (Safe, 1992). In the United States, PCBs were commercially manufactured under the trade name Aroclor. They were synthesized, graded and marketed according to their percentage by weight chlorine content (Safe, 1992). Thus, the Aroclor named 1221, 1232, 1242, 1248, 1254, 1260, 1262 and 1268, contain 21, 32, 42, 48, 54, 60, 62 and 68 % by weight chlorine, respectively, as indicated by the last two numbers of their four-digit name. The first two numbers indicate the 12 carbon atoms in the biphenyl molecule.

Commercially, PCBs found rapid and widespread industrial use as electrical insulators, lubricants, hydraulic fluids, diffusion pump oils, cutting oils, plasticizers, and liquid seals. PCBs found their way into the home as flame retardant in a number of plastic, as preservatives and protectants in rubber, in weatherproof coatings and stucco, in steel coatings, waxes, varnishes, inks, and a host of other everyday items (Hooper et al., 1990; Safe, 1989; Layton et al., 1994).

In the 1970's all open uses for PCBs were discontinued, however, the closed applications as dielectric fluids in capacitors and transformers were continued until 1978 (Cordle et al., 1982; Safe, 1989; Flanagan and May, 1993). New transformers and capacitors now contain alternative dielectric fluids and as older PCB-containing electrical equipment is withdrawn from service or repaired, the PCB fluids are replaced.

Although their use has been discontinued, PCBs remain one of the most serious environmental pollutants because of the characteristics that made them so commercially successful (Hooper et al., 1990). The nonpolar nature of PCBs results in a high dielectric constant, making them an ideal choice for insulating coolant in electrical equipment. Nonpolarity, however, also confers a high lipophilicity and potential for bioaccumulation. The strong electronegativity of chlorine suppresses the normally reactive electron-rich carbon backbone, stabilizing the biphenyl molecule. This results in a heat-resistant fluid with excellent long term stability. Although this is a desirable characteristic in oils and preservatives, these molecules are recalcitrant to physical and biological degradation.

PCBs were first recognized as important environmental pollutants in 1966 when scientists in Sweden reported PCBs in declining eagles populations (Paulson and Brown, 1978), herring and other environmental samples (Hooper et al., 1990; Weimer et al., 1978; Erikson, 1992). PCBs came to world-wide attention in 1968 when five people died and 1,000 others were stricken in Japan after eating cooking oil contaminated by PCBs (Weimer et al., 1978). In 1975, high levels of PCBs were reported in Hudson River fish (Cordle et al., 1982). These compounds were reported as contaminants in almost every

component of the ecosystem including air, water, fish, wildlife and human blood, adipose tissue, and milk (Safe, 1989).

In 1985, researchers exposed healthy bacteria, plankton, benthic macroinvertebrates, fishes, and birds to Detroit River sediments and sediment porewater contaminated with PCBs (Manny and Kenaga, 1991). The negative impacts found included genetic mutations in bacteria; death in macroinvertebrates; accumulation of PCBs in insects, clams, fishes, and ducks; and tumor formation in fish. The biomagnification of PCBs investigated in Southeastern Lake Michigan showed a PCB increase of 12.9 times in average concentration from plankton to fish (Evans et al., 1991). These PCBs are also bioaccumulated into the tissues of populations of fish eating predators (Giesy et al., 1994).

The Bald Eagle population in the United States (Kozie and Anderson, 1991) and the Spanish Imperial Eagle in Europe have declined due to reduced breeding rate caused by high levels of PCBs (Hernandez et al., 1989). Population decline, reproductive effect deformities, behavioral changes, tumors and other effects were reported in Great Lakes wildlife (Colborn, 1991) in species that include the bald eagle, herring gull, mink, beluga whale, lake trout, common terns, and double-crested cormorants (Gilbertson et al., 1991). Reproductive problems including congenital malformations, reduced hatchling success and decreased survival of hatchlings, were also observed in colonial-nestling water birds at the San Francisco Bay National Wildlife Refuge.

Unhatched eggs and deformities occurred at significantly high rate in the common Snapping Turtle collected from four wetlands on the shorelines of Lakes Ontario, Canada (Bishop, 1991) contaminated with PCBs.

Plant life growing in contaminated land also contains PCBs and therefore are another pathway of PCB contamination into the food chain. The dominant route of uptake by plants is via their roots (Sawhney and Hankin, 1984; Bush et al., 1986) although sorption by leaves of volatilized PCBs also occurs. Some authors conducted experiments using crops that were grown in contaminated soil (Sawhney and Hankin, 1984; Webber et al., 1994; O'Connor et al., 1990) and found that virtually all of the PCBs occurs in the peel of vegetables and fruits.

Laboratory studies on mammals indicate that PCBs in the diet can result in death or sublethal effects. The dietary concentrations causing these impacts vary with the species. Rats, mice, and dog survived for one or two years on diets including 100 to 300 mg/l PCB, while dietary levels as low as 3 mg/l were fatal to rhesus monkeys after 245 days (Paulson and Brown, 1978).

PCBs examined during the immediate post-partum of mothers that consumed moderate quantities of contaminated lake fish (Fein et al., 1984) showed shortened gestation, reduced birth size, and smaller head circumference of the infants. These results were consistent with experimental studies in rhesus monkeys.

With the development of high-resolution capillary columns in gas chromatography, many of the toxicological studies of PCBs have been focused in the individuals congeners instead of the total mixture (McFarland and Clark, 1989; Schmidt

and Hesselberg 1992; Hong et al., 1993). These studies have elucidated the profound effect of individual congeners on the toxic response and on induction of drug-metabolizing enzymes in mammalian, avian, and fish species.

There is an evidence that many PCB congeners are relatively nontoxic, whereas a small group can be highly toxic to biota. The most toxic congeners are the non-ortho or mono-ortho chlorine substituted compounds (Colborn, 1991; Gilbertson et al., 1991; Webber et al., 1994) that tend to be planar in geometry and are sterically similar to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD) (McFarland and Clark, 1989; Safe 1992). These include the congeners IUPAC # 77, 81, 126 and 169 (Figure 2). From a human health stand point of view, these congeners are considered to be mixed-function oxidases (MFO) inducers.

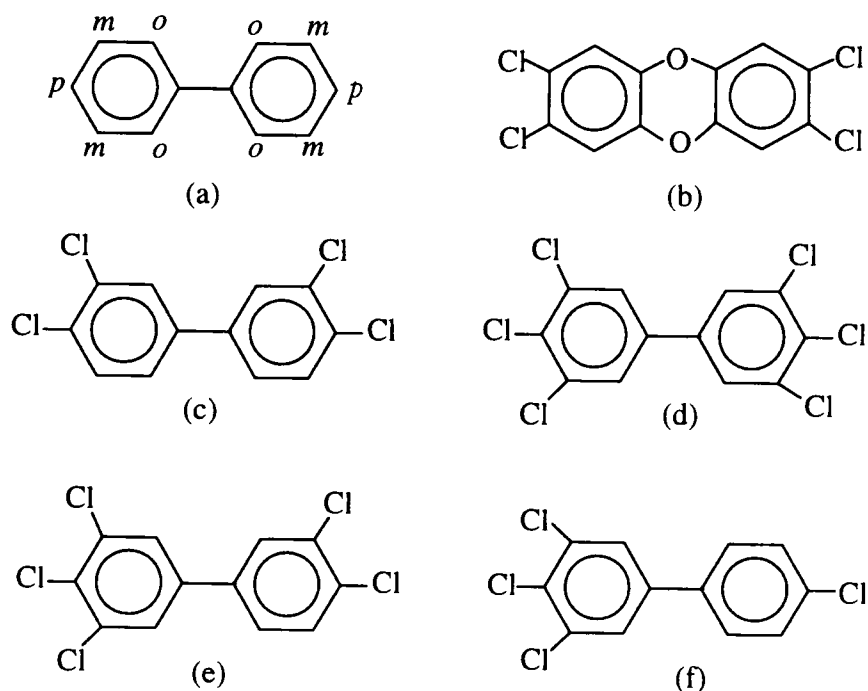


Figure 2 - *o*, *m*, *p* denotes *ortho*, *meta* and *para* respectively (a); Structure of 2,3,7,8-TCDD (b); Structure of PCB congeners IUPAC numbers 77 (c), 169 (d), 126 (e) and 81 (f).

MFO are a category of enzymes that catalyze oxidative biotransformations of aromatic ring-containing compounds. The MFOs that are induced by PCBs are characterized as being phenobarbital-type (PB-type), 3 methylcholanthrene-type (3-MC type), or possessing catalyzing properties of both (mixed-type). The different types of inducers are specific for different substrates and have different region selectivities for catalyzing the insertion of oxygen in the formation of reactive intermediates. The PB-inducible MFOs catalyze insertion of oxygen into conformationally nonhindered sites of globular or noncoplanar lipophilic molecules, facilitating their conjugation and removal. The 3-MC-inducible MFOs insert oxygen into conformationally hindered sites of planar molecules. The results of these reactions may be formation of carcinogenic, mutagenic, or teratogenic biotransformation products from otherwise non toxic parent compounds.

Table 1 shows the grouping of PCBs according to Mixed Function Oxygenases (MFO) induction. PCBs that are 3-Methylcholanthrene-type (3-MC-type) and mixed-type inducers are considered to be potentially more toxic than PCBs that are Phenobarbital-type (PB-type) inducers and the latter are considered potentially more toxic than weak inducers and non-inducers.

Table 1 - PCB groups with high potential environmental significance*

Group 1 3-Methylcholanthrene-Type and Mixed-Type Inducers

Group 2 Phenobarbital-Type Inducers

Group 3 Weak or Non-Inducers Frequent in Environmental Samples

Group 4 Mixed-Type Infrequent in Environmental Samples

*According to McFarland (1989).

Under a variety of conditions PCBs can be degraded by microorganisms. Two classes of bacteria that can partially biodegrade PCBs, include specific aerobic bacteria and anaerobic bacterial processes. The aerobes attack PCBs oxidatively, breaking open the carbon ring and destroying the compounds. Anaerobes on the other hand, leave the biphenyl rings intact while removing the chlorines (Abramowicz, 1990). The aerobic process usually degrades less chlorinated compounds while the anaerobic partially dechlorinates the highly chlorinated congeners. An alternative method of degradation for the highly chlorinated PCBs is the use of UV radiation (Carey et al., 1976; Hawari et al., 1992). In this process the highly chlorinated PCBs are converted into less chlorinated compounds, which can further be degraded by aerobic bacteria.

Microbial degradation of PCBs depends on the degree of chlorination and the position of the chlorine atom on the biphenyl molecule. Lower chlorinated biphenyls are readily transformed by bacteria and *ortho* substitutions decrease rate (Erikson, 1992). In general the steps of aerobic biodegradation of PCBs are an attack involving initial addition of O₂ at the 2,3-position by a dioxigenase enzyme, with subsequent dehydrogenation to the catechol followed by ring cleavage (Abramowicz, 1990; Hooper et al., 1990; Layton et al., 1994) (Figure 3). The formation of chlorinated benzoic acids from chlorinated biphenyls is the most common metabolite produced by aerobic PCB degradation (Abramowicz, 1990; Hooper et al., 1990; Layton et al., 1994; Rochkind et al., 1986).

Bioremediation is a process that uses biological systems or microorganisms such as bacteria, fungi, and yeast or their extracts (Torma, 1994), to transform toxic substances into innocuous compounds (Kremor, 1994). It takes advantage of the natural abilities of

microorganisms to metabolize, oxidize or reduce organic and inorganic compounds. Bioremediation technologies have been developed to treat a variety of media including soil, sediments, water, and sludge (Kremor, 1994). These techniques can be grouped into two general categories: *in situ* techniques, which treat hazardous materials in place without removing contaminated matrix, and *ex situ* which treat contaminated matrix in a confined area (a reactor for example).

Because microorganisms present in contaminated rivers, lakes, and soil can only partially degrade PCBs (Bedard et al., 1986) much attention has been focused on the study of genetically engineered organisms for degradation of these compounds (Bedard et al., 1987; Layton et al., 1994). The Center for Environmental Biotechnology (CEB) at the University of Tennessee, Knoxville (UTK) has developed an *in situ* soil washing/ Bioremediation process for PCB degradation in soils from a TVA power substation, using two genetically engineered organisms.

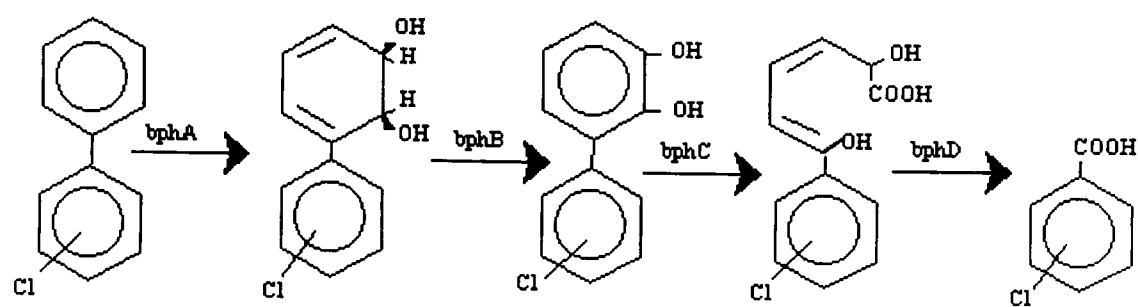


Figure 3 - Degradation of chlorobiphenyl by the 2,3 dioxygenase pathway. Gene designation: bphA = biphenyl 2,3 dioxygenase; bphB = dihydrodiol dehydrogenase; bphC = 2,3 dihydroxybiphenyl dioxygenase; bphD = 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (meta cleavage product) hydrazase.

The ultimate goal of bioremediation is to completely biodegrade environmental toxic substances into carbon dioxide, water, and other harmless byproducts (Kremor, 1994). When a site is being treated by bioremediation, it is usually assumed that the disappearance of the pollutant will produce a reduction in the toxic effects of the contaminants. However, pollutants can undergo partial biodegradation or biotransformation (Schultz and Dawson, 1995) and produce more toxic byproducts than the parent compounds (Bryant and Schultz, 1994).

The hypothesis offered herein is that degradation of PCBs by two genetically engineered microorganisms will not result in the formation of more toxic metabolites. Two main objectives were defined to test this hypothesis: 1) Toxicological evaluation of a bioremediation process for PCBs degradation using two aquatic organisms. 2) Study the relative toxicities of chlorobenzoic acids (the major metabolite produced by aerobic degradation of PCBs) in neutralized and non-neutralized media.

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PART II

ACUTE TOXICITY ASSESSMENT OF AN IN SITU SOIL WASHING/BIOREMEDIATION PROCESS FOR PCBs DEGRADATION

INTRODUCTION

The *in situ* soil washing/ bioremediation process developed at CEB is a two phase system (Layton et al., 1997). In phase 1 a surfactant solution is used to remove PCBs from soil. In phase 2, two surfactant degrading bacterial strains, IPL5::TnPCB (Lajoie et al., 1996) and B30P4::TnPCB (Lajoie et al., 1994), which contain the genes for PCB degradation are added to degrade both the surfactant and PCBs (Figure 4).

The bench scale design for the *in situ* soil washing system consist of a 4 liter aspirator bottle with tubing outlet connected to a 1 liter fermentation vessel by stainless steel tubing and fittings (Figure 5). Stainless steel tubing connects the pump to an individual garden sprinkler which is inserted through a stopper on the aspirator bottle. The fermentation vessel with an agitator shaft is placed on a stir plate of a New Brunswick fermenter Bio

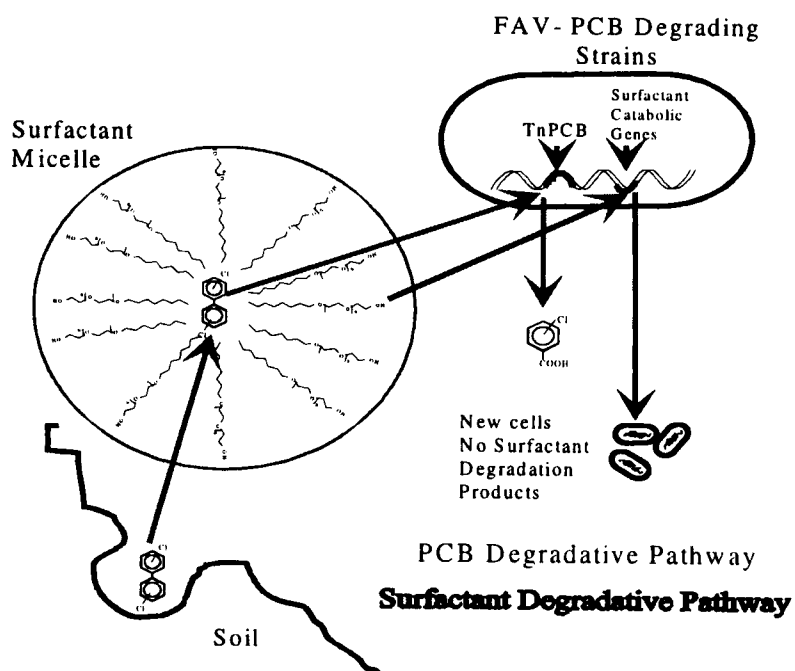


Figure 4- *In Situ* Soil Washing/Bioremediation Process

Flo model C30. A surfactant solution is placed in the fermentation vessel. The aspirator bottle is placed on the platform above the fermentation vessel and soil is added to it. The fermentation vessel is used as both reservoir for the soil washing solution and as the bioreactor for the bioremediation phase. Two matching bioreactors are used so that one experimental and one control treatment can be run simultaneously. The control treatment uses the two surfactant degrading strains with a transposon containing the genes for tetracycline resistance but not the PCB genes.

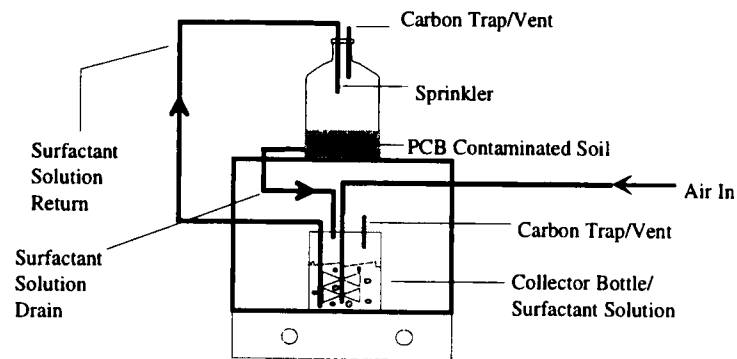


Figure 5 - Bench scale for *in situ* soil washing system

Surfactants are, in the most common form, composed of a hydrocarbon portion and a polar or ionic portion as illustrated schematically in Figure 6 (Ottewill, 1984). The hydrocarbon portion, which can be linear or branched, interacts only very weakly with the water molecules in an aqueous environment. Moreover, the strong interactions between the water molecules arising from dispersion forces and hydrogen bonding act cooperatively to squeeze the hydrocarbon out of the water, hence the chain is usually called hydrophobic. The polar or ionic portion of the molecule, usually termed the head group, however interacts strongly with the water via dipole-dipole or ion-dipole

interactions and is solvated. Consequently the head group is said to be hydrophilic. It is essentially the balance between the hydrophobic and hydrophilic parts of the molecule which gives the special properties associated with these compounds.

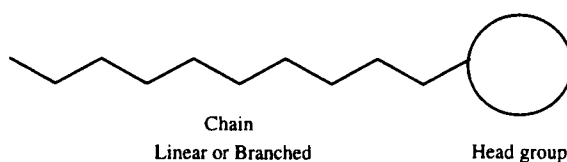


Figure 6 - Schematic diagram of a surface active molecule

There are three major classes of surfactants based on the nature of the head group: The anionic, cationic and nonionic. Lewis (1990) summarized and evaluated the algae toxicity data base for surfactants and assessed their potential for causing an impact in the environment. He studied the three main classes of surfactants and found that in general the nonionic are relatively non-toxic compared to the other classes.

The surfactant used to extract PCBs from the soil, polyoxyethylene 10 lauryl ether (POL), is an alcohol ethoxylate nonionic surfactant. Little, if any, information on the toxicological properties of POL is available in the literature. It was found in our laboratory that when degraded by *Pseudomonas putida* IPL5::TnPCB, 2-(dodecyloxy)-ethanol is formed but it is further degraded by *Alcaligenes eutrophus* B30P4::TnPCB (Figure 7).

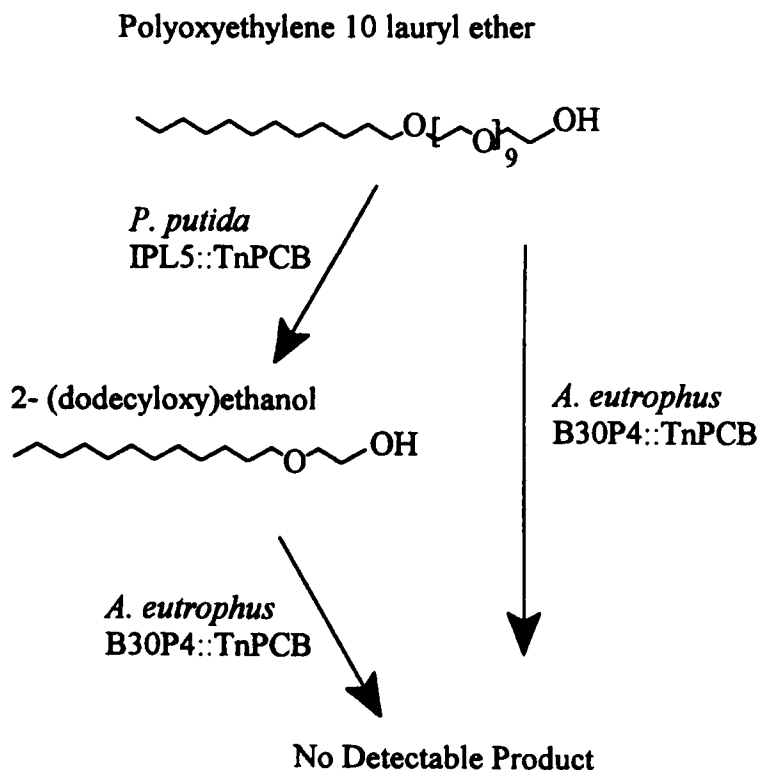


Figure 7 - Degradation of polyoxyethylene 10 lauryl ether by IPL5::TnPCB and B30P4::TnPCB

Nonionic surfactants of the linear alcohol ethoxylate type, are environmentally compatible because they are rapidly biodegradable and show low toxicity in the aquatic environment after passing through treatment plants (Dorn et al., 1993).

Salanitro et al (1989) investigated the primary degradation of linear alcohol ethoxylates and branched nonylphenol ethoxylates in bench scale sludge units treating a synthetic sewage feed. The studies indicated that only the treatment of wastes containing nonylphenol ethoxylates formed toxic effluents. The alcohol ethoxylate surfactant undergo extensive microbial degradation and caused little or no impact on the activated

sludge treatment process. Routledge and Sumpter (1995) used a recombinant yeast screen to assess the estrogenic activity of selected surfactants. They found that only some of the nonylphenol ethoxylates possess estrogenic activity.

Little (1977) and Patoczka and Pulliam (1990) found that the acute toxicity values of alcohol ethoxylates for fish and invertebrates generally range from 1-6 mg/l. It was found that the acute toxicity of some alcohol ethoxylates ranged from 1.5 to 11.4 mg/l to the MicrotoxTM test; 1.3 to 11.6 mg/l for *Daphnia*; and 1.6 to 6.1 for fathead minnow (Dorn et al., 1993).

The aquatic toxicity of this class of surfactants tends to decrease as ethoxylate chain length increases. These findings are in contrast to acute toxicity values in rodents which increase with increasing ethoxylate length.

The results from other mammalian toxicity tests have shown that these surfactants are well tolerated by systemic and dermal exposure (Little, 1977). The use of certain of these surfactants in human therapy as an analgesic has not revealed any untoward reactions after exposure for periods of several months at doses many times those expected for environmental sources. The human experience to date would suggest that continued use of the alcohol ethoxylates pose no threat to environmental quality or to human safety.

In this chapter, the toxicity of the *in situ* soil washing/bioremediation process developed at CEB for degradation of PCBs will be assessed using two aquatic acute toxicity assays: the MicrotoxTM test and the 16-hour *Tetrahymena* Mortality assay.

The Microtox test measures luminescent inhibition in *Vibrio fischeri* and has become a popular test system for toxicity testing. It has been used to test soil leachates

from landfill, hazardous waste sites and industrial effluents and in determining the efficacy of bioremediation and filtration process (Bitton and Koopman, 1992; Hund and Trauspurger, 1994; Lambolez et al., 1994). Toxicity measurements are expressed as median effective concentration (EC_{50}), i.e., the concentration of the test material needed to cause decrease luminescence by 50%.

The 16-hour *Tetrahymena* mortality assay (Schultz et al., 1994) measures lack of ciliary movement and/or cell lysis of the common freshwater ciliate *Tetrahymena pyriformis*. Toxicity measurements are expressed as 50% lethal concentration (LC_{50}), i.e., the concentration of the test material which causes death in 50% of the ciliates.

MATERIALS AND METHODS

Chemical and Soil Selection

The contaminated soil used in this study is a mixture of fine gravel and soil. It was obtained from a Tennessee Valley Authority (TVA) Moccasin Bend power substation in Chattanooga, Tennessee, located adjacent to Lake Nickajack on the Tennessee River. The physical description and other characteristics of the soil are described in Layton (1994). The soil sample is a composite of a number of individual samples from one of the capacitor banks at the transformer substation. The uncontaminated control samples were taken from a walkway region from outside the substation in a grassy area.

Polyoxyethylene (10) lauryl ether (Sigma Chemical, St. Louis, MO) was the surfactant used for removal of PCBs from the contaminated TVA soil. Aroclor 1242 (Monsanto) was used for comparison in the GC analysis and for degradation experiments.

Soil Washing Phase

1 kg of the contaminated soil was placed in the aspirator bottle. 1.1 l of sterile deionized water plus 10.1 gr. of surfactant were placed in the fermentation vessel (to achieve a concentration of 10,000 mg/l) and stirred for 1 hour to solubilize the surfactant. 100 ml of this surfactant wash solution was removed for Cobalt thiocyanate active substances (CTAS) analysis, toxicological analysis, PCB analysis and bacterial enumeration. The pump was turned on to start the soil washing phase. The flow rate for the surfactant solution was 60 ml/min and pump cycle time was set on an automatic timer at 30 minutes on, 30 minutes off (to allow for complete drainage of the surfactant solution). The soil washing phase was performed in two days.

Bioremediation Phase

Using Soil Washing Solution

After 2 days of soil washing the solution was adjusted to pH 7.0 with 10N NaOH and a 100 ml sample was taken for toxicological analysis, and PCB and surfactant concentration analysis. Two 1L Erlenmeyer flasks were used, instead of the fermentation vessel in the original design, to run an experimental and a control treatment simultaneously. The following were added to each flask: 243.3 ml of the soil washing solution; 23.4 ml of phosphate buffer concentrate (PA) (Layton et al., 1977); 3.0 ml of a phosphate buffer minimal salt medium (PAS) (Layton et al., 1977); 0.3 ml of yeast extract (5% wt/vol. deionized H₂O stock) and 1.50 gr. diatomaceous earth. Diatomaceous earth was used to bind residual desolubilized PCBs as the surfactant is being degraded. Into the experimental flask

15 ml of each B30P4::TnPCB (OD₆₀₀ 1.4778) and IPL5::TnPCB (OD₆₀₀ 1.3895) were added. Into the control flask 15ml of each B30P4::TnTc (OD₆₀₀ 0.7330) and IPL5::TnTc (OD₆₀₀ 0.6684) were added. The flasks were put in similar agitators and stirring was kept constant during the 1 week reaction period. Air flow was provided for the first 4 days. The pH was measured daily and adjusted to pH 7.0 with 10N NaOH during the 1 week degradation period. At the end of the experiment 10 ml of the degraded soil washing solution was taken from both reactors for total PCB and surfactant concentration analysis. The remaining solution was transferred to 150 ml corex tubes and centrifuged at 8,000 rpm for 2 hours. PCB and surfactant concentration analysis and toxicological analysis were performed in the supernatant while the pellet was only analyzed for PCBs. The empty 1L flasks were rinsed with ether and this fraction was also analyzed for PCBs.

Using Aroclor 1242

Two other similar PCB biodegradation experiments were conducted using Aroclor 1242 as the PCB source instead of the contaminated soil. Aroclor 1242 resembles the PCB contamination found in the TVA soil. These experiments were designed to serve as a comparison for the degradation of the contaminated soil by using a high initial PCB concentration (approximately 1,000 mg/l) and a low initial PCB concentration (approximately 37 mg/l). For the high PCB concentration duplicate 3 g of surfactant was added to 243.3 ml H₂O, 23.4 ml PA concentrate, 3 ml PAS salts, 0.3 ml yeast extract 0.5% and 1.5 g diatomaceous earth. These solutions were stirred for 1 hour to solubilize the surfactant. 0.3 ml of Aroclor 1242 was added to each flask and let stirring for 1 hour to

solubilize PCBs. 15 ml was added from each of the following cultures: B30P4::TnPCB (OD₆₀₀ 1.8061) and IPL5::TnPCB (OD₆₀₀ 0.8099) to the experimental flask. B30P4::TnTc (OD₆₀₀ 1.4707) and IPL5::TnTc (OD₆₀₀ 1.0534) to the control flask. For the very low PCB concentration 10.5 ml of a 1,000 mg/l solution (0.1 g Aroclor 1242/100 ml POL 10,000 mg/l) was added to each flask. The OD₆₀₀ for each culture were as follow: B30P4::TnPCB (OD₆₀₀ 1.8972) and IPL5::TnPCB (OD₆₀₀ 0.9525) to the experimental flask. B30P4::TnTc (OD₆₀₀ 1.3331) and IPL5::TnTc (OD₆₀₀ 0.9961) to the control flask. Immediately after the addition of the cultures, 50 ml samples were taken from each flask for toxicological analysis and surfactant and PCB concentration analysis. After the 1 week degradation, 10 ml samples were taken from the reactors for total PCB and surfactant concentration analysis. The remaining solution was transferred to 150 ml corex tubes and centrifuged at 8,000 rpm for 2 hours. PCB and surfactant concentration analysis and toxicological analysis were performed in the supernatant while the pellet was only analyzed for PCBs. The empty 1L flasks were rinsed with ether and this fraction was also analyzed for PCBs.

Comparison of Uncontaminated and Contaminated Soils

Four grams of uncontaminated and contaminated soil were placed in triplicate 25 ml corex tubes. 20 ml of a 10,000 mg/l surfactant solution or deionized water was added separately to the tubes and put in a reciprocating shaker at high speed for 48 hours. The tubes were then centrifuged at 8,000 rpm for 2 hours and the water and surfactant leachates analyzed for CTAS, PCBs and toxicological analysis.

Methods for Chemical Analysis

PCB Analysis from Contaminated Soil

The contaminated soil was analyzed for PCBs before the bioremediation process started. Triplicate 2 g contaminated TVA soil were placed in 25 ml corex tubes and extracted with 2 g Na_2SO_4 , 8 ml of deionized H_2O and 10 ml of ether. The tubes were shaken on a reciprocating shaker for 1 hour and then centrifuged on a table top centrifuge at 1,000 rpm for 15 minutes. 1 ml of the ether phase was placed in 15 ml screw cap tubes containing 1 g silica gel and 9 ml hexane. The tubes were shaken for 15 minutes on the reciprocating shaker and centrifuged for 5 minutes at 1,000 rpm on a table top centrifuge. 1ml of the ether/hexane supernatant was transferred to GC vials.

The concentration of PCB congeners were determined according to Layton (1994). A Shimadzu (Kyoto, Japan) model GC-14A gas chromatograph equipped with an AOC-14 auto injector, an electron capture detector and a split/splitless injector both maintained at 300°C. A DB-1 capillary column (30 m X 0.25 mm, J & W Scientific Inc. Folsom, CA USA) was used with nitrogen as the carrier gas (1.3 ml/min) and the make up gas (70 ml/min). Injection of 1 μL samples were performed using the splitless mode. The GC temperature program was as follows: Initial oven temperature at 60°C hold for 2 minutes, ramp at 10°C/min to 180°C, ramp at 6°C/min to 225°C, hold for 18.5 minutes, and ramp at 10°C/min to 250°C hold for 5 minutes. Individual peak numbers were identified by comparison to peaks from Aroclor 1242 as described by Bedard et al (1987).

Total PCB concentrations were calculated by comparing the individual peak areas of the samples to the averages individual peak areas from a 1 mg/l Aroclor 1242 standard. The weight percent values and peak numbering system were obtained from Bedard (1987). The calculated concentration for each individual peak were summed to provide a total mg/l for each soil sample.

PCB Analysis from Contaminated Soil Washing and Aroclor Solutions

The percentage of PCBs degraded was calculated as follow: $\% \text{ degraded} = (1 - (\text{final mg/l})/(\text{initial mg/l})) * 100$. The initial and final PCB concentrations were determined by taking triplicate 2 ml of the contaminated soil washing or Aroclor 1242 solution from both the beginning and end of the degradation experiment. This samples were extracted, analyzed in the GC and calculated as above. 2 ml of the supernatant (aqueous fraction) and 2 g of the pellet (aqueous and diatomaceous earth bound PCBs) were extracted and analyzed in the GC as described above. The ether rinse phase was dried with silica gel and analyzed in the GC. The PCB concentration of each of the fractions was determined as above, converted to mg and summed. This sum was compared to the final total PCB concentration.

Surfactant Analysis

The concentration of surfactant was determined by the Cobalt thiocyanate Active Substances (CTAS) method (Standard methods for the examination of water and wastewater). CTAS are substances that react with aqueous Cobalt thiocyanate solution to give a cobalt containing product extractable into an organic liquid in which it can be

measured. The Cobalt thiocyanate solution was prepared by dissolving 36 g of $\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ and 240 g of NH_4SCN in 1000ml of deionized water.

For each analysis, triplicate 1.5 ml samples were taken from the surfactant solution and contaminated soil washing or Aroclor 1242 solution at the beginning and end of degradation experiment. These samples were centrifuged in Eppendorf centrifuge tubes at 11,000 rpm for 5 minutes. 1 ml supernatants were mixed with 8 ml of methylene chloride and 3 ml of the cobalt thiocyanate solution in 15 ml screw cap tubes. These tubes were placed on the reciprocating shaker for 15 minutes and then centrifuged at 1000 rpm for 5 minutes. 1.2 ml were pipetted from the lower layer (i.e. methylene chloride), the pipettes wiped with tissue and then emptied into quartz cuvetts. The amount of CTAS was determined by measuring the absorbance at 620 nm and comparing the absorbance to a standard curve using 100 mg/l to 10,000 mg/l of surfactant.

Methods for Toxicological Analysis

Two acute tests using aquatic species - the ciliate *Tetrahymena pyriformis* and the luminescent marine bacterium *Vibrio fischeri* (i.e. the MicrotoxTM test) - were used to find out if the bioremediation process could result in the production of toxic metabolites. These tests were also used to evaluate the pure surfactant (polyoxyethylene (10) lauryl ether) toxicity and for comparisons between PCB contaminated and uncontaminated soils.

16-Hour *Tetrahymena* Mortality Assay

Experiments were run in sterile 20 ml 16 x 125 mm pyrex test tubes. A range finding test was first done for the soil washing solution, Aroclor 1242 solutions and for the pure

surfactant before and after degradation. The concentrations ranged from 0.1 to 100% for the first two samples and from 1 to 10,000 mg/l for the surfactant. These solutions were prepared by diluting the samples in 0.1X Hank's Balanced Salt Solution to a total volume of 10 ml. Definitive tests were repeated three times. Stationary-growth-phase cells were added to each tube to a final concentration of 1×10^3 and incubation was made at $26 \pm 1^\circ\text{C}$ for 16 hours (Schultz et al., 1994). Acute toxicity was assessed by lack of ciliary movement and/or cell lysis with the aid of a dissecting light microscope. The endpoint determined with this assay was the 50% Lethal Concentration (LC_{50}).

Microtox Test

The assays were conducted using the Microtox Toxicity Analyzer System manufactured by Azur (Carlsbad, CA). The liquid reagents and freeze-dried bacterial reagent were those produced by Azur. Two test protocols were used according to the toxicity of the sample: The basic test (for highly toxic samples) and the 90% sample concentration basic test (for less toxic samples). Furthermore, the solid-phase protocol was carried out, using both water and surfactant solution to extract the contaminants from the contaminated and uncontaminated soils. The test is accomplished using a suspension of the luminescent bacteria prepared by hydrating a vial of the freeze-dried Microtox Reagent. Aliquots of the cell suspension are transferred to test vials containing 2% NaCl equilibrated to 15°C using a controlled photometer. Light readings are taken for each test vial before and 15 minutes after sample addition. The amount of light lost per sample dilution is proportional to the toxicity of that sample. Light loss is expressed as gamma value and is the ratio of light lost to the light

remaining in each of the test cuvettes. The effective concentration of a compound causing 50% reduction of the light emitted by the luminescent bacteria (EC_{50}) was the endpoint determined in this assay.

RESULTS

Table 2 shows the concentration of the individual congeners present in the contaminated soil and Aroclor 1242. It can be seen that the PCB congeners present in the TVA soil are very similar to the congeners in Aroclor 1242, exception for the first nine congeners which are absent in the contaminated soil. It can also be seen that the less chlorinated congeners in the Aroclor 1242 are at a higher concentration whereas in the TVA soil the higher chlorinated congeners are in higher concentration.

Table 3 shows the degradation of PCBs for the soil washing and Aroclor 1242 low and high concentration experiments. The greatest degradation was obtained for the Aroclor 1242 low concentration experiment, followed by the Aroclor 1242 high concentration and soil washing. These results shows that degradation of PCBs doesn't appear to be influenced by their initial concentration. The results for the control experiments shows that there was a considerable removal of PCBs in all 3 experiments. Since the controls had the bacteria for surfactants degradation but not for PCBs degradation, we believe that losses might have occurred due to problems such as extraction efficiency, adherence of PCBs onto glass wall etc. The loss of PCBs was calculated as $(1 - (\text{mg/l after degradation} / \text{mg/l before degradation})) * 100$ and the conservative estimated percentage degradation given by Experimental % removal -

Table 2 - Composition of Aroclor 1242 and TVA soil

Peak Number ^a	Congener Identification ^a	IUPAC Number ^b	Concentration (mg/l) ^c	
			TVA soil	Aroclor 1242
2	2,2' ; 2,6	4 ; 10	0	1.161
3	2,4 ; 2,5	7 ; 9	0	0.456
4	2,3'	6	0	0.565
5	2,3 ; 2,4'	5 ; 8	0	2.446
6	2,6,2'	19	0	0.446
7	2,5,2'	18	0	3.589
8	2,4,2' ; 4,4'	17; 15	0	1.830
9	2,3,6; 2,6,3'	24; 27	0	0.385
10	2,3,2' ; 2,6,4'	16; 32	0.215	2.038
11	2,5,3'	26	0.028	0.535
12	2,4,3'	25	0.359	3.410
13	2,5,4'	31	0.174	3.136
14	2,4,4'	28	0.883	2.621
15	2',3,4; 2,5,2',6'	33; 53	0.153	1.414
16	2,4,2',6' ; 2,3,4'	51;22	0.143	0.407
17	2,3,6,2'	45	0.134	0.257
18	2,3,2',6'	46	0.169	0.277
19	2,5,2',5'	52	1.845	1.376
20	2,4,2',5'	49	1.735	1.158
21	2,4,2',4'	47	0.916	0.499
22	2,4,5,2'	48	0.502	0.609
23	2,3,2',5'	44	2.903	1.667
24	3,4,4' ; 2,3,2',4'	37; 42	2.765	1.756
25	2,3,4,2' ; 2,3,6,4' ; 2,6,3',4'	41; 64; 71	3.099	1.480
26	2,3,2',3'	40	1.09	0.107
27	2,4,5,4'	74	3.469	0.952
28	2,5,3',4'	70	1.977	1.770
29	2,4,3',4' ; 2,3,6,2',5'	66; 95	10.388	2.211
30	2,3,6,2',4'	91	3.392	0.796
31	2,3,3',4' ; 2,3,4,4'	56; 60	7.238	1.406
32	2,3,6,2',3' ; 2,3,5,2',5'	84; 92	2.385	0.726
33	2,,3,5,2',4' ; 2,4,5,2',5'	90; 101	4.561	0.664
34	2,4,5,2',4'	99	4.148	0.568
35	2,4,5,2',3' ; 2,3,5,6,2',6'	97; 152	4.99	0.622
36	2,3,4,2',5'	87	5.78	0.727
37	2,3,4,2',4'	85	4.658	0.664
38	2,3,6,3',4' ; 3,4,3',4'	110; 77	5.761	0.632
39	2,3,4,2',3'	82	4.343	0.455
40	2,3,6,2',4',5' ; 2,4,5,3',4'	149; 118	5.73	0.439
41	2,3,4,3',4' ; 2,3,4,2',3',6'	105; 132	10.733	0.169
	2,3,4,2',4',5' ; 2,3,4,5,6,3' ;	138;160		

^aIdentification of individual congeners was based on a comparison with the results of Bedard et al (1986).

^bObtained from McFarland and Clark (1989). ^cDetermined in our laboratory.

Table 3- Degradation of PCBs

	Solution Before Degradation		Solution After Degradation		Removal ^a		Degradation ^b
	Experimental ^c mg/l ($\pm$ SD)	Control ^d mg/l ($\pm$ SD)	Experimental ^c mg/l ($\pm$ SD)	Control ^d mg/l ($\pm$ SD)	Experimental ^c %	Control ^d %	
Soil Washing	165.67	165.67	124.66	145.93	25	12	13
Solution	(25.29)	(25.29)	(13.32)	(25.16)			
Aroclor 1242	37.51	37.51	9.06	25.45	76	27	49
(Low Concentration)	(3.60)	(4.77)	(2.58)	(2.33)			
Aroclor 1242	1166.5	1013.89	576.13	753.89	51	26	25
(High Concentration)	(7.49)	(2.25)	(36.90)	(27.68)			

^aCalculated as $(1 - (\text{mg/l after degradation} / \text{mg/l before degradation})) * 100$. ^bCalculated as Experimental % loss - Control % loss.

^cGenes for both PCBs and surfactant degradation. ^dGenes for surfactant degradation.

Control % loss.

A chromatogram of the degradation of Aroclor 1242 low concentration experiment before and after degradation by IPL5::TnPCB and B30P4::TnPCB is shown in Figure 8. The less chlorinated congeners (peaks at the very front part of the chromatogram which include di-chloro substituents) were completely degraded. The tri-, four- and five-chloro substituents (peaks located in the middle of the chromatogram) were lesser degraded. Finally, the higher chlorinated congeners (peaks at the end part of the chromatogram which include hexa-chloro substituents) were not degraded. The identification of individual peaks can be seen in Table 2.

Table 4 shows the degradation of surfactants. The amount of surfactant degraded is comparable in each of the three experiments between the control and experimental. The degradation was approximately 90%, exception for the Aroclor 1242 high concentration experiment which was approximately 80%.

We could not attribute the lower degradation in the Aroclor 1242 high concentration experiment to the high initial amount concentration of PCBs (around 1,000 mg/l). The degradation of surfactant was calculated as $(1 - (\text{mg/l after degradation} / \text{mg/l before degradation})) * 100$.

The percentage recovery of PCBs in all 3 experiments can be seen in Tables 5 to 7. The results are given in mg instead of mg/l to allow for comparison between supernatant, pellet and the walls of the glass in which the experiments were performed. It can be seen that in all experiments the majority of PCBs are concentrated in the pellet in both control and experimental analysis.

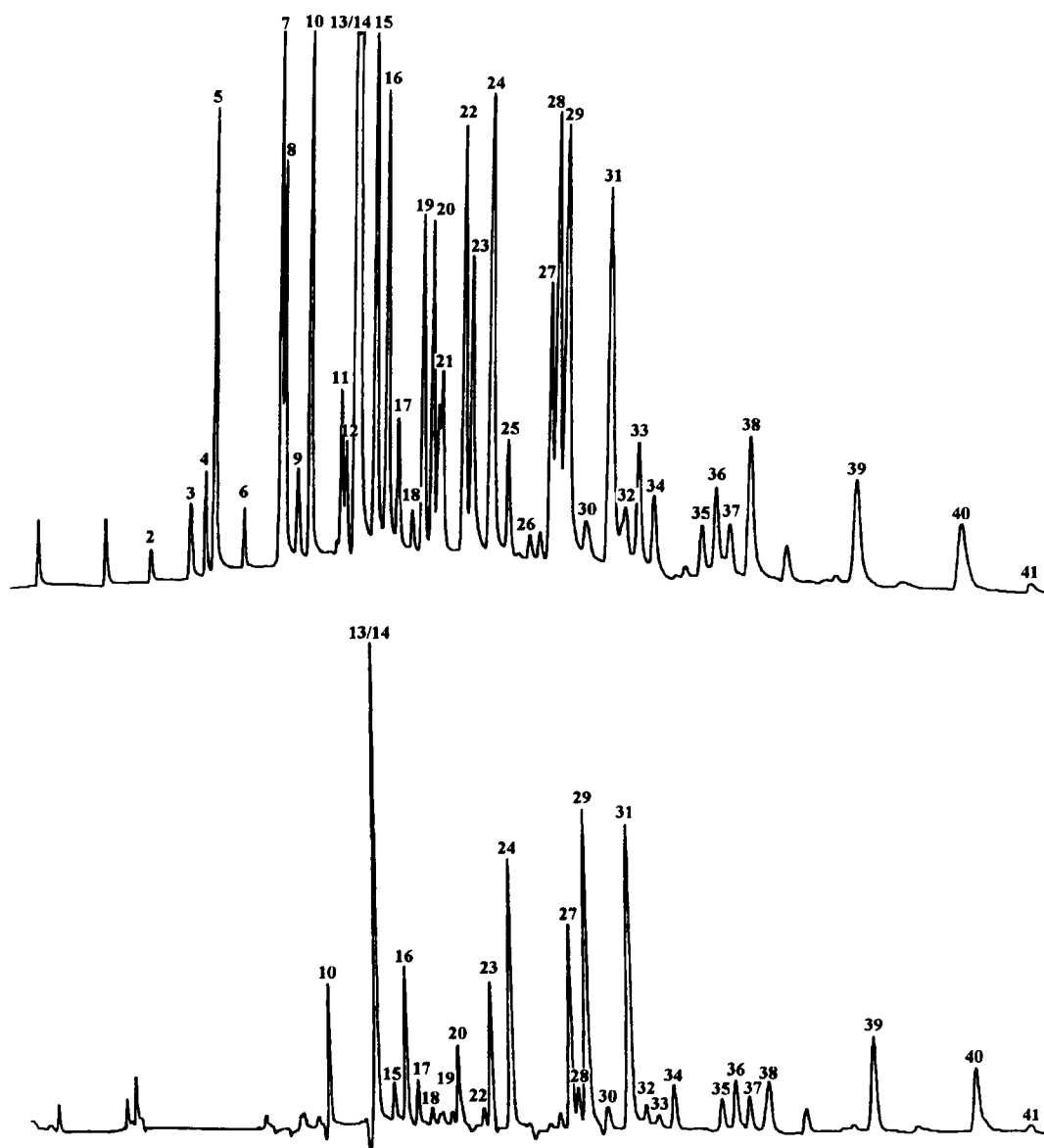


Figure 8 - Degradation of Aroclor 1242 by IPL5::TnPCB and B30P4::TnPCB

Table 4 - Degradation of Surfactant

	Solution Before Degradation		Solution After Degradation		Degradation ^a	
	Experimental ^b mg/l ($\pm$ SD)	Control ^c mg/l ($\pm$ SD)	Experimental ^b mg/l ($\pm$ SD)	Control ^c mg/l ($\pm$ SD)	Experimental ^b %	Control ^c %
Soil Washing	8915.20	8915.20	698.49	646.10	92	93
Solution	(69.37)	(69.37)	(17.98)	(19.34)		
Aroclor 1242	10553.43	9567	851.4	841.19	92	91
(Low Concentration)	(1.13)	(0.98)	(0.23)	(0.11)		
Aroclor 1242	10853.92	12064.63	2426.15	1874.4	78	84
(High Concentration)	(53.54)	(58.25)	(22.81)	(73.61)		

^aCalculated as $(1 - (\text{mg/l after degradation} / \text{mg/l before degradation})) * 100$. ^bGenes for both PCBs and surfactant degradation. ^cGenes for surfactant degradation.

Table 5 - Recovery of PCBs in the soil washing solution experiment

	Experimental	Control
Initial Total PCB (mg) ^a	49.71	49.71
Supernatant (mg) ^b	0.5	0.49
Pellet (mg) ^b	32.42	37.92
Glass (mg) ^c	0.04	0.02
Final Total PCB (mg)	33	38.43
% Recovery	66.39	77.31

^aIn the solution before degradation. ^bAfter degradation the solution was centrifuged and separated into supernatant and pellet. ^cThe walls of the flask in which the degradation was performed was extracted with ether and analyzed for PCBs.

Table 6 - Recovery of PCBs in the Aroclor 1242 (low conc.) experiment

	Experimental	Control
Initial Total PCB (mg) ^a	9.38	9.38
Supernatant (mg) ^b	0.03	0.1
Pellet (mg) ^b	2.46	7.17
Glass (mg) ^c	0.03	0.06
Final Total PCB (mg)	2.49	7.27
% Recovery	26.55	77.5

^aIn the solution before degradation. ^bAfter degradation the solution was centrifuged and separated into supernatant and pellet. ^cThe walls of the flask in which the degradation was performed was extracted with ether and analyzed for PCBs.

Table 7 - Recovery of PCBs in the Aroclor 1242 (high conc.) experiment

	Experimental	Control
Initial Total PCB (mg) ^a	350	304.17
Supernatant (mg) ^b	5.6	18.49
Pellet (mg) ^b	75.79	116.88
Glass (mg) ^c	6.88	13.94
Final Total PCB (mg)	88.27	149.31
% Recovery	25.22	49.09

^aIn the solution before degradation. ^bAfter degradation the solution was centrifuged and separated into supernatant and pellet. ^cThe walls of the flask in which the degradation was performed was extracted with ether and analyzed for PCBs.

recovery in tables 5 to 7 are in agreement with the results of PCB degradation in Table 3.

The toxicity evaluation for contaminated and uncontaminated soil are presented in Table 8. The water leachate for both soils didn't show the presence of PCBs when analyzed by GC, or surfactant, when analyzed by CTAS. Furthermore, the water leachate showed to be not toxic to *Tetrahymena* or to *Vibrio fischeri*. When analyzed by GC and CTAS, the contaminated soil presented a concentration of 150 mg/l of PCBs and 10,000 mg/l of surfactant and the uncontaminated soil had no detectable PCBs and 10,000 mg/l of surfactant respectively. Despite the differences in PCB concentration, both soils presented the same toxicity to *Tetrahymena* and Microtox. One reason for this result might be that the toxicity was due to the surfactant concentration. The acute toxicity of the pure polyoxyethylene 10 lauryl ether to *Tetrahymena* and to Microtox is $LC_{50} = 5.68$ mg/l and $EC_{50} = 5.87$ mg/l, respectively. The results on table 8 shows that 0.06 % of the 10,000 mg/l surfactant solution give us that a concentration of approximately 6 mg/l of surfactant is causing the LC_{50} and EC_{50} . This is the concentration that causes the LC_{50} and EC_{50} in response to the pure polyoxyethylene 10 lauryl ether.

Table 8 - Toxicity evaluation of uncontaminated and contaminated TVA soil

	Uncontaminated Soil ^a		Contaminated Soil ^b	
	Water Leachate ^c	Surfactant Leachate ^d	Water Leachate ^c	Surfactant Leachate ^d
Acute <i>Tetrahymena</i> (LC_{50})	ntd	0.06 ^e (0.02-0.10)	ntd	0.06 ^e (0.02-0.10)
Solid-Phase Microtox (EC_{50})	ntd	0.06 ^e (0.04-0.10)	ntd	0.06 ^e (0.04-0.10)

ntd, no toxicity detected. ^aUncontaminated soil had no detectable PCB in it. ^bContaminated soil had 149 mg/l of PCB. ^cSoil extraction with deionized water. ^dSoil extraction with a 10,000 mg/l surfactant solution. ^eThe result is given in percentage which means that 0.06% of the 10,000 mg/l surfactant solution caused the LC_{50} or EC_{50} .

Tables 9 to 11 show the toxicity evaluation for the 3 degradation experiments. The results obtained in the *Tetrahymena* and Microtox assays are consistent. In all experiments a dilution of approximately 0.06% of the non-degraded solution caused the LC₅₀ and EC₅₀. This percentage represents a surfactant concentration of 6 mg/l, which is approximately the concentration that results in the LC₅₀ and EC₅₀ concentration of the pure polyoxyethylene (10 lauryl) ether. Degradation of PCBs and surfactant resulted in a reduction in toxicity. The supernatant from the Aroclor 1242 (low concentration) and from the TVA soil bioremediation produced a lower toxicity compared to the Aroclor 1242 (high concentration) degraded supernatant. It can be noted that the surfactant concentration in the latter is higher (2426 mg/l in the experimental and 1874 mg/l in the control) compared to the Aroclor 1242 (low concentration) and TVA soil bioremediation (851 mg/l in the experimental, 841 mg/l in the control and 696 mg/l in the experimental, 636 mg/l in the control respectively.) It is important to note, however, that even these lower concentrations, are not even close to the concentration of the pure polyoxyethylene (10) lauryl Ether that caused the LC₅₀ and EC₅₀.

Table 9 - Toxicity evaluation of Aroclor 1242 (low concentration) degradation

	Experimental		Control	
	Solution Before Degradation ^a	Supernatant After Degradation ^b	Solution Before Degradation ^c	Supernatant After Degradation ^d
Acute <i>Tetrahymena</i> - LC ₅₀	0.06 ^e	67.31 ^e	0.06 ^e	66.33 ^e
(95% Confidence Range)	(0.02-0.10)	(63.29-71.33)	(0.02-0.10)	(65.16-67.50)
Acute Microtox - EC ₅₀	0.07 ^e	54.94 ^e	0.07 ^e	34.93 ^e
(95% Confidence Range)	(0.04-0.10)	(53.13-56.82)	(0.04-0.10)	(32.86-37.12)

^aPCBs = 38 mg/l and surfactant = 10.553 mg/l. ^bPCBs = 1 mg/l and surfactant = 851 mg/l. ^cPCBs = 38 mg/l and surfactant = 9567 mg/l. ^dPCBs = 3 mg/l and surfactant = 841 mg/l. ^eThe results are given in percentage which means that x % of the Aroclor solution caused the LC₅₀ or EC₅₀.

Table 10 - Toxicity evaluation of Aroclor 1242 (high concentration) degradation

	Experimental		Control	
	Solution Before Degradation ^a	Supernatant After Degradation ^b	Solution Before Degradation ^c	Supernatant After Degradation ^d
Acute Tetrahymena - LC ₅₀	0.06 ^e	10.21 ^e	0.06 ^e	9.78 ^e
(95% Confidence Range)	(0.02-0.10)	(8.63-11.17)	(0.02-0.10)	(8.22-10.67)
Acute Microtox - EC ₅₀	0.07 ^e	3.06 ^e	0.07 ^e	0.61 ^e
(95% Confidence Range)	(0.04-0.10)	(0.95-9.82)	(0.04-0.10)	(0.04-9.25)

^aPCBs = 1167 mg/l and surfactant = 10854 mg/l. ^bPCBs = 132 mg/l and surfactant = 2426 mg/l. ^cPCBs = 1014 mg/l and surfactant = 12065 mg/l. ^dPCBs = 172 mg/l and surfactant = 1874 mg/l. ^eThe results are given in percentage which means that x % of the Aroclor solution caused the LC₅₀ or EC₅₀.

Table 11 - Toxicity evaluation of the TVA soil bioremediation

	Experimental		Control	
	Solution Before Degradation ^a	Supernatant After Degradation ^b	Solution Before Degradation ^c	Supernatant After Degradation ^d
Acute Tetrahymena - LC ₅₀	0.06 ^e	78.33 ^e	0.06 ^e	77.44 ^e
(95% Confidence Range)	(0.02-0.10)	(77.39-79.27)	(0.02-0.10)	(76.35-78.53)
Acute Microtox - EC ₅₀	0.07 ^e	82.90 ^e	0.07 ^e	68.68 ^e
(95% Confidence Range)	(0.04-0.10)	(75.28-101.79)	(0.04-0.10)	(54.95-77.24)

^aPCBs = 166 mg/l and surfactant = 8915 mg/l. ^bPCBs = 1 mg/l and surfactant = 696 mg/l. ^cPCBs = 166 mg/l and surfactant = 8915 mg/l. ^dPCBs = 1 mg/l and surfactant = 636 mg/l. ^eThe results are given in percentage which means that x % of the PCB solution caused the LC₅₀ or EC₅₀.

DISCUSSION

Soil, sediments, and waters contaminated with PCBs present both ecological and human health hazards. PCBs are of great concern because of the toxic effects they may cause in humans and in wildlife. The skin and liver are the major sites of pathological change, and other targets include the gastrointestinal tract, the immune system and the nervous system (Mckinney and Waller, 1994; Safe, 1989). The non-*ortho*- and mono-*ortho*-substituted congeners are usually implicated in such toxic effects (McFarland and Clark, 1989). Studies in animals also suggest that these type of congeners are able to

disrupt the endocrine system because of their ability to mimic thyroid and other steroidal hormones (Mckinney and Waller, 1994).

Twelve congeners present at high concentration in the TVA soil are either non-ortho- or mono-ortho-substituted congeners and are of human health concern (McFarland and Clark, 1989. See introduction for explanation). These include the highly chlorinated congeners IUPAC numbers 77, 105 and 118 which are 3-methylcholanthrene-type inducers, and congeners IUPAC numbers 87, 99, and 101 which are phenobarbital-type inducers. Congeners IUPAC numbers 18, 44, 49, 52, 70, and 74 are considered to be weak inducers and congener 37 is considered a mixed-type inducer.

Landfilling and incineration of PCB contaminated materials remains the most used method of PCB remediation (Hickey et al., 1993; Robinson and Lenn, 1994). However, the demonstration that some sites are undergoing natural aerobic and anaerobic attenuation of PCBs and the high cost of incineration are encouraging for the development of bioremediation strategies (Layton et al., 1997).

Although the purpose of bioremediation processes is to transform toxic substances into innocuous compounds, toxic metabolites may accumulate during the process. Svenson and Kaj (1989) using the Microtox assay found products from photochemical conversion of 2,4,6-trichlorophenol were more toxic than the parent compound. Similarly, Middaugh et al. (1993) using the silverside fish (*Medidia beryllina*) embryo assay demonstrated that intermediate metabolites of pentachlorophenol degradation were more toxic than the original substance. It has been also observed that

the hydroxylated metabolites of PCBs are several times more toxic than the parent compound (Sawhney and Hankin, 1985).

The *in situ* soil washing/bioremediation process developed at CEB, uses two genetically engineered bacterial strains to degrade PCBs. These strains only contain the genes necessary to convert PCBs to chlorobenzoic acids, and thus we would expect chlorobenzoic acids and surfactant metabolites to be present in the final effluent. However, even though the predominant metabolite of the less chlorinated PCBs are chlorobenzoic acids, the more highly chlorinated PCBs congeners can be also subject to partial degradation resulting in the formation of toxic metabolites (Robinson and Lenn, 1994).

Two acute ecotoxicological tests, *Tetrahymena* and MicrotoxTM were used to evaluate the toxicity of the *in situ* soil washing/bioremediation process. These two tests represent both a prokaryotic and an eukaryotic tests, respectively, complementing each other. The advantages of the *Tetrahymena* assay include easy of handling and culture, limited laboratory space requirements (Giesy and Hoke, 1989), inexpensive and rapid (Schultz et al., 1990). The MicrotoxTM assay is simple, replicable, has a relative toxicity to many compounds which are similar to most species of insects, crustaceans, protozoans, mollusks and fish (Giesy and Hoke, 1989) and has been recommended as the first stage in a tiered testing scheme (Sloof, 1985). Furthermore, the MicrotoxTM solid-phase test was used to measure the toxicity of the contaminated and uncontaminated soils.

The results obtained in this study showed that after degradation there was not an increase in toxicity of the final degraded effluent compared to the solution before

degradation (see Tables 9-11). However, although a good degradation of PCBs was achieved (see Table 3 and Figure 8) the toxicological results could not be attributed to the metabolites produced by PCB degradation. It seems that the results of toxicity in both degraded and non-degraded solutions were due to the surfactant used to extract the PCBs from the soil. Both *Tetrahymena* and *Vibrio fischeri* were very sensitive to the surfactant.

CTAS analysis showed that almost complete degradation of the surfactant was achieved in all experiments (see Table 4). The concentration of the pure surfactant that causes the LC_{50} and EC_{50} are 5.87 and 5.68 mg/l, respectively, so contrary to the finds of this study, we would expect a concentration of approximately 600 or 800 mg/l to be still highly toxic to both *Tetrahymena* and *Vibrio fischeri*. The biodegradation of alcohol ethoxylate nonionic surfactants can involve attack at the far end of either the hydrophobe or the ethylene oxide chain, or it can be a central fission separating the hydrophobe and the ethylene oxide chain (Patoczka and Pulliam, 1990). Toxicity increases with increasing hydrophobe (AE) chain length and decreases in the ethylene oxide (EO) groups (Dorn et al., 1992 and Ribosa et al., 1993). For example, a C_{16} -AE with one EO would be more toxic than a C_{14} -AE with seven EO. One explanation for the results found in our study could be that in the CTAS assay the ethylene oxide group is being measured, which is less toxic than the hydrophobe chain.

The MicrotoxTM solid-phase test was also not adequate for measuring the toxicity of contaminated and uncontaminated soils. In this assay, a water extraction put the toxicant bound to the soil, sediment or sludge in contact with the test organisms. In our case, PCBs are bound to the soil, but they are not water soluble. A surfactant solution

could be used to extract the PCBs from the soil but, as discussed above, the surfactant distorts the results.

Because surfactants interferes with the evaluation of the toxicity of metabolites that could have been produced in the bioremediation process and because chlorobenzoic acids are the major metabolites formed by aerobic PCB degradation, the next chapter will be dedicated to the study of the relative toxicity of chlorobenzoic acids.

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PART III

QUANTITATIVE STRUCTURE-ACTIVITY RELATIONSHIPS

FOR

CHLOROBENZOIC ACIDS (CBAs)

INTRODUCTION

It has long been recognized that the activity of a chemical towards living organisms depends upon a physical or chemical action on biological tissues (Turner et al., 1986), and the nature of such action will depend ultimately on the molecular structure of the chemical. Many attempts have been made to relate biological activity to molecular structure in a quantitative way.

Mathematical models which relate some physical, chemical, biological, or environmental activity of interest to some quantitative structural descriptor (e.g. a physicochemical property) are collectively known as quantitative structure-activity relationships (QSAR) models. QSAR models are usually developed for a group(s) of structural congeners and the primary objective in creating them is to use them to predict the activities of untested congeners. Such studies also serve to gain a better understanding about mechanisms of action of structures under study (Sabljić, 1990). The statistical procedure used to derive QSAR models is the linear regression analysis and it can be either single or multivariable depending on the number of structural descriptors used in a particular analysis.

The use of QSARs include estimations of the biological effects of chemicals which have not been tested (Jones and Schultz, 1995) which is important for evaluations of new chemicals during development; priority setting of chemical substances; and regulatory activities (Hermens, 1989)

McFarland (1970) stated that the manifestation of chemical toxicity is preceded by two circumstances: 1) The penetration of the chemical into the cell membrane and 2)

The interaction of the chemical with the molecular site of action. The first is modeled customarily by the hydrophobic term $\log K_{ow}$. The interactions have been modeled by electronic and/or steric parameters. This two step process can be modeled by the generic QSAR:

$$\log 1/\text{toxic effect} = A \log (\text{penetration}) + B \log (\text{interaction}) + \text{intercept}$$

The majority of industrial organic chemicals act via the nonpolar narcosis mode of toxic action (Bradbury and Lipnick, 1990). Hydrophobicity, quantitated by $\log K_{ow}$, is the most universal molecular descriptor for the nonpolar narcotic mechanism. QSARs for this mechanism serve to estimate baseline or minimum toxicity in the absence of any stereoelectronic or bioreactive interactions (Schultz, 1989).

A high-quality penetration-dependent quantitative structure-activity relationship for this mechanism is very important, as it is to this mechanism of action that bioreactive class of chemicals are to be compared, via excess toxicity, T_e (Lipnick et al., 1987). Excess toxicity is defined as the ratio of predicted toxicity (as predicted by the baseline model) and to the observed toxicity. It has been shown (Schultz et al., 1995) that T_e values greater than 2.0 are indicative of bioreactivity for a chemical or group of chemicals.

Because chlorobenzoic acids are the main metabolite produced by aerobic bacterial PCB degradation (Abramowicz, 1990; Hooper et al., 1990; Layton et al., 1994; Rochkind et al., 1986) and because their toxicity could not be assessed in the *in situ* soil washing/bioremediation process due to the presence of surfactants, we attempted to assess their relative toxicities. In this chapter, QSARs are developed for chlorobenzoic

acids using both an acute mortality and a population growth inhibition toxicity assays employing *Tetrahymena pyriformis*.

Tetrahymena is a ubiquitous, pear-shaped, uniformly ciliate protozoan that measures approximately 50 μm in length and 30 μm in width and has a generation time of 3 to 4 hours. This ciliate protozoan provides an organism that is appropriate as an *in vitro* eukaryotic toxicity assessment model and it is also inexpensive, rapid and well characterized (Schultz et al., 1990). The use of *Tetrahymena pyriformis* for toxicity assessment has been demonstrated for a variety of materials including pharmaceuticals, food additives, metals, and industrial organics (Schultz et al., 1990).

MATERIALS AND METHODS

Chemical Selection and Physicochemical Parameters Determinations

Benzoic acid, 4-bromobenzoic acid, and a total of 10 chlorobenzoic acid derivatives with mono-, di-, or tri-chlorination were obtained commercially (Aldrich Chemical Co., Milwaukee, Wisconsin, USA).

Logarithms of the 1-octanol/water partition coefficient ($\log K_{ow}$) of each derivative were obtained as measured values from LOGKOW databank (Sangster, 1993) or as estimated values from MEDCHEM CLOGP version 3.55 (Leo and Weingier, 1989). The ionization constant (pK_a) values for each derivative were obtained as measured values from Dean (1992) or as estimated values from Hunter (1988).

16-Hour *Tetrahymena* Mortality Assay

Acute toxicity in this assay was assessed by lack of ciliary movement and/or cell lysis of the common freshwater ciliate *Tetrahymena pyriformis* with the aid of a dissecting light microscope and results were given in percent mortality. Fresh stock solutions of each benzoic acid (BA) were prepared in dimethyl sulfoxide (DMSO). A series of dilutions were done in sterile 20 ml 16 x 125 mm Pyrex test tubes containing 10 ml of a 0.1X Hank's Balanced Salt Solution or 10 ml of a 0.1X Hanks/Hepes solution for non-neutralized and neutralized BAs, respectively. A range finding test was first done for each chemical with concentrations ranging from 5 to 50 mg/l for the non-neutralized BAs and from 50 to 4200 mg/l for the neutralized BAs. Then definitive tests were repeated three times. Stationary-growth-phase cells were added to each tube to a final concentration of 1×10^3 and incubation was made at 26 ± 1 °C for 16 hours (Schultz et al.,1994). The endpoint determined with this assay was the 50% Lethal Concentration (LC₅₀).

40-Hour *Tetrahymena* Growth Impairment Assay

Chronic toxicity in this assay uses axenic cultures of *Tetrahymena pyriformis* to study population growth inhibition induced by chemical stress (Schultz, 1996). Fresh stock solutions of each BA were prepared in DMSO. A series of dilution ranging from 100-600 mg/l for the non-neutralized BAs and from 100-2400 mg/l for the neutralized BAs was done in foam stoppered 250 ml Erlenmeyer flasks containing 50 ml of proteose-peptone media (Schultz, 1996). In the neutralized analysis each solution was neutralized to pH 7.0 with 0.1N NaOH using an automatic titrator (Mettler-Toledo DL50 Titrator). A range finding test

was first done for each chemical in order to determine the chemical concentration that covered from no effect to complete inhibition of growth. The definitive tests were then repeated three times in duplicate runs with seven or more step concentration series. For inoculation, log-growth-phase cells were added to each flask to a final concentration of 1.5×10^3 and incubation was done at 27 ± 1 °C for 40 hours. Positive (*Tetrahymena* grown in media without chemical) and negative (flasks with neither cells or chemical) controls were performed with each experiment. Population density was estimated spectrophotometrically as absorbance at 540. The 50% inhibitory growth concentration (IGC₅₀) was the endpoint determined in this assay.

Statistical Procedures

The IGC₅₀ was determined for each acid using Probit Analysis of Statistical Analysis System (SAS) software (SAS Institute Inc. 1989) with the dependent variable being the absorbency normalized as percentage of control and the independent variable being the toxicant concentration in mg/l.

The Statistical Analysis System (SAS) software (SAS Institute Inc., 1989) was used to perform simple and multiple regression analysis for the development of QSARs. Log K_{ow} and pK_a, either separated or together, were used as the independent variable. The dependent variables were determined by the log of the inverse of a particular endpoint (LC₅₀ or IGC₅₀) in mM.

The models were measured for quality by the coefficient of determination (r^2). The square root of the mean square for error (s) was reported as an error of estimate of the

coefficient of regression. Moreover, the ratio of model mean square or the Fisher Statistic (f) and the level of significance ($Pr > f$) were also noted.

Aliphatic Monocarboxylic Acid Model

Toxicity values for seven aliphatic monocarboxylic acids were secured from TETRATOX, an accumulation of the toxicity testing results of Schultz and co-workers for over 1,500 different industrial organic chemicals. These data along with hydrophobicity properties were used to construct via the general linear model of regression analysis (SAS Institute Inc., 1989) a QSAR here after referred to as the aliphatic monocarboxylic acid model. This acid model and related statistics are presented in Table 1.

Excess Toxicity

Excess toxicity (T_e) evaluations were conducted for each chemical by calculating the ratio of predicted toxicity to observed toxicity (Lipnick et al., 1987). For acute effects the toxicity was determined from the log K_{ow} dependent baseline model for the LC_{50} endpoint (Bearden, 1996). These models and corresponding statistics are presented in Table 1. For chronic effects toxicity was measured as the IGC50 in mM. Predicted toxicity was determined from the log K_{ow} dependent baseline model for this endpoint (Schultz and Tichy, 1993). Excess toxicity was also evaluated using the acid model.

RESULTS

Table 12 present a list of the baseline models for acute and chronic endpoints and the chronic acid model. They were used for comparisons to the models obtained in this study and for calculation of the T_e values.

Table 12 - Baseline models for acute and chronic *Tetrahymena pyriformis*

Endpoint	Equation	n	r ²	s	f	Pr>f	Equation Number
LC ₅₀ ^a	0.89*Kow-2.43 ^c	14	0.99	0.21	914	0.0001	1
IGC ₅₀ ^b	0.80*Kow-2.04 ^d	34	0.98	0.17	1745	0.0001	2
IGC ₅₀ ^b	0.36*Kow-0.86 ^e	07	0.97	0.10	151	0.0001	3

^a50% Lethal Concentration. ^b50% Inhibitory Growth Concentration. ^cObtained from Bearden (1996).

^dObtained from Schultz and Tichy (1993). ^eAliphatic monocarboxylic acid model.

Acute Toxicity

The physicochemical parameters and toxicity data for both neutralized and non-neutralized BAs are listed in Table 13. The neutralized acids are consistently less toxic than the non-neutralized acids. It was also noted that in both neutralized and non-neutralized tests the most toxic acids are the 3,4- and 3,5- dichloro and the 4-chloro and 4-bromobenzoic acids. The less toxic are the benzoic acid, 2-chloro and 2,6-dichlorobenzoic acids. This result can also be noted in Figure 9 which shows a plot of toxicity versus hydrophobicity for non-neutralized benzoic acids. For halogenated benzoic acids toxic potency decreases as hydrophobicity decreases, an exception is the non-neutralized 4-bromobenzoic acid. This acid is less hydrophobic than either the 3,4- and 3,5- dichlorobenzoic acids but it is still more toxic. However, it should be noted that the ionization constant for the 4-bromobenzoic acid is higher than for the 3,4- and 3,5 dichloro derivatives.

Table 14 shows the aT_e values for non-neutralized and neutralized acids. It can be seen in Table 14 that acute toxicities of non-neutralized acids are in excess when compared to the estimated potency based on the nonpolar narcotic QSARs, whereas the

Table 13 - Physicochemical parameters and acute toxicities for *Tetrahymena pyriformis*

Name	CAS Number ^a	Log K _{ow}	pK _a	Non-Neutralized LC ₅₀ ^b	Neutralized LC ₅₀ ^b
benzoic acid	65-85-0	1.87m	4.20m	0.91	-1.03
2-chlorobenzoic acid	118-91-2	1.99m	3.85m	0.89	-1.36
3-chlorobenzoic acid	535-80-8	2.60m	3.83m	1.01	-0.88
4-chlorobenzoic acid	74-11-3	2.65m	3.85m	1.19	-0.41
2,3-dichlorobenzoic acid	50-45-3	2.67e	2.55e	0.98	-0.72
2,4-dichlorobenzoic acid	50-84-0	3.44e	2.68e	1.10	-0.42
2,5-dichlorobenzoic acid	50-79-3	2.82m	2.55e	1.10	-0.90
2,6-dichlorobenzoic acid	50-30-6	1.80m	1.64e	0.80	-1.35
3,4-dichlorobenzoic acid	51-44-5	3.46m	3.68m	1.28	-0.20
3,5-dichlorobenzoic acid	51-36-5	3.00m	3.46e	1.28	-0.20
2,4,6-trichlorobenzoic acid	50-43-1	2.86e	1.40e	1.05	-1.03
4-bromobenzoic acid	586-76-5	2.80e	3.99e	1.30	-0.30

^aChemical Abstract Service registry number. ^b50% Lethal Concentration. m-measured Log K_{ow} and pK_a values obtained from Sangster (1993) and Dean (1992) respectively. e-computer estimated Log K_{ow} and pK_a values obtained from Leo and Weingier (1989) and Hunter (1988) respectively.

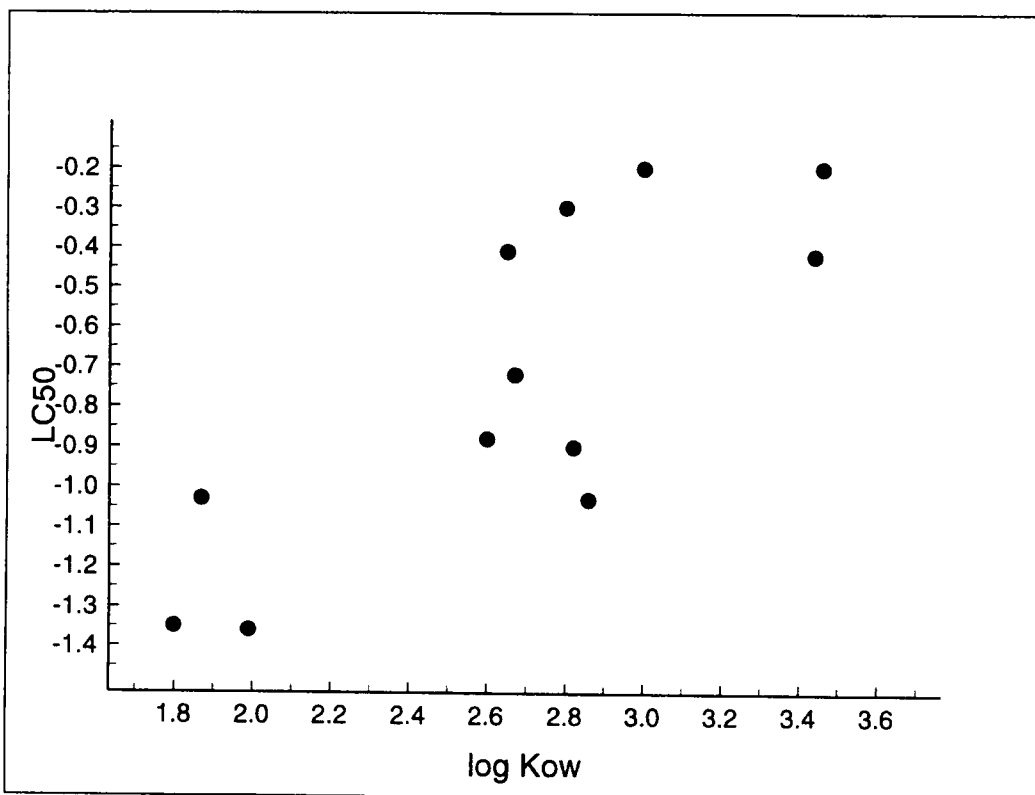


Figure 9 - A scatter plot of toxicity (LC₅₀) versus hydrophobicity (log K_{ow}) for non-neutralized benzoic acids

neutralized acids are less toxic.

When non-neutralized, the benzoic acid , 2-chloro-, and 2,6-dichlorobenzoic acids exhibit the highest excess toxicity. The pK_a values for the first two acids are the greatest among the 12 acids tested in this study and the $\log K_{ow}$ values for the three acids are among the lowest ones (see Table 13). The pK_a value for the 2,6-dichlorobenzoic acid is estimated which may explain it being out of this pattern. 2,4- and 3,4-dichlorobenzoic acids exhibited the least excess toxicities. Their $\log K_{ow}$ values are among the highest ones, and the pK_a values are intermediate.

When neutralized, the benzoic acids exhibit toxicity less than predicted by the baseline model. However, toxicity of the parent compound benzoic acid is predicted by the baseline models. When the acids are neutralized there is no correlation between excess toxicity and pK_a values and the excess toxicity seems to decrease with decrease in $\log K_{ow}$.

Table 14 - Excess acute toxicity of non-neutralized and neutralized benzoic acids

Name	Non-neutralized aT_e^a	Neutralized aT_e^a
benzoic acid	47.4674	0.54770
2-chlorobenzoic acid	35.6926	0.19829
3-chlorobenzoic acid	13.6338	0.17042
4-chlorobenzoic acid	18.4590	0.46148
2,3-dichlorobenzoic acid	10.8075	0.21615
2,4-dichlorobenzoic acid	2.9742	0.08922
2,5-dichlorobenzoic acid	10.5966	0.10597
2,6-dichlorobenzoic acid	42.8484	0.30246
3,4-dichlorobenzoic acid	4.2821	0.14274
3,5-dichlorobenzoic acid	10.9915	0.36638
2,4,6-trichlorobenzoic acid	8.6425	0.07202
4-bromobenzoic acid	17.4277	0.43569

^aexcess toxicity with predicted toxicity determined by the LC_{50} baseline model.

The QSARs and associated statistics generated for both neutralized and non-neutralized acids are shown in Table 15. Neither log K_{ow} nor pK_a are a quality descriptor of toxicity. None of the QSARs presented in Table 15 are of sufficient merit to be used for predictive purposes. In an effort to improve predictability multiple regression analyses using both log K_{ow} and pK_a as independent variables were performed. These results are shown in Table 16. Overall the resulting models showed an improvement in the r^2 value. However, in equations 9 and 10 the pK_a terms were not statistically significant at the 0.05 level. Despite this fact, it is felt that these equations are of sufficient quality to be used for predictive purposes.

Chronic Toxicity

Table 17 shows the physicochemical parameters and chronic toxicity data for both neutralized and non-neutralized BAs. The m and e letters beside log K_{ow} and Pk_a values stands for measured and estimated values, respectively. However, both measured and estimated values were obtained from the literature. The neutralized acids are consistently less toxic than the non-neutralized acids.

Table 15 - Linear regression models for acute toxicity

Condition	Dependent Variable	Independent Variable	Slope	Intercept	r^2	s	f	Pr > f	Equation Number
Non Neutralized	LC ₅₀ ^a	Log K_{ow}	0.24	0.45	0.62	0.11	16	0.0025	4
Neutralized	LC ₅₀ ^a	Log K_{ow}	0.06	0.89	0.12	0.16	18	0.0019	5
Non Neutralized	LC ₅₀ ^a	pK_a	0.62	-2.38	0.64	0.27	1	0.2794	6
Neutralized	LC ₅₀ ^a	pK_a	0.16	-1.23	0.13	0.41	1	0.2548	7

^a50% Lethal Concentration.

Table 16- Multiple regression models for acute toxicity

Condition	Dependent Variable	Independent Variables	Slope	f	Pr > f	Intercept	r ²	s	F	Pr > F	Equation
Non-Neutralized	LC ₅₀ ^a	Log K _{ow}	0.24	23	0.0009						
		pK _a	0.06	5	0.0518	0.23	0.75	0.09	14	0.0018	8
Neutralized	LC ₅₀ ^a	Log K _{ow}	0.63	28	0.0005						
		pK _a	0.17	6	0.3260	-2.95	0.79	0.22	17	0.0009	9

^a50% Lethal Concentration.

Table 17 - Physicochemical parameters and chronic toxicities for *Tetrahymena pyriformis*

Name	CAS Number ^a	Log K _{ow}	pK _a	Non-neutralized		Neutralized	
				log (IGC ₅₀ ⁻¹) ^b		log (IGC ₅₀ ⁻¹) ^b	
benzoic acid	65-85-0	1.87m	4.20m	-0.30		-0.69	
2-chlorobenzoic acid	118-91-2	1.99m	3.85m	-0.45		-0.84	
3-chlorobenzoic acid	535-80-8	2.60m	3.83m	-0.13		-0.25	
4-chlorobenzoic acid	74-11-3	2.65m	3.85m	0.11		-0.15	
2,3-dichlorobenzoic acid	50-45-3	2.67e	2.55e	-0.41		-0.69	
2,4-dichlorobenzoic acid	50-84-0	3.44e	2.68e	-0.39		-0.63	
2,5-dichlorobenzoic acid	50-79-3	2.82m	2.55e	-0.46		-0.90	
2,6-dichlorobenzoic acid	50-30-6	1.80m	1.64e	-0.67		-1.31	
3,4-dichlorobenzoic acid	51-44-5	3.46m	3.68m	0.31		0.23	
3,5-dichlorobenzoic acid	51-36-5	3.00m	3.46e	0.27		0.23	
2,4,6-trichlorobenzoic acid	50-43-1	2.86e	1.40e	-0.41		-0.78	

^aChemical Abstract Service registry number. ^b50% Inhibition Growth Concentration. m-measured Log K_{ow} and pK_a values obtained from Sangster (1993) and Dean (1992) respectively. e-computer estimated. Log K_{ow} and pK_a values obtained from Leo and Weingier (1989) and Hunter (1988) respectively.

than the non-neutralized acids. In both neutralized and non-neutralized acids tests, the most toxic BAs are the 3,4-, 3,5- and 4-chloro derivatives. The less toxic BAs are the 2-, 2,5- and 2,6- derivatives. The potency decrease for these acids as hydrophobicity decreases, an exception is 2,5-dichlorobenzoic acid. The most toxic derivatives show in general higher pK_a values compared to the less toxic.

Table 18 shows the chronic excess toxicity (cT_e) for non-neutralized and neutralized acids, respectively. The cT_e was calculated with predicted toxicity determined by the chronic baseline model (cT_{e1}) and by the acid model (cT_{e2}).

The cT_{e1} values in Table 18 shows that when non-neutralized, benzoic acid and 2-, 3-, 4-, 3,4- and 3,5-chloro derivatives are not in excess when compared to the estimated potency based on the nonpolar narcotic QSARs. The 2,3-, 2,4-, 2,5-, 2,6- and 2,4,6- derivatives appears to be less toxic than predicted. Potency seem to increase for these derivatives as $\log K_{ow}$ and pK_a increase. The cT_{e2} values shows that when compared

Table 18 - Excess chronic toxicity of non-neutralized and neutralized benzoic acids

Name	Non-neutralized		Neutralized	
	cT_{e1}^a	cT_{e2}^b	cT_{e1}^a	cT_{e2}^b
benzoic acid	1.75	0.77	0.71	0.31
2-chlorobenzoic acid	1.00	0.49	0.41	0.20
3-chlorobenzoic acid	0.68	0.62	0.51	0.47
4-chlorobenzoic acid	1.07	1.04	0.59	0.57
2,3-dichlorobenzoic acid	0.31	0.31	0.16	0.16
2,4-dichlorobenzoic acid	0.08	0.17	0.05	0.10
2,5-dichlorobenzoic acid	0.21	0.24	0.08	0.09
2,6-dichlorobenzoic acid	0.38	0.35	0.19	0.08
3,4-dichlorobenzoic acid	0.85	0.84	0.32	0.70
3,5-dichlorobenzoic acid	0.81	1.12	0.74	1.02
2,4,6-trichlorobenzoic acid	0.22	0.26	0.09	0.11

^aExcess toxicity with predicted toxicity determined by the chronic baseline model. ^bExcess toxicity with predicted toxicity determined by the acid model.

to the estimated potency based on the acid QSAR approximately the same results are obtained.

After neutralization the T_e values decreased and only benzoic acid, 3-, 4-, and 3,5-derivatives are not in excess when compared to the estimated potency based on the nonpolar narcotic QSARs. Similar results were found for comparison to the acid QSAR.

The QSARs generated for both neutralized and non-neutralized acids are shown in Table 19 along with their statistics. Again, neither $\log K_{ow}$ nor pK_a alone is a quality descriptor of toxicity. None of the QSARs presented in Table 19 are worth being used for predictive purposes. In an effort to improve predictability, multiple regression analysis using both $\log K_{ow}$ and pK_a as the independent variable were performed. The results on Table 20 shows that there was an improvement in the r^2 values. Although these are not high quality coefficients they are of sufficient quality to be used for predictive purposes.

Tables 21 and 22 shows a theoretical calculation of the expected metabolites (Bedard et al., 1990) that would be obtained if 100 mg/l of Aroclor 1242 or PCBs from a contaminated soil were degraded by IPL5::TnPCB and B30P4::TnPCB. 1 Toxic Unit (TU) is defined as the amount of a compound that causes 50% Inhibitory Growth Concentration (IGC_{50} in mg/l) of a particular organism (in our case, *Tetrahymena*) (Logan and Wilson, 1995). Assuming additivity, we obtained a TU of 0.18 for the amount of chlorobenzoic acids produced by Aroclor 1242 degradation and 0.05 for the amount of chlorobenzoic acids produced by TVA soil Bioremediation. This means that if 100 mg/l of PCBs is degraded in both cases we are not producing toxic metabolites.

Table 19 - Linear regression models for chronic toxicity

Condition	Dependable Variable	Independable Variable	Slope	Intercept	r ²	s	f	Pr > f	Equation Number
Non Neutralized	IGC50 ^a	Log K _{ow}	0.30	-1.03	0.27	0.29	3.44	0.0965	10
Neutralized	IGC50 ^a	Log K _{ow}	0.51	-1.88	0.36	0.41	5.14	0.0496	11
Non Neutralized	IGC50 ^a	pK _a	0.20	-0.84	0.34	0.28	4.71	0.0581	12
Neutralized	IGC50 ^a	pK _a	0.30	-1.43	0.34	0.42	4.63	0.0599	13

^a50% Inhibitory growth concentration.

Table 20 - Multiple regression models for chronic toxicity

Condition	Dependable Variable	Independable Variables	Slope	f	Pr > f	Intercept	r ²	s	F	Pr > F	Equation number
Non Neutralized	IGC ₅₀ ^a	Log K _{ow}	0.32	7	0.0340						
		pK _a	0.21	9	0.0170	-1.73	0.66	0.21	7.84	0.0130	14
Neutralized	IGC ₅₀ ^a	Log K _{ow}	0.55	12	0.0166						
		pK _a	0.32	12	0.0078	-2.94	0.75	0.27	12.06	0.0039	15

^a50% Inhibitory growth concentration.

Table 21 - Expected metabolites resulting from aerobic metabolism of aroclor 1242

PCB Congener ^a	mg/l ^b	% Degraded ^b	CBA ^c	mg/l produced ^d	IGC ₅₀ (mg/l) ^e	T.U. ^f
2,2'	2.7	95	2-	1.79	438.01	0.0041
2,4	0.45	100	2,4-	0.38	472.39	0.0008
2,5	0.45	100	2,5-	0.38	547.09	0.0007
2,3'	1.2	100	3-	0.84	212.33	0.0039
2,4	5.8	95	4-	3.83	122.96	0.0312
2,6,2'	0.9	60	2,6-	0.40	884.03	0.0004
2,5,2'	8.7	100	2,5-	6.38	547.09	0.0117
2,4,2'	4.3	95	2,4-	3.00	472.39	0.0063
2,6,3'	0.7	90	2,6-	0.46	884.03	0.0005
2,6,4'	4.8	70	2,6-	2.46	884.03	0.0028
2,5,3'	1.1	100	3-	0.66	212.33	0.0031
2,4,3'	7	100	2,4-	5.13	472.39	0.0109
2,5,4	7	100	4-	4.21	122.96	0.0342
2',3,4	3.3	100	3,4-	2.42	93.83	0.0258
2,3,6,2'	0.5	55	2,3,6-	0.21	584.23	0.0004
2,5,2',5'	3.1	95	2,5-	1.90	547.09	0.0035
2,4,2',5'	2.6	95	2,4-	1.59	472.39	0.0034
2,4,5,2'	1.4	85	2,4,5-	0.91	584.23	0.0016
2,3,2',5'	3.4	95	2,3-	2.09	492.41	0.0042
2,3,4,2'	3	55	2,3,4-	1.26	584.23	0.0022
2,5,3',4'	3.9	95	3,4-	2.39	93.83	0.0255
2,3,6,2',5'	4.8	45	2,3,6-	1.47	584.23	0.0025
2,3,5,2',5'	0.5	35	2,3,5-	0.12	584.23	0.0002
2,4,5,2',5'	1.1	85	2,4,5-	0.64	584.23	0.0011
2,3,4,2',5'	1	70	2,3,4-	0.48	584.23	0.0008
Total						0.1816

^aOnly the PCB congeners which are degraded to chlorobenzoic acids were used in the calculations. ^bObtained in this study. ^cObtained from Bedard et al. ^dCalculated as (mg/l of PCB x MW of CBA x %degraded)/ MW of PCB). ^eObtained in this study. ^fCalculated as mg/l of CBA/IGC₅₀.

Table 22 - Expected metabolites resulting from aerobic metabolism of TVA soil

PCB Congener ^a	mg/l ^b	% Degraded ^b	CBA ^c	mg/l produced ^d	IGC ₅₀ (mg/l) ^e	T.U. ^f
2,4,2'	4.3	53.75	2,4-	1.70	472.39	0.0036
2,6,3'	0.7	0.00	2,6-	0.00	884.03	0.0000
2,6,4'	4.8	45.01	2,6-	1.58	884.03	0.0018
2,5,3'	1.1	43.35	3-	0.29	212.33	0.0014
2,4,3'	7	40.67	2,4-	2.09	472.39	0.0044
2,5,4	7	40.64	4-	1.71	122.96	0.0139
2',3,4	3.3	32.82	3,4-	0.79	93.83	0.0085
2,3,6,2'	0.5	25.18	2,3,6-	0.10	584.23	0.0002
2,5,2',5'	3.1	33.72	2,5-	0.67	547.09	0.0012
2,4,2',5'	2.6	31.78	2,4-	0.53	472.39	0.0011
2,4,5,2'	1.4	29.36	2,4,5-	0.31	584.23	0.0005
2,3,2',5'	3.4	28.77	2,3-	0.63	492.41	0.0013
2,3,4,2'	3	40.58	2,3,4-	0.93	584.23	0.0016
2,5,3',4'	3.9	32.20	3,4-	0.81	93.83	0.0086
2,3,6,2',5'	4.8	29.22	2,3,6-	0.96	584.23	0.0016
2,3,5,2',5'	0.5	27.69	2,3,5-	0.09	584.23	0.0002
2,4,5,2',5'	1.1	100.00	2,4,5-	0.75	584.23	0.0013
2,3,4,2',5'	1	40.76	2,3,4-	0.28	584.23	0.0005
Total						0.0517

^aOnly the PCB congeners which are degraded to chlorobenzoic acids were used in the calculations. ^bObtained in this study. ^cObtained from Bedard et al. ^dCalculated as (mg/l of PCB x MW of CBA x %degraded)/ MW of PCB). ^eObtained in this study. ^fCalculated as mg/l of CBA/IGC₅₀.

DISCUSSION

Study on the toxicity of organic acids and bases to aquatic organisms has received limited investigation (Zhao et al., 1996). A literature review found that few carboxylic acids, either aromatic or aliphatic, have been evaluated for any of the major ecotoxicological endpoints such as fish mortality, daphnid immobility, *Tetrahymena* population growth or microbial bioluminescence (Swann et al., 1995).

While both ionized and non-ionized forms of a chemical contribute to its toxicity, the non-ionized molecules cross biological membranes faster than ionized molecules, and, thus, have a greater contribution (Broderius et al., 1977; Zhao et al., 1996). The

results obtained in our study are in agreement to this statement. The non-neutralized BAs showed to be more toxic than the neutralized BAs. When non-neutralized, the excess of H^+ ions in the solution drives the equilibrium reaction to the formation of the non-ionized form of the acid, which have a greater contribution to toxicity.

Excess toxicity (T_e) was evaluated in order to verify if BAs could be predicted by the baseline models. Excess toxicity is defined as the ratio of predicted toxicity (as predicted by the baseline model) to the observed toxicity. It has been shown (Schultz et al., 1995) that T_e values greater than 2.0 are indicative of bioreactivity for a chemical or group of chemicals. The observed toxicity of acute non-neutralized benzoic acids are greater than 2.0 indicating bioreactivity. When neutralized, the observed toxicity was below that predicted by the baseline QSAR. This is thought to be due to the formation of ionized form of the acid which effected bioavailability. No explanation could be found for explain some of the chronic non-neutralized benzoic acids having a toxicity below that predicted by the baseline QSAR.

The toxic response of the majority of industrial chemical which are non-reactive and non-ionic can be quantitatively modeled by the 1-octanol/water partition coefficient ($\log K_{ow}$). Despite the success in predicting narcotic effects of chemicals by using hydrophobicity, the observed toxicity of some chemicals is significantly greater than that predicted by $\log K_{ow}$. The toxicity of ionizable compounds does not model by the same QSAR (Schultz, 1987).

Toxic units of 0.18 and 0.05 were obtained in a theoretical calculation of the formation of chlorobenzoic acids resulting from degradation of Aroclor 1242 and

contaminated TVA soil. However, in this theoretical calculations the possible formation of other metabolites were not taken into account. The predominant mechanism of aerobic PCB biodegradation is via a 2,3-dioxygenase attack resulting mainly in the formation of the corresponding chlorobenzoic acid (Flanagan et al., 1993). Some *Pseudomonas* and *Alcaligenes* strains degrade most of the PCB congeners by the 2,3-dioxygenase pathway, but can degrade others, particularly those containing a 2,5-chlorobiphenyl ring, via a 3,4-dioxygenase attack (Bedard et al., 1987). These two strains can also generate ring-chlorinated acetophenones from attack on a 3-, 2,5-, or 2,4,5,-chlorobiphenyl ring (Bedard and Haberl, 1990; Robinson and Lenn, 1994). Flanagan and May (1993) found qualitative evidence for the presence of 2,3-dihydro-2,3-dihydroxy-2'chlorobiphenyl and 2,3-dihydroxy-2'chlorobiphenyl in sediment analysis. It has been observed that the hydroxylated metabolites of PCBs are several times more toxic than the parent compound (Sawhney and Hankin, 1985).

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PART IV

SUMMARY AND CONCLUSIONS

Surfactants solutions have been shown to be effective in desorbing hydrophobic organic pollutants from soil (Chu and Jafvert, 1994). In the particular process developed at CEB, the surfactant also had the additional benefit of serving as growth substrate for the genetically engineered strains. Approximately 50% of PCBs and 90% of surfactants were degraded in the bioremediation process and there was not an increase in the toxicity in the final degraded effluent. Although *Tetrahymena* and *Microtox*TM assays gave very consistent results. Nevertheless, the use of surfactants masked the toxicity of the system. A better analysis is required for surfactant analysis.

The relative toxicity of chlorobenzoic acids was studied because these are the major metabolites produced by aerobic PCB degradation and because the toxicity of the bioremediation process was masked by the surfactants. We found in this study that non-neutralized chlorobenzoic acids are more toxic than the neutralized ones and chlorine substituents at *meta* and *para* positions render benzoic acids more toxic than benzoic acids with chlorines at *ortho* position.

Using both log K_{ow} and pK_a as descriptors, substantially improved the models. Although these are not high quality descriptors, at present they are of sufficient quality to be used for predictive purposes.

By studying the relative toxicities of chlorobenzoic acids we were able to state that if the degradation of PCBs by the engineered strains IPL5::TnPCB and B30P4::TnPCB would result in the accumulation of chlorobenzoic acids, these would not be toxic to the *Tetrahymena*.

VITA

Maurizia Muccini was born in Sao Paulo - SP, Brazil on April 24, 1967. She attended elementary school in the Colegio Nossa Senhora Auxiliadora (Manaus-AM-Brazil) and graduated from Escola Tecnica Federal do Amazonas (Manaus-AM-Brazil) high school in December, 1983. The following February she entered the Universidade Federal do Para (Belem-PA-Brazil) and in December, 1988 received the degree of Industrial Chemist. The following May she entered the Universidad Simon Bolivar in Caracas, Venezuela and received her master degree of Food Chemistry in July, 1991. The following year she worked for Coca-Cola Company in Manaus as a Quality Assurance Chemist. On June, 1995 she entered the University of Tennessee, Knoxville and received her Master degree of Ecology in August, 1997.