

ISOLATION AND PARTIAL CHARACTERIZATION  
OF PCB AND PAH-DEGRADING BACTERIAL CONSORTIA  
FROM CONTAMINATED SITES IN STEPHENVILLE AND  
ARGENTIA, ISLAND OF NEWFOUNDLAND

CENTRE FOR NEWFOUNDLAND STUDIES

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DEBORAH V. SQUIRES-PARSONS







ISOLATION AND PARTIAL CHARACTERIZATION OF PCB AND PAH-  
DEGRADING BACTERIAL CONSORTIA FROM CONTAMINATED SITES IN  
STEPHENVILLE AND ARGENTIA, ISLAND OF NEWFOUNDLAND

BY

DEBORAH V. SQUIRES-PARSONS, B.Sc.

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## **Abstract**

Soil and sediment samples collected from PCB contaminated sites in Stephenville (soil) and Argentia (sediment) were used to isolate several microbial consortia capable of growth on biphenyl medium. These cultures were enriched by repeated transfer on biphenyl medium, and laboratory scale experiments were carried out to determine the ability of the Argentia consortia to degrade naphthalene, phenanthrene, phloroglucinol and toluene, as well as Aroclor 1254. The Stephenville cultures were also tested for the ability to degrade Aroclor 1254, using test tube, flask experiments (both soil-free and soil slurries) and bioreactor experiments.

Results show that all of the cultures tested grew on biphenyl medium and several of the cultures were able to also degrade PAHs and PCBs. Results of soil slurry experiments showed that the addition of enriched consortia plus biphenyl as cosubstrate stimulated biodegradation of Aroclor 1254. Growth on Aroclor 1254 was also shown in soil-free microcosms by an increase in optical density at 600 nm, as compared with controls. Dry weight of cells also increased when compared with controls. A sequential anaerobic/aerobic treatment regime was also found to be successful in degrading Aroclor 1254.

These results show that indigenous bacteria, enriched by growth on biphenyl medium, can be useful in treatment of PCB-contaminated soils and sediments.

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## List of Abbreviations and symbols

acidity	pH
Agency for Toxic Substances and Disease Registry	ATSDR
ammonium	NH <sub>4</sub>
Argentia Management Authority	AMA
Argentia Remediation Group	ARG
Argentia samples	AR
British Columbia	B.C.
biphenyl	Bp
benzene, toluene, ethylbenzene, xylene	BTEX
Canadian Environmental Protection Act	CEPA
carbon dioxide	CO <sub>2</sub>
chloride	Cl
copper	Cu
degree Celsius	°C
Department of Fisheries and Oceans	DFO
eosin-methylene blue agar	EMB agar
environmental site assessment	ESA
ferrous sulfate	FeSO <sub>4</sub>
gas chromatograph with electron capture detector	GC-ECD
gas chromatography - mass spectroscopy	GC-MS
gram(s)	g
Henry's Law constant	K <sub>h</sub>
Hewlett Packard	HP
high performance liquid chromatography	HPLC
hydrochloric acid	HCl
kilogram	kg
manganese	Mn
microgram	µg
microlitre	µL
milligram	mg
millilitre	mL
millimetre	mm
minimal salts agar	MSA
minimal salts medium	MSM
molybdenum	MO
methyl red/Voges-Proskauer tests	MR/VP
New Brunswick	N.B.

New Jersey	N.J.
optical density	OD
oxidation-fermentation test	OF test
parts per million	ppm
phloroglucinol	PG
polyaromatic hydrocarbon	PAH
polychlorinated biphenyl	PCB
Public Works and Government Services Canada	PWGSC
revolutions per minute	rpm
Royal Military College	RMC
Science Applications International Corporation	SAIC
sodium chloride	NaCl
Stephenville samples	SV
two-phase partitioning bioreactor	TPPB
trypticase soy agar	TSA
University of British Columbia	UBC
United States of America	U.S.A.
water	H <sub>2</sub> O

## **Chapter 1**

### **Introduction**

#### **1.1. Polyaromatic compounds in soils and sediments**

Polycyclic Aromatic Hydrocarbons, or PAHs, are organic compounds which contain two or more fused benzene (aromatic) rings, hence the term polyaromatics. These organic chemicals are produced by a wide range of human activity, such as industrial pollution, chemical spillage from manufacturing plants, and incomplete combustion of fossil fuels, wood and charcoal (Young and Cerniglia, 1995). Many industrial processes produce large amounts of PAHs synthetically, such as the petrochemical industry, which produces gasolines and other petroleum products. PAHs are also found in coal tars, wood treating chemicals (such as creosote) and refinery wastes (Baker and Herson, 1994; Norris *et al.*, 1994).

PAHs are ubiquitous in the environment, mainly as a result of the production of petrochemicals. The combustion of fossil fuels can make many of these compounds airborne, which can transport them miles away from their source. Compounds which are more hydrophobic, once deposited in the soil via precipitation, tend to become adsorbed to soil particles (Gibson, 1984). Other compounds are more volatile, and are transported through the atmosphere in the gaseous phase. The major sources of PAHs in Canada are aluminum smelters and residential wood stoves. Natural sources, such as forest fires, can

contribute up to 2,000 tonnes of PAHs to the environment each year.

Some eukaryotic microbes use mono-oxygenase enzymes to degrade aromatic hydrocarbons, and this leads to carcinogenic intermediates. This pathway is also used by mammals in detoxifying these compounds. (Ribbons and Eaton, 1982). PAHs have been declared toxic under CEPA (the Canadian Environmental Protection Act, 1999) ([www.ec.gc.ca/pdb/npri](http://www.ec.gc.ca/pdb/npri)). As a result, much attention has been placed on reducing the amounts of these compounds in the environment, as well as minimizing the effects of human exposure.

The polyaromatic hydrocarbons selected for use in this study are shown in figure 1.1. The following sections briefly describe the chemical characteristics for each, as well as their incidence and toxicity to humans and animals.

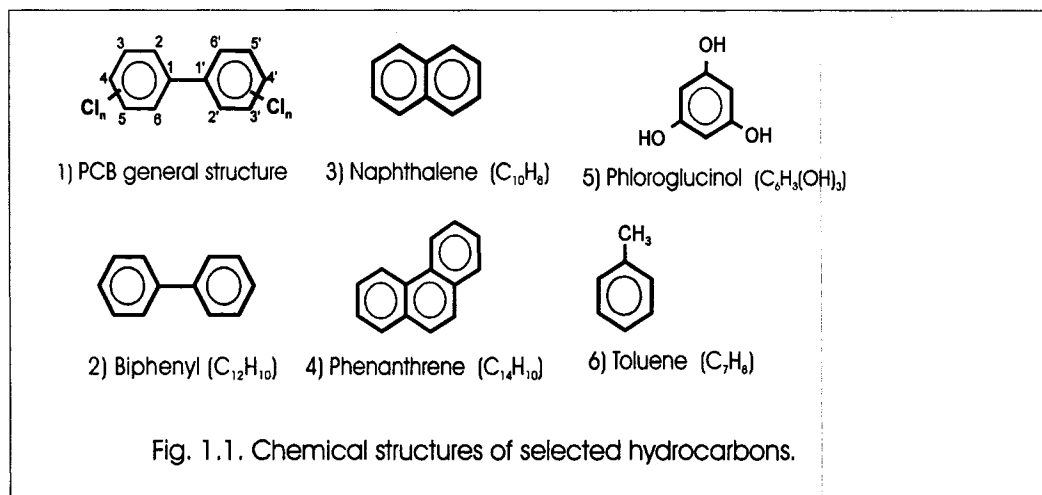
#### **1.1.1: Toluene in soils and sediments**

Toluene (  $C_7H_8$  ) is a clear, colourless liquid at room temperature. It is highly volatile and evaporates easily. It occurs naturally in crude oil and is found in the tolu tree. Also it is produced commercially and used as an industrial solvent in the production of paints, paint thinners, lacquers, adhesives, rubber and nail polish. Toluene is also used in the production of gasoline and other fuels, and the making of coke from coal (Smith, 1990; [www.atsdr.cdc.gov/toxprofiles](http://www.atsdr.cdc.gov/toxprofiles) ). It has been classified as a priority pollutant due to its carcinogenic effects as well as depressing the nervous system

(Coschigiano and

Young, 1997).

Toluene is also highly soluble in water, so can enter soils and sediments via accidental spills, or leakage, from gasoline storage tanks. Gasoline spills and petrochemical sludge from industrial plants are the primary sources of environmental toluene in soils and sediments (Gibson, 1984; Young and Cerniglia, 1995). Because of its high volatility, toluene can rapidly become airborne and deposited elsewhere, hence the ubiquitous distribution in soils and sediments.



### **1.1.2: Phloroglucinol in soils and sediments**

Phloroglucinol ( $C_6H_3(OH)_3$ ) exists as a naturally occurring compound within several plant polymers such as flavones, anthocyanins and catechins (Armstrong and Patel, 1992). It consists of a benzene ring with hydroxyl groups substituted at positions 1, 3 and 5 on the benzene ring, so is often called 1,3,5 trihydroxybenzene.

Phloroglucinol is released into soils and sediments via the decomposition of plant material (Patel *et al.*, 1981; Armstrong and Patel, 1994). It is manufactured for research purposes only and occurs as an off-white powder with a faint odour.

### **1.1.3: Naphthalene in soils and sediments**

Naphthalene ( $C_{10}H_8$ ) is a dicyclic aromatic hydrocarbon, consisting of two fused benzene rings. It is a white, crystalline solid at room temperature. It is highly volatile and is commonly used in the production of moth repellants, such as mothballs and moth flakes (Young and Cerniglia, 1995). It is also a common component in wood preservatives, such as creosote, so can enter soils and sediments through leakage or accidental spills at wood treatment sites, or from discarding moth control products in municipal dumps (Baker and Herson, 1994; Young and Cerniglia, 1995). This compound is relatively insoluble in water, so tends to adhere to soil particles, rather than dissolve in groundwater (Leisinger *et al.*, 1981).

Human exposure to naphthalene occurs via inhalation of tobacco smoke, exhaust

from burning wood or fossil fuels, and exposure to mothballs and moth flakes. Exposure to large amounts of naphthalene can cause hemolytic anemia (the destruction of red blood cells) leading to fatigue, pale skin and lack of appetite ([www.atsdr.cdc.gov](http://www.atsdr.cdc.gov)). However, there is no direct evidence that naphthalene exposure leads to cancer in humans.

#### **1.1.4: Phenanthrene in soils and sediments**

Phenanthrene ( $C_{14}H_{10}$ ) is a tricyclic compound, consisting of three fused benzene rings with no added substituents. Due to the three fused rings, it is far less volatile than toluene or naphthalene. It is relatively insoluble in water, so tends to bind to soil particles in the environment. It is a major component of creosote, a wood preservative, and is also found in smaller amounts in crude oils and petroleum products (Baker and Herson, 1994). It is an off-white, fine crystalline powder with a faint odour at room temperature.

#### **1.1.5: Biphenyl in soils and sediments**

Biphenyl ( $C_{12}H_{10}$ ) is an aromatic compound consisting of two benzene rings which are joined at a C-C bond, thus are not fused. Biphenyls can occur in soils and sediments via dechlorination of lower molecular weight PCBs, as a component of petrochemical sludge, and via industrial spillage or dumping of used chemicals (Baker and Herson, 1994; Gibson, 1984). Biphenyl is a white, crystalline powder at room

temperature, with a mild chemical odour. It is relatively insoluble in water, so tends to adsorb to soil particles. This is the parent molecule for PCBs, which are produced by direct chlorination of biphenyl.

#### **1.1.6: PCBs in soils and sediments**

Polychlorinated biphenyls, or PCBs, are a class of aromatic compounds consisting of two benzene rings, joined by a carbon-carbon bond, which have one or more chlorine atoms as added substituents. They are produced by direct chlorination of biphenyl and are named based on the percentage of chlorine in the mixture. For example, Aroclor 1254, which is used in this study, has 54% chlorine. The number 12 refers to the number of carbon atoms in the two benzene rings of the parent compound (biphenyl). PCBs can be lightly or heavily chlorinated. Direct chlorination of biphenyl leads to a mixture of different congeners, each containing from 1-10 chlorine atoms per molecule. Mono- and dichlorobiphenyls occur as an off-white powder, while the heavier, highly chlorinated mixtures are oily in texture. This oily texture leads to adsorption of the PCBs to soil particles, increasing the difficulty in remediating contaminated soils and sediments.

PCBs are ubiquitous in the environment. They occur in soils and sediments as a result of improper disposal of used electrical equipment, such as transformers and capacitors, and due to incineration of PCB-laden materials. Due to their hydrophobic nature, PCBs usually adsorb strongly to soil particles (Baker and Herson, 1994; Young



and Cerniglia, 1995). PCBs released into the environment can have one of several fates. They can be weathered, over time, in exposed areas, causing changes in the composition of PCB mixtures in the environment. PCBs can become mobile via adsorption to particles which become airborne, such as soot, or via water droplets containing dirt particles coated with PCBs and thus can be transported long distances from their origin (Baker and Herson, 1994).

PCBs in soils and sediments tend to bioaccumulate in the food chain. PCBs have been detected in organisms ranging from bacteria and fungi to fish, mammals and humans. As it moves up the food chain, bioaccumulation may lead to toxicity in the affected organism. Coplanar PCBs are considered the most toxic, having effects similar to dioxins and furans (de Voogt *et al.*, 1993). PCBs are also lipophilic, and as such tend to accumulate in fatty tissues. Finley *et al.* (1997) showed elevated levels of Aroclors 1248, 1254, and 1260 concentrations in muscle and hepatopancreas of striped bass and blue crab sampled from the Passaic River, New Jersey. Surface sediments were contaminated with all three Aroclors.

Winter flounder (*Pleuronectes americanus*), living near a contaminated site in Argentea, Newfoundland and Labrador, have been found to suffer from stress and increased parasitism as a result of exposure to PCBs in the sediment (Khan, 1999). Leonards *et al.* (1998) showed bioaccumulation of PCBs in four species of mustelids (weasel, stoat, polecat and otter) in the Netherlands. The otter was the most sensitive,

because of its diet, that consists largely of fish (ibid). Watanabe *et al.* (1999) showed that PCBs are accumulated in the blubber of Caspian seals (*Phoca caspica*).

Human exposure to PCBs occurs via inhalation of particles containing PCBs, ingestion of soil particles in partially washed vegetables grown in contaminated soil, and by consumption of contaminated fish or shellfish. The effects of exposure to PCBs in human adults include fatigue, acne, swelling of arms and legs, and increased incidence of cancer (Danse *et al.*, 1997). Guo *et al.* (1995) showed that children exposed *in utero* and early after birth to PCBs and dibenzofurans displayed physical deformities and mental retardation. Due to their lipophilic nature, PCBs can be passed from mother to child via human breast milk, thus posing a great risk to developing fetuses. Hack and Selenka (1996) used a digestive tract model to show mobilization of PAHs and PCBs via ingestion. They found that the addition of lyophilized milk doubled the fraction of PAH and PCB mobilized. These results show that intake of contaminated food is directly related to PCB exposure and subsequent bioaccumulation.

## **1.2: Microbial degradation of hydrocarbons in soil**

### **1.2.1: Factors influencing degradation of polyaromatic hydrocarbons in soils and sediments**

The degradation of hydrocarbons in soil is dependant on a number of factors, such as (1) temperature of optimal enzyme activity, (2) pH of the soil matrix, (3) nutrient

composition of the soil, (4) moisture content of the soil, (5) bioavailability of the substrate to be degraded, and (6) the mixture of contaminants present and their concentration in the soil. PAHs occur in soils and sediments as complex mixtures of many different compounds (Baker and Herson, 1994).

The temperature of optimal enzyme activity differs for each bacterial species and is directly related to the temperature of its natural environment. It was predicted that the samples collected from Argentia and Stephenville, Newfoundland and Labrador would be psychrotolerant - that is, they would be able to live and grow at low temperatures, but also at room temperature (22-28°C). The consortia used in these experiments were isolated at room temperature, and degradation experiments were also carried out at this temperature. The rationale for this is that enzyme activity doubles with each ten degree rise in temperature. Since the compounds being tested are considered, for the most part, recalcitrant, or difficult to degrade, the warmer temperature may induce a faster rate of degradation than incubation at lower temperatures.

As temperature decreases, enzymes display a decreased catalytic rate due to a reduction in structural flexibility, eventually causing cold denaturation. The rigid secondary structure and disulfide bridges, present in enzymes of psychrotrophic organisms, are absent in those of psychrophiles, thus making psychrophilic enzymes more thermolabile (Nichols *et al.*, 1999). Morita (1975) used the term psychrotrophic for cold-tolerant microorganisms previously referred to as facultative psychrophiles, the

maximum temperature for growth being above 25°C. Psychrotrophs are more abundant than psychrophiles at low temperatures and are numerous in even permanently cold environments. They are also more adapted to changes in temperatures than psychrophiles and are more resistant to environmental fluctuations in oxygen and nutrient availability, which makes them useful in biotechnological applications (Gounot, 1991; Mohn *et al.*, 1997). Master and Mohn (1998) showed that PCB degradation rates were higher in cultures incubated at 20°C than at lower temperatures, even in samples collected from Arctic and subarctic sites. Due to the recalcitrant nature of some PAHs and PCBs, any factor, such as increased temperature that can increase the rate of degradation, would be beneficial to the treatment of contaminated sites.

The pH of the soil also plays a role in degradation of organic pollutants in the matrix. If the pH of the soil becomes too acidic, because of the buildup of intermediates of hydrocarbon metabolism, the bacteria in the soil may not be able to function as effectively in remediating the selected compounds. Research has shown that the rate of biodegradation of PCBs and PAHs in soil is higher at neutral pH than in acidic conditions (Baker and Herson, 1994). The nutrient composition of the soil is also important in providing a basic carbon source to allow the proliferation of hydrocarbon-degrading microbes. Psychrotrophic organisms tend to be zymogenous, moving into and colonizing a contaminated area to exploit the carbon source. Once the population has increased, the presence of the hydrocarbons could then stimulate the enzyme systems necessary to

degrade the compound in question. Co-metabolites are often added under laboratory conditions to stimulate growth of the desired microorganisms. During the research carried out in this study, biphenyl was added as cometabolite in some of the PCB-degradation experiments to stimulate the bacterial consortia to produce the enzymes necessary to degrade the PCBs. This is a very useful cometabolite for use in the lab but can be toxic to living systems. In the environment, nutrients can be added to the soil (a process called biostimulation) to help the degradative process. Master and Mohn (1998) demonstrated that the addition of linoleic acid or pyruvate to the culture medium stimulated an increased percentage removal of PCBs in laboratory microcosms.

The moisture content of the soil is also a major factor affecting the rate of biodegradation. If the soil is too dry, the bacteria cannot travel easily to the source of the pollution to effect degradation (Baker and Herson, 1994). Instead, they become localized, or stuck to soil particles, which slows down the rate of degradation. The use of soil slurries in the laboratory allows for maximum exposure of the microbes to the compounds of interest. Bioavailability of the compounds in the soil to the degraders is one of the most confounding factors to environmental biologists and engineers. Because the higher molecular weight compounds are extremely hydrophobic, and are often tightly bound to soil particles, conditions such as low water content in the soil reduces the bioavailability of the substrate. Some bacterial species produce biosurfactants, or emulsifying agents, which can render the compounds more soluble and thus increase their

rate of degradation (Alexander, 1994; Baker and Herson, 1994). Surfactants have also been used in degradation experiments in laboratory microcosms and were found to aid the degradation process (Baker and Herson, 1994). Triton X-100, a nonionic surfactant, is also used in extraction of PCBs for GC analysis. The addition of this surfactant increases the extraction efficiency for high molecular weight PCBs (Bedard *et al.*, 1986).

The mixture of contaminants present in the soil also affects the rate of biodegradation. Complex mixtures, such as pesticides, BTEX compounds, creosote and coal tar are much more recalcitrant to degradation than a soil contaminated with a single type of hydrocarbon. Some compounds may be too concentrated, making it toxic for the naturally occurring biota. Lower molecular weight compounds are more easily degraded than those of high molecular weight. Also, the number of substituents appended to the ring(s), especially highly halogenated compounds (such as PCBs) are more resistant to biodegradation (Alexander, 1994; Baker and Herson, 1994; Gibson, 1984). Thus, it is important for the researcher to know the chemical composition of the soil before attempting any remediation strategy.

### **1.2.2: Microbial degradation of polyaromatic hydrocarbons in soils and sediments**

Microbial degradation of polyaromatic compounds has been shown to occur under laboratory conditions as well as *in situ* (Leisinger *et al.*, 1981; Young and Cerniglia,

1995). Compounds with fused rings, such as naphthalene and phenanthrene, are more difficult to degrade than toluene which is composed of a single benzene ring with an added methyl group. Toluene is also much more volatile than naphthalene or phenanthrene. Volatility is measured by Henry's Law constant, or  $K_H$ . The higher the value, the higher the volatility of the compound. Henry's Law constant is given by the following equation:

$$K_H = p/c$$

Where  $p$  = partial pressure of the gas, and  $c$  = molar concentration. Toluene is the most volatile of the compounds used in this study, with a Henry's Law constant of  $6.3 \times 10^{-3}$ . Naphthalene is next, with a value of  $4.1 \times 10^{-4}$ , and phenanthrene is least volatile, with a Henry's Law constant of  $2.5 \times 10^{-5}$  (Norris *et al.*, 1994). Di- and tricyclic aromatic hydrocarbons are extremely insoluble, and tend to adhere to soil particles, making them less available for degradation (Leisinger *et al.*, 1981). Increasing the number of aromatic rings also makes them more difficult to degrade, thus naphthalene is easier to degrade than phenanthrene (Baker and Herson, 1994, Leisinger *et al.*, 1981).

Cometabolism also plays an important role in bioremediation of higher molecular weight compounds. Some of the bacteria will utilize one intermediate compound, but not others in the degradative pathway. Enrichment of an indigenous culture by growth on a similar parent molecule also has the potential to increase the rate of degradation of that compound (Alexander, 1994; Young and Cerniglia, 1995). Benzene is the parent

molecule from which all aromatic molecules arise. Important intermediates of benzene metabolism are catechols, which are bright yellow compounds consisting of two hydroxyl groups on the benzene ring. These chemicals and their derivatives can be used to make synthetic flavours, such as vanillin (Smith, 1990).

### **1.2.3: Microbial degradation of PCBs and biphenyls in soils and sediments**

Biodegradation of biphenyl, the parent molecule of PCBs, is relatively easy to accomplish because of the lack of added substituents. Bacteria from sediments in the Hudson River have been shown to degrade mono- and dichlorobiphenyls (Harkness *et al.*, 1993). The more chlorine atoms added to the benzene rings in the nucleus, the lower the rate of degradation, with the pentachlorobiphenyls, the major component of Aroclor 1254, and hexachlorobiphenyls, the major component of Aroclor 1260, being more resistant to degradation (Furukawa, 1982). Coplanar PCBs are the most resistant to degradation because the folding of the molecule leads to steric hindrance, preventing the degradative enzymes from reaching the appropriate binding sites (de Voogt *et al.*, 1993). Thus, degradation of higher molecular weight Aroclors requires a sequential anaerobic/aerobic treatment (Baker and Herson, 1994; Young and Cerniglia, 1995). The anaerobic treatment allows for reductive dechlorination, followed by aerobic degradation of the low molecular weight PCB or biphenyl molecule.



Reductive dechlorination by methanogenic bacteria in soils and sediments produces a wide range of mono-, di- and trichlorinated PCBs which can easily be degraded by oxidative bacteria (Brown *et al.*, 1987). The predominant pathway is to cleave the parent molecule (benzene ring) using a 2,3 dioxygenase. This produces chlorobenzoic acid, which can then be degraded by other aerobic bacteria in the consortium (Harkness *et al.*, 1993). Catechols are often produced as central intermediates in PCB degradation. These are brightly coloured compounds (yellow or brown) which are common products from ring fission via ortho or meta ring cleavage (Leisinger *et al.*, 1981). The ultimate goal of biodegradation is to achieve total mineralization of the PAH or PCB. This can be achieved when utilizing a bacterial consortium which produces intermediates that can be fed into the Krebs's Cycle, then totally oxidized to CO<sub>2</sub> and H<sub>2</sub>O.

One of the limiting factors in the bioremediation of PCBs in soils and sediments is the availability of indigenous microbes to do the work. If the numbers are low, added nutrients can induce biodegradation. Bioaugmentation, achieved by addition of enriched consortia, can raise the bacterial numbers to a level which could increase the rate of degradation. Bioavailability of the substrate can also be a limiting factor. As PCBs are extremely hydrophobic they tend to become tightly bound to soil particles. Addition of a surfactant can make the PCBs more soluble and thus bioavailable to the native consortia (Norris *et al.*, 1994; Young and Cerniglia, 1995). Some bacteria produce their own

biosurfactants which act in the same manner and allow *in situ* bioremediation to proceed (Baker and Herson, 1994).

### **1.3: Research objectives**

Because of the persistent nature of PAHs and particularly PCBs in the environment and their resulting toxicity to animals and humans, it is important to find ways to reduce the concentrations of these compounds in the environment (Danse *et al.*, 1997, de Voogt *et al.*, 1993). The most cost-effective means of treating an area contaminated with these compounds is via bioremediation (Young and Cerniglia, 1995). Other methods, such as incineration, involve costly transport of contaminated materials to the incinerator with additional costs involved in the incineration process (Danse *et al.*, 1997). Microbes that are capable of degrading PAHs and PCBs are ubiquitous in the environment (Abramowicz, 1990; 1994). It is important to determine the range of specificity of the native consortia for the contaminants in question.

To that end, the objectives of this research are:

- (1) to isolate bacterial consortia from two PCB- and PAH-contaminated sites in Newfoundland and Labrador (Argentia and Stephenville) and to enrich these cultures by growth on biphenyl as sole carbon and energy source;
- (2) to determine the ability of these enriched cultures to degrade toluene,

phloroglucinol, naphthalene, phenanthrene, and Aroclor 1254;

- (3) to use these consortia in laboratory experiments to determine their ability to enhance biodegradation of PCBs by use of bioaugmentation. This was done by incubating the enriched consortia with soil slurries containing PCB mixtures , or by inoculating the enriched consortia into soil-free microcosms spiked with Aroclor 1254;
- (4) to investigate the effect of biostimulation in enhancing the rate of PCB degradation in soil slurry experiments. This was done by adding a cosubstrate (biphenyl) to soil slurries containing Aroclor 1254 and enriched consortia;
- (5) to examine the potential of using a sequential anaerobic/aerobic route for PCB degradation. The anaerobic portion involved reductive dechlorination, done using bioreactors, with subsequent aerobic treatment to further degrade the dechlorinated mixture;
- (6) to investigate the use of a Two-Phase Partitioning bioreactor to degrade PCBs by native consortia, and

- (7) to partially characterize the bacteria found to have the ability to degrade PCBs and PAHs.

Because native consortia are already adapted to local conditions, it was decided early on in this research to study mixed populations of indigenous bacteria. It has been shown in previous studies that microbes capable of biodegradation of a given hydrocarbon are most readily found in sites already contaminated with a given pollutant (Whyte *et al.*, 1996). In addition, the recalcitrance of the compounds, as well as the complex pathways required to achieve complete mineralization, would almost certainly require the efforts of several different species of bacteria, each able to degrade a specific intermediate in the degradation pathway (Young and Cerniglia, 1995).

#### **1.4: Sampling sites**

##### **1.4.1: Argentia site description**

The town of Argentia is located on the western side of the Avalon peninsula, in Placentia Bay on the island of Newfoundland, province of Newfoundland and Labrador, Canada. It is the site of a former U.S. air and naval base, which was active in the Second World War and thereafter used as a base for search and rescue operations. The base consisted of a northside, which contained the airstrip, maintenance hangars and supply shops; and a southside which contained all housing, a shopping centre, school, library, garages and warehouses. During the Second World War, Argentia played a vital role in

the protection of convoys crossing the Atlantic with troops and supplies to Britain. It served as a terminal for allied warships, cargo ships and tankers. Because fuels and supplies were distributed via this site, accidental fuel spills were common. Nearby landfill sites were also used to dump used transformers, capacitors, and other PCB-laden equipment.

The U.S. airbase officially closed in 1973, and the Canadian government took control of the northside of the base in 1981. The U.S. navy relinquished its remaining buildings on the southside in 1994. Since that time, the Argentinia Management Authority (AMA) was set up to remediate the site and sell all marketable structures and assets from the former base. The Department of Public Works and Government Services Canada (PWGSC) is the current landlord of the site, and is investigating remedial options for contaminated soil, groundwater, and sediment. Preliminary assessment of the site, from a remedial perspective, began with a Phase II environmental assessment carried out in 1994, in which highly contaminated sites targeted for remediation were identified. A Phase III/IV Environmental Site Assessment was carried out in 1995 by the Argentinia Remediation Group (ARG), which was contracted by PWGSC to determine the extent of pollution at each site. The ARG found extensive contamination by fuels, heavy metals and PCBs, which affected groundwater, soils and sediments. The report was released in October, 1995.

#### **1.4.2: Stephenville site description**

The Pinetree Radar site is a former U.S. Military base located at an elevation of 350 metres on Table Mountain in Western Newfoundland, province of Newfoundland and Labrador, Canada. It is located 20 km northwest of the town of Stephenville, so the samples collected and used in this study were designated SV. The Pinetree site was used to monitor air traffic during the war, serving as an early warning against enemy aircraft entering British/Canadian airspace.

Upon closure of the site following the Second World War, ownership of the site was transferred back to Canada, and between 1993-1997 the Department of Public Works and Government Canada (hereafter designated as PWGSC) carried out an Environmental Site Assessment (ESA) where three areas were found to contain high levels of PCB contamination. These included an abandoned dump site, containing used transformers and other electrical equipment, an adjacent swampy bog and parts of the site sewage system. All impacted soil and debris was transferred to a containment cell lined with an 80 mil geomembrane. The cell was filled in two phases; Area 1 in 1998 and Area 2 in 1999. PCB contaminated water was treated with activated carbon and the used carbon was also placed in the containment cell. Once the material, approximately 1000 m<sup>2</sup>, was in place, the containment cell was sealed by closing the geomembrane and covering it with soil. Monitoring wells were drilled around the site to monitor PCB levels in the surrounding groundwater, and soil samples were taken for analysis. (PWGSC project

summary, 1998).

Since then, PWGSC, in conjunction with Environment Canada, has been looking into remedial options for the site. To this end, Science Applications International Corporation (hereafter designated SAIC Canada) was contracted to investigate bioremediation and other modalities for treatment of the impacted soil in the containment cell. SAIC Canada contacted MUN in July of 2000 to undertake research into aerobic degradation of biphenyl and PCBs using contaminated soil samples collected from the containment cell.

## **Chapter 2: Materials and methods**

### **2.1: Collection, isolation and characterization of the microbial consortia**

#### **2.1.1 Sample collection : Argentina**

A total of twelve sediment samples were collected in January 2000 around the periphery of Shag Pond, located on the south side of the base (Figure 2.1) and were designated as AR1 to AR12. Samples were collected using a grab sampler, collecting the top 10 cm of sediment. Samples were then refrigerated, and sent to a local company (Servco) for analysis of hydrocarbon contamination levels. The samples were found to be contaminated with a wide range of PAHs and PCBs. The predominant Aroclor present in these samples was Aroclor 1260, which has a high concentration of hexachlorobiphenyls. The source of the PCBs can be traced to an adjacent transformer shop and landfill site, where used transformers were dumped (Glen Troke - PWGSC, Argentina, personal communication). The samples were stored at 5°C until transferred to MUN in March of 2000. The samples contained a mixture of fine sediment, small rocks, and liquid. All sediment samples were grey-black in colour with no discernible odour.



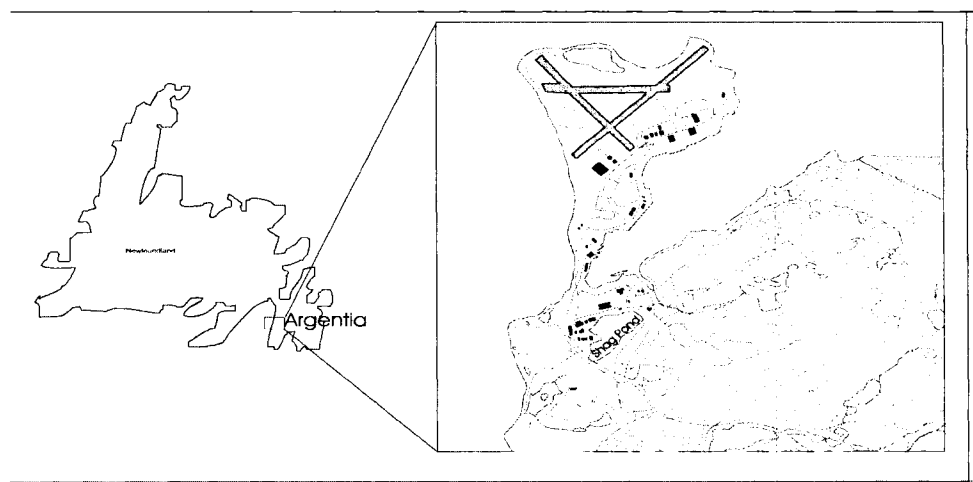


Fig. 2.1. Map of Newfoundland with reference to the Argentia site.

### **2.1.2: Sample collection - Stephenville**

A total of ten soil samples were collected by Jacques Whitford Environmental Services from the Pine Tree site on Table Mountain (Fig. 2.2). The sampling involved digging through the surface soil covering the containment cell, then opening the geomembrane to remove soil samples. Samples were taken from three different locations within the containment cell. From each location, they attempted to remove four samples from different depths. The deepest samples were taken at three feet below the surface. This was only possible for location number two. Each of the other locations, one and three, were immersed in groundwater at that depth, so soil samples could not be obtained below two feet.

Samples were collected with stainless steel trowels disinfected with 95% ethanol, and placed in sterile glass bottles for transport. Samples were shipped on ice to MUN for culture isolation. Soil samples mostly consisted of fine grained particles, grey-black in colour with a moderately strong hydrocarbon odour. The samples taken > 1 foot below the surface had a higher water content and resembled sediment. They had a strong odour of hydrocarbons. Soil analysis by SAIC showed that the soil contained high levels of PCBs, primarily Aroclors 1254 and 1260, as well as numerous other hydrocarbons, such as toluene.

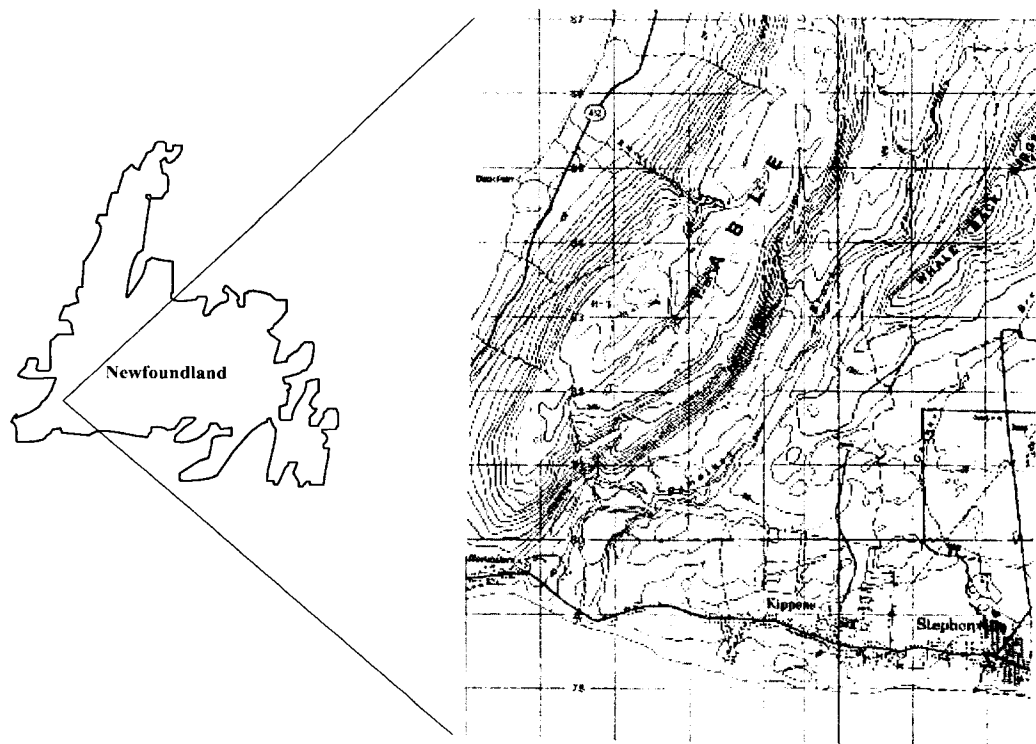


Fig.2.2. Map of Newfoundland with reference to the Stephenville site.

### **2.1.3: Source of the chemicals used in this study**

Chemicals used to make Minimal Salts Medium (MSM) and Trace Elements Stock solutions were obtained from Fisher Scientific or Sigma Aldrich Canada. Toluene, phloroglucinol, naphthalene, phenanthrene and biphenyl were obtained from Fisher Scientific. Aroclors 1254 (neat, or undiluted) and 1254 (200 µg/ml in methanol) were obtained from Supelco Canada. Acetone was used to dilute the Aroclor 1254 (neat), which is extremely hydrophobic. A concentrated solution of Aroclor 1254 (50 mg/mL) was used in the test tube and soil slurry experiments. This solution was stored at 5°C in a tightly capped, brown glass vial to minimize volatilization of the solvent between experiments.

### **2.1.4: Minimal salts medium**

The medium used to isolate the consortia from both sites was Minimal Salts Medium (MSM), as described by Armstrong and Patel, 1992. It contained (g/L); 0.5 g  $(\text{NH}_4)_2\text{SO}_4$ , 0.1 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , and 6.8 g  $\text{KH}_2\text{PO}_4$  in 1L distilled water. This mixture was autoclaved, then 0.1 mL of trace elements solution and 0.1 mL of yeast extract (0.001% yeast extract) were added. The trace elements solution contained (per 100 mL); 0.002 g  $(\text{NH}_4)_6\text{MO}_7\text{O}_{24}$ , 0.002 g  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.013 g  $\text{MnCl}_2 \cdot \text{H}_2\text{O}$ , 0.012 g  $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ , and 0.05 mL concentrated HCl. The trace elements and yeast extract solution were filter-sterilized before addition to the cooled MSM. If a solid medium was required (MSA), 20 g of agar per litre was added to the MSM base medium before autoclaving.

#### **2.1.5: Enrichment of the cultures using biphenyl**

For each set of samples, 1 g of soil or sediment was added to 50 mL of MSM, along with 0.5 g of crystallized biphenyl, in 125 mL screw- or metal-capped Ehrlenmeyer flasks. The flasks were placed on a gyrotary shaker at 150 rpm, and incubated at 22°C for 28-30 days. Growth was measured by an increase in turbidity of the medium, with a colour change from clear to yellow or brown. Cultures were transferred to fresh MSM with biphenyl once a month, using 5 mL aliquots of culture, 50 mL MSM, and 0.5 g biphenyl per flask. After several transfers, sediment-free cultures were available for testing on Aroclors and other hydrocarbons of interest in this study. Flask cultures were also streaked onto solid MSM in petri plates, with biphenyl crystals placed on a filter paper in the lid to serve as the sole carbon source.

#### **2.1.6: Tests used in bacterial characterization**

Initial observations of bacteria in the Argentia sediment samples were made by recording colony characteristics using dilution and spread-plating technique. One gram of sediment was added to 99 mL of physiological saline, shaken, then 0.1 mL spread-plated in triplicate on Typticase Soy Agar (TSA). The plates were incubated, inverted, at 22°C and were checked at 24 and 48 hrs for growth of different colony types. A total of eighteen isolates were obtained by visual observation of differences in colony characteristics. These isolates were collected by picking well-isolated colonies from the

plates and streak-plating onto fresh TSA until a pure culture of each was obtained. These isolates were further characterized by use of standard microbiological and biochemical tests, such as those for Gram reaction, shape, motility, OF- test (glucose), urease, caseinase, lipase, hemolysis on blood agar, citrate utilization, acid and/or gas production from maltose, lactose, glucose and sucrose (using phenol red as indicator), cytochrome oxidase, the MR/VP test, catalase, nitrate reductase, growth in thioglycollate medium (to check oxygen requirements) and growth on eosin-methylene blue (EMB) agar (Morris *et al.*, 2000). In a similar fashion, isolates were obtained in the Stephenville samples. However, time did not permit a more thorough examination of their biochemical characteristics.

Following the Argentia experiments on growth of various consortia on naphthalene, phenanthrene, phloroglucinol, biphenyl and toluene, those plates showing growth had a portion of the growth scraped off and streak-plated onto TSA for colony isolation. Each pure isolate was then Gram stained to determine Gram reaction, shape and arrangement. Most of the isolates were also tested for motility and oxygen requirements (using OF medium with glucose). These isolates were then stored frozen in glycerol ( 0.15 mL glycerol + 0.85 mL culture in 1.5 mL Eppendorf tubes).

The Stephenville samples were primarily used for PCB degradation experiments, so characterization of the bacteria was limited to describing the consortia SV2-4 and SV3-2, which showed the most promise in degradation of Aroclor 1254. These consortia were

streak-plated from MSM/biphenyl medium onto TSA for separation of colony types, then well isolated colonies were picked off and streaked onto fresh TSA until pure colonies were obtained. The SV3-2 and SV2-4 consortia were separated into different isolates, using differences in colony morphology, Gram stain, shape, and cell size. The SV2-4 consortium isolates were also tested for growth on MacConkey agar.

## **2.2: Bacterial degradation of the hydrocarbons**

### **2.2.1: PAH biodegradation experiments**

For both the Argentina and Stephenville samples, initial cultures were obtained by first enriching the consortia by growth on biphenyl as sole carbon source. This was achieved by adding one gram of soil or sediment to 50 mL of MSM, and adding 0.5 g of crystallized biphenyl in 125 mL Erlenmeyer flasks. Once growth on biphenyl was established, growth being determined by an increase in turbidity and a colour change of the medium to yellow, these cultures were used to streak onto MSA (MSM to which agar is added - see recipe in section 2.1.3 above) for use in degradation experiments.

Naphthalene, phenanthrene, and phloroglucinol were provided to the bacteria by placing 0.5 g of the compound onto Whatman #9 filter paper and placing the filter paper with the growth substrate on the inverted lid of the inoculated petri plate. These plates were incubated at 28°C for two weeks and checked for growth daily. Growth was confirmed by (1) physical growth of visible colonies on the plates and (2) a colour change

of the bacterial growth or the surrounding medium, usually yellow or brown, indicating the production of coloured intermediates of metabolism.

Because of its high chemical reactivity and volatility, growth on toluene was tested using sterilized glass petri plates containing MSA. Toluene is a liquid at room temperature, so 0.5 mL of toluene was pipetted into a glass Durham tube and stoppered with sterile cotton wool to slow down the rate of evaporation and allow exposure of the compound to the inoculated bacteria on the plate. The filled Durham tube was placed in the inverted lid of each petri plate. These plates were prepared in duplicate for each sample tested. The plates were incubated at 28°C in a chemical fume hood for 30 days, with observations for growth daily. Toluene was replaced if the contents of the Durham tube evaporated to dryness. Again, growth was confirmed by the appearance of yellow or brown colonies on the MSA.

#### **2.2.2: PCB degradation: Two-phase partitioning bioreactor**

##### **(TPPB) experiments**

The two-phase partitioning bioreactor (TPPB) method was used in a series of experiments set up by myself and SAIC staff at SAIC labs, Ottawa, February 26-March 3, 2001. Four bioreactors were set up to test the ability of the SV 3-2 consortium, previously enriched by repeated transfer on biphenyl medium, to degrade Aroclor 1254. The two phases were created by mixing the Aroclor 1254 in dodecane, which is not miscible with



water. Thus, the bacterial consortium within the growth medium was separated from the contaminant. In this way, the level of contamination was not a factor, as it allows for slow diffusion of the contaminant into the aqueous phase.

Reactor A consisted of a two litre fermenter with sampling ports (Bioflo, N.B. Scientific, Edison, N.J). It contained one litre of modified MSM (recipe for MSM was doubled with 1 g tryptone and 1 g benzoic acid added), 30 mL bacterial culture and 200 mL Aroclor 1254 in dodecane (100 ppm). Reactor A was run anaerobically, using nitrogen gas, and the others aerobically. All air/gas hoses contained Nalgene air filters. Reactors B, C and D were constructed using one litre Mason jars, with two holes in the lid. One was for sampling and the other for an aeration tube with an aquarium stone. All reactors and media used in these experiments were autoclaved before use. Reactors B and D contained 500 mL modified MSM with benzoic acid, as described above, 30 mL culture, except 20 mL for reactor D, and 100 mL Aroclor 1254 in dodecane (100 ppm). Reactor C contained 500 mL modified MSM with biphenyl as cometabolite (1 g added), 30 mL culture, and 100 mL Aroclor 1254 in dodecane (100 ppm).

All reactors were agitated to allow mixing of the culture with the contaminant phase. Reactor A had a built-in agitator, while reactors B, C and D were placed on magnetic stirrers, with stir bars inserted prior to addition of the reactants. The experiment was run for 8 days, with samples taken daily for GC analysis and OD readings.

### **2.2.3: PCB degradation: Flask experiments using soil-free media**

The first flask experiment to test for growth on PCBs was set up on March 20, 2001. This experiment used SV3-2 and AR2 cultures which were positive for growth on biphenyl. The Stephenville 3-2 culture was also used in the TPPB experiments set up in late February, 2001 at SAIC labs in Ottawa.

The flask experiment was set up in duplicate, with the experimental flasks containing 50 mL MSM, 200  $\mu$ L Aroclor 1254 (200  $\mu$ g/mL in methanol), and 400  $\mu$ L culture. In this experiment, AR2 and SV3-2 consortia were tested. The control flasks contained 50 mL MSM and 400  $\mu$ L culture. The experiment was conducted for 22 days, with optical density at 600 nm taken every 2-3 days.

The SV3-2 consortium showed growth in the experimental flask and was further characterized by streaking onto TSA to achieve colony isolation. Colonies showing differences in morphology were picked off and transferred to fresh TSA until four different colony types were isolated. The consortium from the flask was also observed via a wet mount to observe the cells for motility.

These pure cultures, as well as the consortium, were also tested for their ability to grow on the methanol solvent used to dissolve the PCBs. Experimental flasks contained 50 mL MSM, 200  $\mu$ L Aroclor 1254 (200  $\mu$ g/mL in methanol), and 1 mL culture. Single colony isolates SV3-2A, B, C and D were harvested from TSA and suspended in 0.89% NaCl before addition to the flasks. The SV 3-2 consortium was streaked onto TSA from

the MSA/bp flask, then harvested as for the single colony isolates. Two controls were set up for this experiment: the first control contained 50 mL MSM, 200  $\mu$ L methanol (analytical grade) and 1 mL culture; the second set contained 50 mL MSM and 1 mL culture. The flasks were incubated at 25°C on a gyrotary shaker for 48 days with optical density readings taken every three to four days.

#### **2.2.4: PCB degradation: Test tube experiments using soil-free media**

Test tube experiments were typically set up in duplicate, with 9.5 mL MSM, 0.5 mL bacterial culture (soil-free cultures were obtained from MSM-biphenyl flasks), and 200  $\mu$ L Aroclor 1254 (50 mg/mL in acetone) in the experimental tubes. For each experiment two controls were set up. One (labeled C1) contained 9.5 mL MSM, 0.5 mL culture and 200  $\mu$ L acetone (solvent used to dissolve PCBs). The other set of controls (labeled C2) contained 9.5 mL MSM and 0.5 mL culture, with no added carbon source. The first set of controls was designed to test whether or not the bacteria could grow on the solvent alone, and the second was to show that the bacteria would not grow in the absence of a carbon source. All tubes were incubated at room temperature (22°C) for 54-60 days, with optical density measurements taken every two to three days.

Early test tube experiments using SV 2-1 and SV1-2 consortia showed that the bacteria grew as well on the acetone solvent as in the experimental tubes. Because of this result, it was not possible to confirm growth on the Aroclor tested. As a result, further test

tube experiments were set up in which all 22 flask cultures grown on MSM-biphenyl (ten from Argentia samples and twelve from Stephenville samples) were tested for the ability to grow on acetone as sole source of carbon. The experimental tubes contained 10 mL MSM, 100  $\mu$ L bacterial culture (to minimize any autophagy which would confuse the results), and 100  $\mu$ L acetone. Controls contained 10 mL MSM and 100  $\mu$ L bacterial culture. Each set of tubes was prepared in duplicate and incubated at 22°C for 19 days. Those cultures positive for growth on acetone were eliminated from further testing on Aroclors. Thirteen cultures (five from Argentia and eight from Stephenville samples) were negative for growth on acetone. Three of these consortia, SV3-1, SV2-2 and SV3-3 were selected for subsequent testing for growth on AR1254.

Experimental tubes contained 9.5 mL MSM, 0.5 mL culture (unwashed, from MSM/biphenyl flasks) , and 200  $\mu$ L Aroclor 1254 (50 mg/mL in acetone). Two sets of controls were set up. The first set contained 9.5 mL MSM, 0.5 mL culture and 200  $\mu$ L acetone and the second set contained 9.5 mL MSM and 0.5 mL culture. All tubes were prepared in duplicate and incubated on a gyrotary shaker at 150 rpm for 29 days at 22°C. Optical density was measured every two to three days using the Bausch and Lomb Spectronic 20 Spectrophotometer (Milton Roy Co., Rochester, N.Y.).

In order to determine whether any biodegradation had occurred, total bacterial counts (cfu/mL) were performed on all cultures on day one and day 29 for each consortium. Dry weights were also determined for all experimental and control replicates

by pouring the aqueous fraction of each sample into sterile Pyrex test tubes and drying in a hot air oven for two days. Samples were then weighed using the Sartorius B120s analytical balance (Sartorius-Werke, Germany).

#### **2.2.5: PCB degradation: Soil slurry experiments**

Five different soil slurry experiments were set up to examine the ability of native consortia to degrade biphenyl and Aroclor 1254. The cultures used were SV mixed consortium and SV2-4, previously enriched by repeated transfer on biphenyl medium. Three different soil samples were used in these experiments. Experiments 1 and 2 used a low-PCB soil (20 mg/kg), experiments 3 and 4 used a high-PCB soil sample (80 mg/kg), and experiment 5 used a soil slurry from a bioreactor in Kingston, Ontario, which had been dechlorinated using cultures provided by Dr. W. Mohn at the University of British Columbia. The setup for the experimental flasks is outlined in Table 2.1. Some of the experimental flasks were spiked with biphenyl as cosubstrate, while others were spiked with Aroclor 1254 (50 mg/mL in acetone). The biphenyl was added in crystal form, while the liquid Aroclor/acetone mixture was delivered to the flasks using 50  $\mu$ L glass Hamilton microsyringes. The controls contained the same elements (MSM, culture and soil, or just MSM and soil) without the addition of a carbon source.

The soil used in these experiments was collected from the Pinetree site and

delivered to MUN in January 2002 (experiments 1 and 2) and August 2002 (experiments 3 and 4). The soil was sieved to 2 mm particle size using a metal mesh sieve. It was sieved while still wet to preserve the natural microbial biota. The rationale for sieving the soil was that SAIC Canada labs had performed PCB analyses on the soil fractions, and found that >80% of the PCBs were contained in the fines (ie.  $\leq 2$ mm particle size) fraction (Monique Punt, SAIC, Personal communication). A dechlorinated soil slurry from a bioreactor at Royal Military College (RMC), Kingston, Ontario, was used in experiment 5.

A dilution series was set up for the soil before and after sieving, using 0.1 mL aliquots spread-plated in triplicate on TSA. Dilutions of  $10^{-2}$  to  $10^{-8}$  were prepared for enumeration. Dilution series were prepared for the experimental and control flasks at the beginning and the end of each experiment, and for the SV consortium culture added at the beginning of each experiment. Each experiment used 500 mL Ehrlenmeyer flasks with plastic screw caps lined with teflon. All flasks were incubated at 22°C on a gyrotary shaker at 150 rpm for 21 to 22 days. Following each experiment all samples were shipped, on ice, to SAIC labs in Ottawa for GC (PCBs) and HPLC (biphenyl) analysis.

<b>Table 2.1. Contents of experimental flasks for TPPB : Experiments 1-5</b>					
Experiment #	1	2	3	4	5
# of exptl. replicates	3	3	4	4	4
type of soil	2 mm (low pcb)	2 mm (low pcb)	2 mm (high pcb)	2 mm (high pcb)	dechlorinated soil slurry
amt. of soil added/flask	10 g	10 g	20 g	20 g	20 mL (~2 g)
amt. of MSM	100 mL	100 mL	200 mL	200 mL	20 mL
amt. of consortium culture added	10 mL	10 mL	10 mL	10 mL	10 mL
amt. of pcb added (50mg/mL in acetone)	0.0	0.0	200 µl (E1-E4)	0.0	0.0
Amt. of biphenyl added (g)	0.0	0.01 (E1-E3 + control)	1.0 (E3 and E4 only)	1.0 (E1 and E2 only)	0.5 (E1 and E2 only)

Control flasks contained either soil + MSM (expts. 1,4,5) or soil + MSM + consortium culture (expt 2,3). The control for expt. 2 was also spiked with 0.01 g biphenyl.

All cultures were incubated at room temperature (@ 22°C) on a gyrotary shaker at 150 rpm for 21-22 days. Total bacterial counts were performed using dilution and spread-plating technique on day 1 and day 22 of each experiment. Samples were analysed using GC-MS (HP Gas Chromatograph 5900 Series II with electron capture detector, Hewlett Packard) for PCBs and HPLC (High Performance Liquid Chromatography with autosampler, Varian) for biphenyl.

#### **2.2.6: Analytical methods**

In the case of the TPPB experiments, samples were analyzed using an HP5890 series II gas chromatograph with an autosampler (model 7673) and electron capture detector. Samples of the solvent phase were collected from each bioreactor daily. Aliquots of 10  $\mu$ L were added to 10  $\mu$ L of internal standard (2,4,5,6 tetrachlorometaxylene + tributylchloroendate) and 980  $\mu$ L isooctane. These samples were run at time 0 and every 24 hrs until completion of the experiment.

Samples of test tube experiments using soil-free microcosms (SV3-1, SV2-2, and SV3-3) could not be run through the GC due to technical problems with the machine. In order to determine whether or not any growth had occurred in these samples, total bacterial counts were taken on the first and last days of the experiment. Dry weights (mg) were also calculated for both control and experimental tubes, as described in section 2.2.4.

For the soil slurry experiments both PCB and biphenyl analyses were undertaken. PCBs were analyzed using an HP 5900 Series II Gas Chromatograph equipped with autosampler (HP7673) and electron capture detector, all by Hewlett Packard. Biphenyls were analyzed using a Varian High Performance Liquid Chromatograph (HPLC) equipped with solvent delivery system model 9012 and autosampler model 9100.

PCB samples from soil slurry experiments were extracted using equal volumes of



hexane and the hexane layer analyzed for total PCBs. Total PCBs was calculated by looking at changes in the ratios of hexa- to nonachlorobiphenyls, which were more resistant to degradation, to the mono-, di-, and trichlorobiphenyls, which were more easily degraded, in the PCB mixture over time. Changes in the ratio can then be attributed to losses of PCBs because of microbial activity (Harkness *et al.*, 1993). All data provided by analysis of PCBs is in  $\mu\text{g/g}$ , unless otherwise stated.

Biphenyl samples were extracted using equal volumes of methanol, ultrasonicated, then centrifuged and the solvent phase injected directly into the HPLC. Biphenyl concentrations are reported in ppm. Both liquid and soil fractions of the samples were analyzed for PCB and biphenyl.

#### **2.2.7: Measurements of bacterial growth**

Growth of cultures was measured in several ways. First, the testing of Stephenville and/or *Argentia* consortia for growth on phenanthrene, phloroglucinol, toluene, naphthalene and biphenyl was done by streaking flask cultures onto MSA in petri plates. The compounds were provided by (a) placing powdered substrate on Whatman's #9 filter paper, with the exception of toluene, which is a liquid at room temperature. Toluene was supplied in a Durham tube stoppered with sterile cotton wool to allow the toluene vapour to reach the bacterial cells inoculated on the agar. The powdered and liquid substrates were placed on the inside of the inverted lid. Growth of brown, yellow,

or white colonies indicated the consortium tested was able to grow on the test compound as sole carbon and energy source. Each colony type was then picked off and streaked on TSA to confirm viability of the bacterial cells and to use them for biochemical tests used in characterization.

Growth of the consortia on biphenyl was measured by growth on solid media, as described above, and, for liquid media, by increases in optical density over time. *Argentia* flask cultures were set up by addition of 1 g of soil to 50 mL MSM and 0.5 g biphenyl. The flasks were incubated at 22°C on a gyrotary shaker at 150 rpm for 30 days, then streaked on TSA and grown for 24 hrs, after which growth was scraped off and transferred to fresh MSM with biphenyl. For the Stephenville cultures, the initial flasks were set up as for the *Argentia* cultures, but these cultures were maintained by transferring 5 mL of each flask culture directly to fresh MSM with biphenyl. This process was repeated monthly, until soil-free cultures were available for microcosm experiments. Growth of these soil-free cultures was measured by recording optical density at 600 nm using the Shimadzu UV 260 Spectrophotometer (Shimadzu Corp., Kyoto, Japan). These enriched cultures were then used in test tube and flask experiments.

Growth in test tube experiments was measured by increases in optical density at 600 nm, using a Bausch and Lomb Spectronic 20 Spectrophotometer (Milton Roy Co., Rochester, N.Y.). Optical density readings for experimental cultures containing Aroclor 1254 or other source of carbon was compared with those for controls, where no carbon

source was provided, to show growth on the selected compound.

Growth in these cultures was also shown by measuring their dry weights. This was done by placing the aqueous fraction, approximately 9.5 mL per test tube, into preweighed Pyrex test tubes. The test tubes were placed in a Pyrex beaker in a 100°C oven for 24 to 48 hrs. Test tubes were weighed every 24 hrs until constant weight was obtained. Weight measurements were performed using the Sartorius B120S analytical balance (Sartorius-Werke, Germany). Increases in dry weights of the experimental tubes, as compared with controls, were used as an indicator of growth on Aroclor 1254.

Growth in soil slurry experiments was measured by an increase in the cell numbers following incubation with the selected substrate. Enumeration of the cells in each culture flask, including experimental and controls, was carried out on day one and day twenty-two of each experiment. Because of the addition of soil to these flasks, it was not possible to measure increases in optical density. A dilution series was prepared for each flask, as well as for the culture being used in the experiment (SV 2-4) by addition of 1 mL culture fluid to 99 mL dilution blank, shaking vigorously to mix, then transferring 1 mL of this to a 9 mL dilution blank, vortexing and continuing until the cultures were diluted between  $10^{-2}$  to  $10^{-8}$ . Aliquots of 0.1 mL were spread-plated in triplicate on TSA and incubated for 24 to 48 hrs before counting the colonies. Only those plates containing between 30 and 300 colonies were considered in the calculation of the number of colony forming units (cfu) per milliliter of culture. The calculation for cfu/mL (or cfu/g) is as follows:

$$\text{cfu/mL} = \text{average \# colonies} * \text{dilution factor} * 1/\text{amount transferred}$$

Growth on the selected substrate was also shown by measuring the concentration of PCBs (using GC -ECD) and biphenyl (using HPLC) in the mixtures, both before and after the experiment. A decrease in concentration of the substrate in the experimental flasks, as compared with the controls, was also used as a measure of bacterial growth (see section 2.2.6 for analytical methods).

For the TPPB experiments optical density was measured daily by sampling the aqueous phase from each bioreactor. Growth was shown by an increase in turbidity of the liquid medium. Samples were also removed daily from the solvent phase for GC analysis. (See section 2.2.6 for analytical methods).

## **Chapter 3: Results and discussion**

### **3.1. Characterization of the consortia**

#### **3.1.1: Argentia cultures**

The characteristics of the Argentia sediment consortium are outlined in Table 3.1. Eighteen isolates were obtained using dilution and spread-plating technique. Ten of the isolates were Gram negative while eight were Gram positive. All isolates were rod-shaped, except isolate 5a, which was coccus. It is important to note that these characteristics were taken from diluted sediment samples prior to enrichment on biphenyl, so not all of the isolates would be capable of biodegradation of hydrocarbons. Some of the isolates are probably saprophytes, feeding on dead cells and other detritus.

Based on the information in Table 3.1, the isolates can be grouped into possible genera. For example, isolates 1b, 1c, 2b, 2c, 11b and 12a can be placed in the Enterobacteriaceae, with 2b, 2c and 11b being in the genus *Escherichia* and 1b, 1c and 12a probably belonging to the genus *Enterobacter*. This is shown by the results of the EMB test, which is a selective medium used to isolate Gram negative enteric bacteria. This medium also differentiates between the enteric bacteria, based on the colour of the colonies. Isolates 1b, 1c and 12a showed pink or purple colonies, indicating the genus *Enterobacter* or *Klebsiella*, while isolates 2b, 2c and 11b all showed blue-black colonies

Table 3. 1. Observations of morphology and physiology of 18 bacterial isolates obtained from Shag Pond, Argentina, Newfoundland.

ID#	colony characters	cell shape	Gram reaction	motility	OF (glucose)	urease	caseinase	lipase	hemolysis	citrate
1a	off-white, raised, shiny	rod	-	-	no growth	-	-	-	-	-
1b	white, slimy, shiny, raised	rod	-	+	OF (weak in oiled tube)	-	-	-	-	+
1c	beige, raised, shiny	rod	-	+	OF (weak in oiled tube)	-	-	-	-	+
2a	white, filamentous, flat, rough	rod	+	+	no growth	+	-	-	-	-
2b	off-white, raised, shiny	ovoid rods	-	+	OF (weak in oiled tube)	-	+	-	-	+
2c	beige, flat, rough	rod	-	+	OF (weak in oiled tube)	+(w)	+(w)	-	-	+
4a	orange, flat, smooth, shiny	rod	-	-	OF (weak in oiled tube)	-	-	-	-	+
4b	off-white, raised, smooth	rod	-	-	no growth	-	-	-	-	+
5a	white, flat, rough	cocci	+	-	no growth	+	-	-	-	-
9a	white, rough, raised	rod (pleomorphic)	+	+	no growth	+	+	-	-	-
9b	white, filamentous, flat, rough	rod	+	+	no growth	+	+	-	-	-
9c	orange, flat, rough	rod	-	no rxn	no growth	-	-	-	-	-
9d	white, shiny, mucoid, raised	rod	+	no rxn	no growth	-	+	-	-	-
10a	orange/beige, dry, raised	rod	+	+	no growth	-	+	-	beta	+

10b	orange, flat, rough	rod	+	no rxn	no growth	-	-	-	-	-
11a	fried egg-light beige centre, clear edges, mucoid	rod with clear capsule	+	-	no growth	-	+	-	-	-
11b	off-white, raised, shiny with bumpy surface	rod	-	+	OF (weak in oiled tube)	-	-	-	-	+
12a	orange, raised, bumpy surface, shiny	rod	-	-	no growth	-	-	-	alpha	-
Notes: OF = oxidation-fermentation test; A = acid; AG = acid and gas; w = weak; NT = not tested										

Table 3.1. Observations on morphology and physiology of 18 bacterial isolates obtained from Shag Pond, Argentina, Newfoundland (continued)

	Acid and/or gas from									
ID#	maltose	lactose	glucose	sucrose	cyt. Oxidase	MR/VP	catalase	nitrate reduction	thioglycollate	EMB
1a	–	–	–	–	–	–	no growth	+ (N <sub>2</sub> )g	no growth	–
1b	–	–	–	–	+ (w)	–	+	+ (N <sub>2</sub> )g	facultative	+ (pink)
1c	–	–	–	–	+ (w)	–	+	+ (N <sub>2</sub> )g	facultative	+ (pink)
2a	–	AG (w)	AG (w)	AG	–	–	no growth	+ (N <sub>2</sub> )g	facultative	–
2b	–	–	AG	–	–	+/-	+	–	facultative	+ (metallic green)
2c	–	–	–	–	–	+/-	+	+ (N <sub>2</sub> )g	facultative	+ (metallic purple)
4a	–	AG (w)	AG (w)	–	–	–	no growth	+ (N <sub>2</sub> )g	aerobe	+ (clear)
4b	–	A (w)	A (w)	–	–	NT	NT	NT	aerobe	–
5a	–	AG (w)	AG	AG	–	+/-	+	+ (N <sub>2</sub> )g	facultative	–
9a	–	A (w)	–	AG	–	+/-	+	+ (N <sub>2</sub> )g	facultative	–
9b	–	–	AG	AG	–	+/-	+	+ (N <sub>2</sub> )g	facultative	–
9c	–	–	–	A	–	–	+	–	aerobe	–
9d	–	–	–	–	–	–	+	–	facultative	–
10a	A (w)	A (w)	AG (w)	A	–	-/+	+	+ (N <sub>2</sub> )g	facultative	–
10b	AG (w)	AG (w)	AG (w)	A	–	–	no growth	–	no growth	–
11a	–	–	–	–	–	+/-	+	–	aerobe	–
11b	–	–	–	–	–	–	+	+ (N <sub>2</sub> )g	facultative	+ (metallic)
12a	–	A (w)	A (w)	–	–	–	no growth	+ (N <sub>2</sub> )g	aerobe	+
Notes: A = acid; AG = acid and gas; w = weak; NT = not tested										



with a metallic sheen, indicating the genus *Escherichia* (Zimbro and Power, 2003).

Further evidence is shown by other characteristics, such as Gram reaction (all above isolates tested Gram negative), all were motile (except for isolate 12a, which did not grow in the motility test medium) and all were facultatively anaerobic (again, with the exception of isolate 12a, which was an aerobe). It is understandable that these genera would be found in the soil, as the samples were collected near sites of human habitation. Leakage from septic tanks into surrounding groundwater would allow these organisms to mobilize into the soil.

Isolates 4a and 4b also grew on EMB agar, but produced clear colonies. This would indicate that these isolates probably belong to the genus *Proteus*, *Salmonella* or *Shigella*, which do not ferment lactose and thus do not produce a colour change on this medium (Zimbro and Power, 2003). Isolate 5a also grew very slightly on this medium, producing small, clear colonies. This isolate tested Gram positive, with cocci - shaped cells which were non-motile, urease positive, produced acid and gas in sucrose medium, had a positive Methyl Red and negative Voges-Proskauer test, was positive for catalase, was able to reduce nitrate to nitrogen gas, and is a facultative anaerobe. These are all characteristics consistent with the genus *Staphylococcus* (Sneath *et al*, 1986).

Isolates 2a, 9a and 9b were very similar in their biochemical reactions, so will also be considered as a group. These isolates were all Gram positive, facultatively anerobic motile rods, urease and caseinase positive, negative for lipase and citrate utilization, and

positive for nitrate reduction. These characteristics correspond to the genus *Oerskovia*, an actinomycete often found in soil (Sneath *et al.*, 1986). Further evidence to support this choice was the colony appearance (rough surface with filamentous edges) and cell arrangement (pleomorphic rods with coccoid elements), which are also common in actinomycetes (Sneath *et al.*, 1986).

Isolates 1a and 9c were more difficult to characterize, as they didn't grow in many of the media used for classification in Table 3.1. They were both Gram negative, non-motile rods which showed negative results for all tests except a positive nitrate reduction test (isolate 1a) and a positive catalase test and acid production in sucrose media (isolate 9c). These organisms are probably saprophytes which require a more fastidious diet.

Isolates 10a and 10b were both Gram positive, non-sporeforming rods which formed orange colonies on solid media. They were both urease and lipase negative and produced acid and/or gas from maltose, lactose, glucose and sucrose. These are probably species of the genus *Arcanobacterium*. It is interesting to note that isolate 10a demonstrated beta hemolytic activity when grown on sheep's blood agar, a characteristic common to the species *Arcanobacterium hemolyticus* (Sneath *et al.*, 1986). These species are also known for production of orange-pigmented colonies. Isolate 10b did not demonstrate this ability to hemolyse blood agar, and also differed from isolate 10a in that it was negative for caseinase, citrate reductase and nitrate reductase (positive for 10a). Thus, isolate 10b could also belong to the genus *Brevibacterium*, also known to produce

orange colonies on solid media (Sneath *et al.*, 1986).

The remaining two isolates, 9d and 11a, were both Gram positive, non-motile rods which produced mucoid colonies on solid media. Both were negative for urease, lipase, citrate reductase, cytochrome oxidase, acid and/or gas production from carbohydrates, and nitrate reductase. They were both positive for casein hydrolysis and were catalase positive. Isolate 9d produced white, mucoid colonies while 11a produced colonies which had a “fried egg” appearance, with light beige centres and clear edges. This isolate also had a clear capsule around the cells which was easy to see when Gram-stained. These characteristics, especially the formation of a capsule or slimy colonies, are often found in strains of *Arcanobacterium* or *Brevibacterium*, both of which are Gram positive, aerobic rods which are catalase positive (Sneath *et al.*, 1986). Both of these genera are commonly isolated from soil, and are nutritionally nonexacting chemoorganotrophs (*ibid.*).

Of the isolates obtained from the Argentina samples, several genera, such as *Arcanobacterium*, *Brevibacterium*, and the actinomycetes are capable of biodegrading PAHs and PCBs. Also of interest is the observation that isolates 2b, 2c and 11b grew quite readily at 5 degrees Celsius. As described above, these organisms are probably strains of the genus *Escherichia*, and as such have an optimum temperature of 37 degrees. If these isolates are of this genus, they are exhibiting psychrotrophic tendencies.

### 3.1.2: Stephenville cultures

Similar colony types were observed in the soil samples from Stephenville, but time and funding constraints prevented a more thorough examination of their biochemical characteristics. The characterization of these consortia was limited to those which showed growth on Aroclor 1254. The results of characterization of the SV 3-2 consortium are shown in table 3.2. Gram stains were not done on these isolates, as confirmation of Aroclor degradation was not obtained via GC analysis. However, since the consortium grew in the presence of Aroclor 1254, and was shown to also degrade methanol, the colony characteristics are included here. Colonies were either beige or white in colour, and were all smooth except for SV3-2C, which had a rough surface. Isolate SV3-2A also showed growth at 5 degrees when stored in the refrigerator.

Following the experiments using this consortium, visual observations of the consortium were made from a wet mount of the culture taken from the experimental flask. The wet mount showed motile, rod-shaped bacteria, which moved about using a spinning and twirling motion, indicating the presence of peritrichous flagella. Using a wet mount prepared and stained using crystal violet, four types of cells were observed: long thin rods, long, fat rods, pleomorphic (or bent) rods, and coccoid elements. These observations are consistent with the colony and cell descriptions of the actinomycetes, which display a marked rod/coccus cycle, and often show pleomorphic rods. Some genera which fall into this category are the *brevibacteria*, *corynebacteria*, and *Rhodococcus*, all commonly found

in soil and all of which are capable of biodegrading hydrocarbons (Sneath *et al.*, 1986). In addition, the flask culture which grew on the Aroclor 1254/methanol mixture showed the presence of star-shaped, yellowish colonies flocculating the liquid media. This is a common characteristic of the genus *Pseudomonas*, especially *P. rhodos* and *P. echinoides* (Kreig and Holt, 1984), which use their pili to form these aggregates in liquid media. *Pseudomonas* strains are also capable of degrading aromatic compounds using a 1,2 or 2,3 dioxygenase for ring cleavage (ibid.).

Table 3.2. Colony characteristics for SV3-2 consortium used for AR1254 (in methanol) flask and TPPB experiments.

Sample ID	Colony characteristics
SV3-2A	white, mucoid, raised, lobose, 4-5 mm
SV3-2B	beige, smooth, raised, entire, 1-2 mm
SV3-2C	off-white, rough, raised, entire, 1-2 mm
SV3-2D	beige, smooth, raised, entire, pinpoint

Note: The colony descriptions are given as colour, texture, elevation, margin and size. The strain SV3-2D produced a brown pigment which diffused into the surrounding medium.

The results of characterization of the SV2-4 consortium, used in the soil slurry experiments, are shown in table 3.3. This consortium consisted of six strains of bacteria, four of which were Gram negative and two were Gram positive. The two Gram positive isolates, SV2-4A and B, exhibited colony and cell morphology consistent with the

corynebacteria and brevibacteria, which have pleomorphic rods, and grew only slightly on MacConkey agar. MacConkey agar is a selective and differential medium used for the isolation and differentiation of Gram negative, enteric bacilli. It contains bile salts and crystal violet, which inhibit the growth of Gram positive organisms while allowing the growth of Gram negative organisms (Zimbro and Power, 2003). It also contains an indicator dye, neutral red, which causes a colour change in the medium when lactose is fermented. The rest of the isolates, SV2-4B-F, showed moderate to profuse growth on MacConkey agar, with a colour change of the medium to orange or yellow, indicating that these organisms belong to the Gram negative, lactose fermenting bacilli. Typical genera belonging in this group are *Escherichia*, *Enterobacter*, *Klebsiella*, *Citrobacter* and *Serratia*. These organisms belong to the Enterobacteriaceae, and are often found in clinical specimens, the intestinal tracts of man and animals, and are also commonly isolated from soil. All produce acid from lactose fermentation (Kreig and Holt, 1984). These organisms are also chemoorganotrophic, which would allow growth on minimal media.

Table 3.3. Characteristics of bacterial strains isolated from SV2-4 consortium used in soil slurry experiments.

Sample ID	Gram reaction	cell shape and arrangement	cell size (L x W in $\mu\text{m}$ )	* colony morphology	growth on MacConkey agar	
SV2-4A	+	pleomorphic rods - pairs and short chains	2.5-3.5L x 0.5W	Off-white, smooth, convex, entire, 2mm	0/+ (brick red growth)	
SV2-4B	+	rods - singles and pairs	1.5-2.5L x 0.5W	white, rough, raised, entire, pinpoint	+	(purple colonies)
SV2-4C	—	rods - singles and pairs	1.5-2L x 0.25-0.5W	beige, smooth, convex, entire, pinpoint	+++	(pink/purple colonies with colour change of medium to orange)
SV2-4D	—	rods - singles and pairs	1.5-2L x 0.5-0.75W	yellow, smooth, convex, entire, 1-2 mm	++	(purple colonies with colour change of medium to orange)
SV2-4E	—	rods - singles, pairs and chains	2-4L x 0.5W	beige, smooth, raised, entire, pinpoint	++	(pink/purple colonies with colour change of medium to yellow/orange)
SV2-4F	—	rods - singles	2L x 0.5W	beige, smooth, raised, entire, 2 mm	+++	(pink/purple colonies with colour change of medium to orange)

Note:\* Colony morphology is given as colour, texture, elevation, margin, and size; 0/+ = scant growth, ++ = moderate growth, +++ = profuse growth; L = length, W = width.

### **3.2: Biodegradation of PAHs**

#### **3.2.1: Petri plate experiments : Growth of soil consortia on**

##### **biphenyl, toluene, phloroglucinol, phenanthrene, and naphthalene**

Prior to testing the soil and sediment consortia for the ability to grow on the above listed compounds, the consortia were enriched by repeated transfer into liquid MSM + 1% biphenyl. All soil (Stephenville) and sediment (Argentia) samples showed growth in liquid MSM + biphenyl, as shown by an increase in turbidity and by the production of catechols, which turned the medium yellow.

The results of soil-free Argentia flask cultures streaked on MSA and incubated with the above substrates for 25 to 30 days at 28°C are shown in Table 3.4. For toluene, glass plates were used for the MSA, and toluene was supplied in the form of a vapour, dispensed via a durham tube in the lid of the inverted dish. The plates were incubated at 28°C for 30 days. Seven of the twelve flask consortia produced colonies on MSA when exposed to toluene. Colonies were cream - coloured or brown, 1-3 mm in size. They were raised with entire edges. When these colonies were picked off and streaked on TSA, the colonies were creamy white with a pinkish tinge, and were mucoid with a shiny surface, while others were more granular in appearance. This may be due to mixtures of cell types on the plate. Gram stains showed that all but one were Gram negative, and all were rod-shaped cells. OF and motility tests showed all were aerobic, and most were motile.

Toluene can be readily degraded by strains of *Pseudomonas putida* (Zylstra *et*



*al.*, 1988; Zylstra and Gibson, 1989) and *Pseudomonas aeruginosa*, *Achromobacter*, and *Nocardia corallina* (Ribbons and Eaton, 1982). The mucoid colonies obtained when toluene-positive isolates were grown on TSA were Gram negative, aerobic, motile cells, consistent with *Pseudomonas* spp. The Gram positive isolate may belong to the nocardioforms, which are mostly strict aerobes, with motile and nonmotile species in the group (Sneath *et al.*, 1986). Anaerobic metabolism of toluene has been demonstrated in the genus *Azoarcus* (Coschigiano and Young, 1997) and in mixed microbial cultures in sediments (Zeyer *et al.*, 1986).

Table 3.4. Results of testing of Argentia consortia for growth on selected hydrocarbons

Substrate	Number of isolates*	Shape	Gram reaction
toluene	7	rod	- (6), + (1)
phloroglucinol	7	rod	- (6), + (1)
naphthalene	5	rod	- (4), + (1)
phenanthrene	10	rod	- (9), + (1)

\* 12 samples were tested for each compound.

Seven isolates were obtained when the flask consortia were streaked on MSA and incubated with phloroglucinol (PG) as sole source of carbon. Seven of the flask cultures were positive for growth on PG when incubated at 28°C for 30 days. Colonies were

mostly creamy yellow, beige or brown in colour, raised, with entire edges. Two of the samples (from flasks 2 and 7) produced white colonies with a filamentous edge. Flasks 2 and 5 each produced three distinct species of bacteria capable of growth on PG. Flask 2 colonies were beige or white, raised and smooth, with the filamentous-edged colonies being flat with a rough surface. Flask 5 colonies were yellow and mucoid white (both types were raised with entire edges). OF and motility tests showed that all of the isolates were motile, and most were aerobic. Gram stains showed that all were Gram negative except for the white, filamentous colonies, which were Gram positive.

Phloroglucinol can be degraded by both aerobic and anaerobic organisms. Aerobic degradation has been demonstrated in *Pseudomonas* and *Arthrobacter*, as well as *Mycobacterium*, *Flavobacterium*, and fungi such as *Penicillium spp.* (Patel *et al.*, 1981). The Gram positive strain isolated in this experiment corresponds to *Oerskovia spp.*, a facultatively anaerobic rod which tends to produce filamentous colonies on solid media (Sneath *et al.*, 1986). This strain was also isolated from the Argentina sediment consortia before enrichment on biphenyl. The Gram negative isolates most closely correspond with *Pseudomonas spp.* (Kreig and Holt, 1984). Anaerobic degradation of phloroglucinol can be accomplished using *Eubacterium oxidoreductans*, *Pelobacter acidigallici*, *Streptococcus spp.*, and *Coproccoccus spp.*, which is found in the rumen of cattle (Armstrong and Patel, 1992; Patel *et al.*, 1981).

The results show that five out of twelve consortia tested were positive for growth

on naphthalene. Colonies were generally smooth and raised, and were yellow or brown in colour. One filamentous white colony grew, but slowly. Isolates grown on naphthalene were obtained from flasks 1, 5, 7, 9, and 11. Gram staining showed that four out of the five isolates were Gram negative, while one was Gram positive (the filamentous strain). All were rod-shaped organisms. Two of the isolates were both oxidative and fermentative, three were strict aerobes, and most were also motile.

Naphthalene can be degraded aerobically by species of *Pseudomonas putida* (Patel and Gibson, 1974; Samanta and Jain, 2000), *Pseudomonas fluorescens* (Whitman *et al.*, 1998) and *Pseudomonas* spp. (Whyte *et al.*, 1997). The Gram negative organisms isolated in this experiment may be strains of *Pseudomonas*, which are aerobic, Gram negative, motile rods (Kreig and Holt, 1984). The Gram positive isolate again most closely corresponds to *Oerskovia* spp., which are Gram positive, motile rods with filamentous edges to the colonies on solid media (Sneath *et al.*, 1986). Anaerobic degradation of naphthalene can be achieved under nitrate-reducing conditions using a microbial consortium (Rockne *et al.*, 2000).

Samples streaked onto MSA with phenanthrene (Phen) as sole carbon source showed growth in ten out of twelve samples tested. Colonies were generally white or brown, 1 mm in size, raised and smooth in appearance. Samples 1, 6 and 8 showed growth of the white, filamentous-edged colonies seen in the tests with other compounds. This strain may correspond to *Oerskovia* spp, being Gram positive with filamentous edges

to the colonies (Sneath *et al.*, 1986). It is interesting to note that sample 10 produced 20 orange colonies with a raised, wrinkled surface. This is the only compound showing this colony type. This strain may be a species of *Pseudomonas*, such as *P. aureofaciens*, or *Xanthomonas* spp. which are Gram negative, aerobic rods that produce an orange pigment (Kreig and Holt, 1984). Gram stains showed that all were Gram negative rods, except the filamentous white colonies, which were Gram positive rods. OF and motility tests showed that all were aerobic, and most were motile.

Many of the enzymes used by bacteria to degrade naphthalene can also be used in phenanthrene degradation. Biochemical and genetic plasticity of these organisms provides them with a range of metabolic options for degradative pathways. For example, some species can utilize more than one pathway for initial ring cleavage (Ribbons and Eaton, 1982). Phenanthrene degradation has been shown to occur in the Antarctic, by bacteria isolated from fuel spill sites (Smith, 1990). To further show the plasticity of degradative enzymes, a recent study has shown that naphthalene degrading enzymes can also be used in the degradation of biphenyl (Barriault *et al.*, 1998).

All of the flask samples tested were also grown on MSA with biphenyl in the lid. This served as a positive control. Since all of the flask consortia were positive for growth on biphenyl in liquid MSM, they were also positive for growth on MSA with biphenyl crystals in the lid. Colonies were yellow or brown in colour, and all were Gram negative rods. Flask samples were also inoculated onto MSA and incubated at 28°C without a

carbon source to ensure growth which occurred was due to the substrate provided. All plates not supplied with a carbon source did not grow.

### **3.3: Biodegradation of PCBs**

#### **3.3.1: Two-phase partitioning bioreactor (TPPB) experiments: Growth of soil-free consortia on Aroclor 1254**

The two-phase partitioning bioreactor (TPPB) was developed in the early 1990s by researchers at Queen's University, Ontario, Canada for use in bioremediation of organic compounds. It was developed by Dr. Andrew Daugulis and his colleagues for use as a bioreactor in the treatment of BTEX and other polycyclic aromatic hydrocarbons (Daugulis, 2001). It works by maintaining a level of partitioning, which separates the aqueous layer (containing the bacterial culture of biodegraders) from the immiscible solvent layer (containing the contaminant to be degraded). Because the solvent layer is floating on top of the aqueous, the contaminant slowly diffuses into the aqueous layer, where degradation occurs. Thus, the level of contamination can be high, without becoming toxic to the microbes. This technology has been shown to be highly successful in degrading polycyclic aromatic hydrocarbons such as naphthalene, phenanthrene and pyrene (Janikowski *et al.*, 2002, MacLeod and Daugulis, 2003). A diagram of the bioreactor configuration is shown in figure 3.1. This set of experiments was conducted at SAIC labs in Ottawa, Ontario from February 26-March 2, 2001. Four bioreactors were set up to test the ability

of SV3-2, a consortium enriched by growth on biphenyl medium, to degrade Aroclor 1254. Three of the reactors were aerated aerobically, and the fourth was anaerobic (nitrogen gas). The four bioreactors are shown in figures 3.2 and 3.3. In each bioreactor, a cometabolite was added to stimulate the enzymes necessary for biphenyl, and subsequently, Aroclor degradation to occur. Three of the bioreactors (A, B, and D) had benzoic acid as cometabolite, and one (bioreactor C) had biphenyl. The reactors were set up on magnetic stirrers for 8 days, with samples taken daily for optical density and GC analysis. The results in Figure 3.4 show the changes in total PCBs over the length of the experiment. Total PCBs were calculated by measuring the ratios of six PCB congeners within the Aroclor mixture in each sample. Any changes in the ratio of these peaks indicates changes in the composition, including changes in degree of chlorination, of the Aroclor. Changes in these ratios, then, would provide evidence of bacterial action (Punt *et al.*, 2002).

Optical density readings, taken daily, showed that the bacterial consortia grew in all bioreactors, including the anaerobic reactor. This result shows that the bacteria could grow with or without O<sub>2</sub> as terminal electron acceptor. The PCB results show that the ratios of the peaks in the Aroclor mixtures remained relatively constant. However, the figure does show a decrease in PCB concentrations for Reactor A, which was run anaerobically. This may be due to some anaerobic dechlorination which changed the

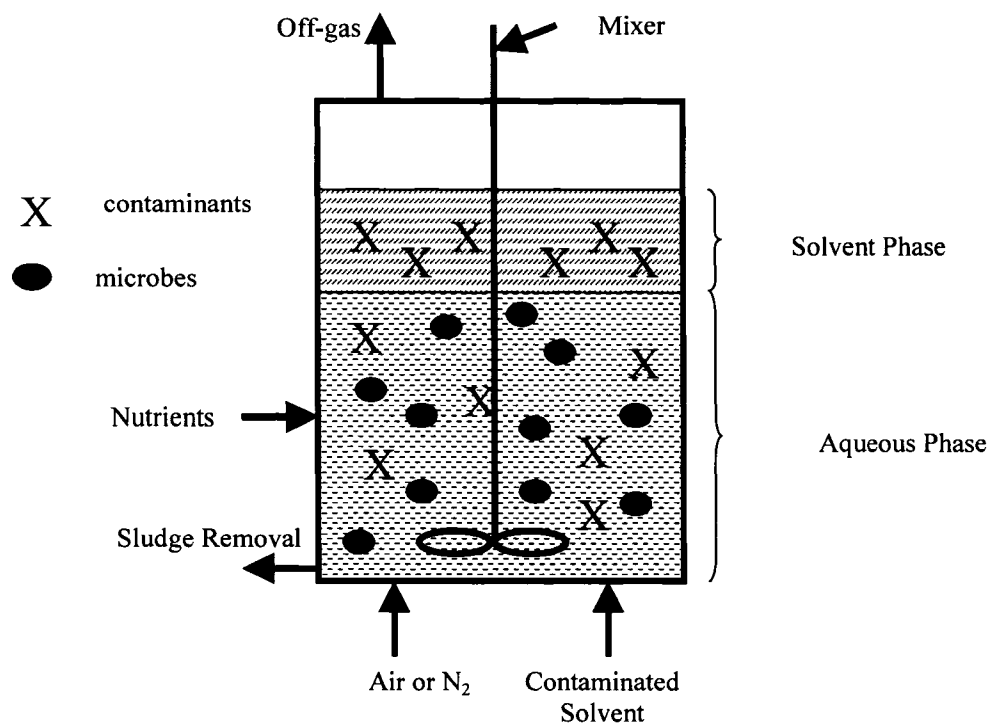


Fig. 3.1. Schematic diagram of a TPPB bioreactor. (From Punt *et al.*, 2002)

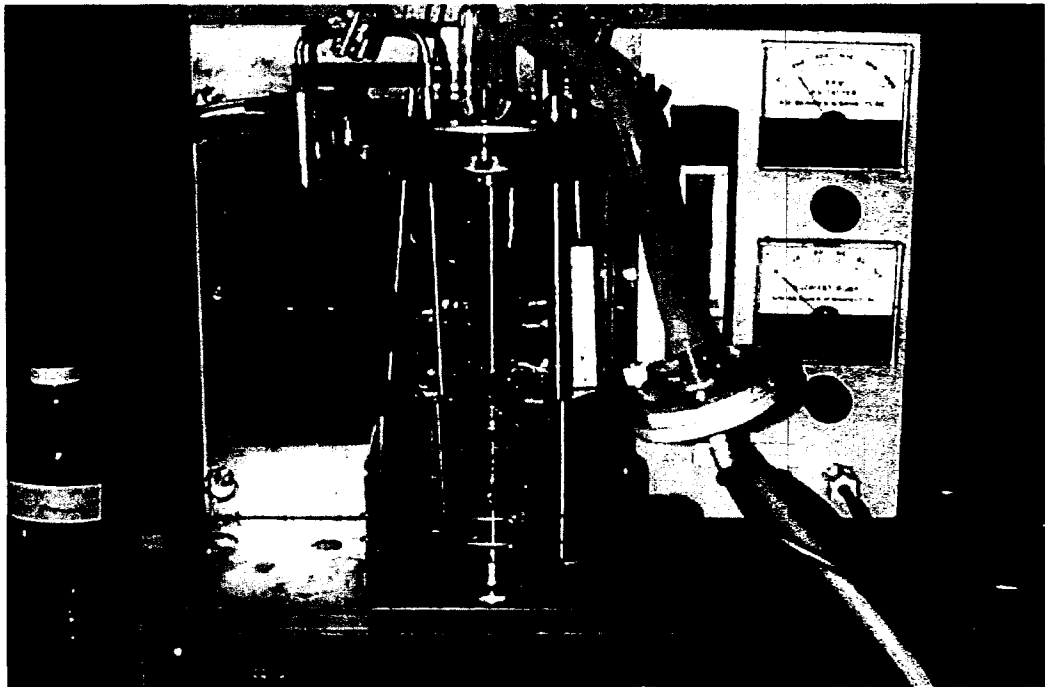


Fig. 3.2. Bioreactor A used in Two-phase partitioning bioreactor (TPPB) experiments - Anaerobic (nitrogen gas). Notes: Reactor A was spiked with 0.1% benzoic acid and 100 ppm Aroclor 1254 and augmented with SV3-2 bacterial consortium.





Fig. 3.3 (L-R). Bioreactors B, C, and D used in TPPB experiments (aerobic).  
Notes: Reactors B and D spiked with 0.1% benzoic acid and 100 ppm Aroclor 1254; Reactor C spiked with 0.2% biphenyl and 100 ppm Aroclor 1254. All reactors were augmented using SV3-2 bacterial consortium.

ratios of the congener peaks. Also, reactors C (aerobic, biphenyl as cometabolite) and D (aerobic, benzoic acid as cometabolite) show an initial increase in PCB concentration, followed by a steady decrease in concentration towards the end of the experiment. This initial increase in the PCB concentration could be due to the production of biosurfactants by the bacterial consortia during their metabolism, which emulsified the PCBs, making them more bioavailable. Agitation of the mixtures within the reactors also allowed the bacteria optimal access to the Aroclor in the solvent phase.

As the experiment was run for only 8 days, it is not conclusive whether extensive degradation of Aroclor 1254 could be achieved using this method. A longer incubation time, typically 3-6 weeks, is usually required to achieve extensive degradation of PCBs (Young and Cerniglia, 1995). Also, the additional carbon source in the form of tryptone may have caused competition for limited resources (Aroclor 1254). The preliminary results, however, are encouraging. Since the consortium was shown to grow in anaerobic conditions, and was able to lower the level of PCB by approximately 10% in 8 days, it is possible that it may be employed in a sequential anaerobic/aerobic treatment process for degradation of Aroclor 1254. More research on this method is required to establish its effectiveness in degrading PCBs.

From Punt *et al*, 2002

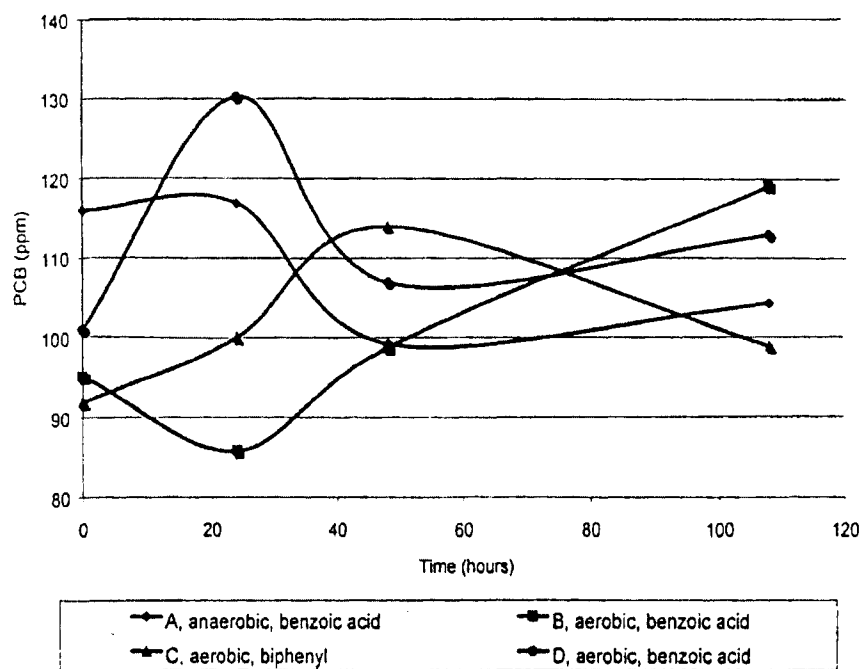


Figure 3.4 Bioreactor PCB Concentrations in Four TPPB Systems

### **3.3.2: Flask experiments using soil-free microcosms: Growth of soil consortia on methanol and Aroclor 1254**

The first flask experiment was conducted using a weak Aroclor 1254 mixture (200 µg/mL in methanol). The experimental flask contained 200 µL of this mixture in 50 mL MSM + 400 µL culture (AR2 and SV3-2 were used). After three weeks incubation, optical density readings increased for SV3-2, but not for AR2 (Figures 3.5 and 3.6). Because only one control was set up (MSM + Culture, with no methanol control), it was not possible to determine whether the growth observed in the experimental SV3-2 flask was due to growth on the Aroclor or the solvent. Wet mounts prepared from the successful SV3-2 culture showed that the cells were motile, exhibiting a twirling and spinning motion. The culture was streaked onto TSA to obtain isolated colonies. Four different colony types were found, based on colony characteristics. Each of these was purified by repeated streaking on TSA, then all were tested for the ability to degrade Aroclor 1254 in methanol.

The results of this second flask experiment are shown in Figures 3.7 - 3.11. The results show that the individual bacterial species were unable to grow on the Aroclor 1254 or on the methanol solvent. In contrast, the consortium did grow on both the Aroclor (200 µg/mL in methanol) and on the solvent alone. These results indicate that a cometabolic process may be involved in the metabolism of methanol (CH<sub>3</sub>OH). It has been shown in

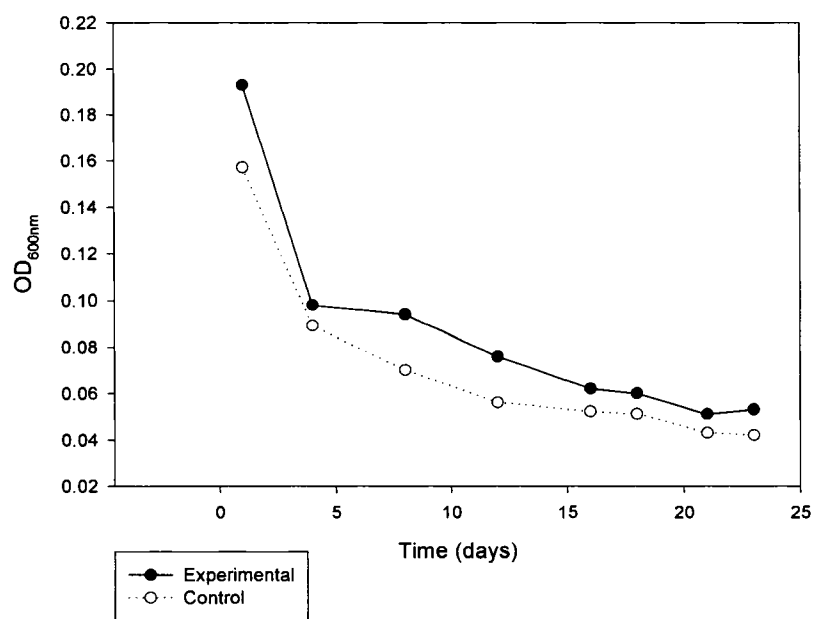


Fig. 3.5. Growth of AR2 soil-free consortium spiked with Aroclor 1254 in Methanol.

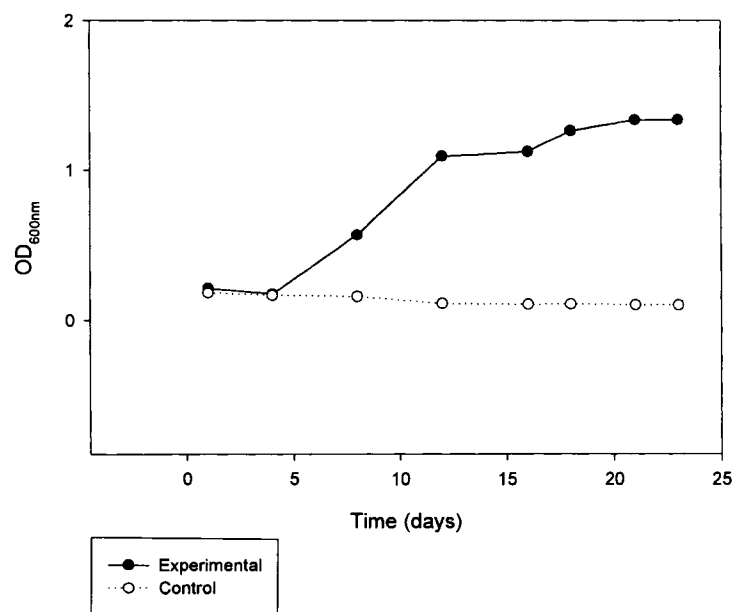


Fig. 3.6. Growth of SV3-2 soil-free consortium spiked with Aroclor 1254 in Methanol.

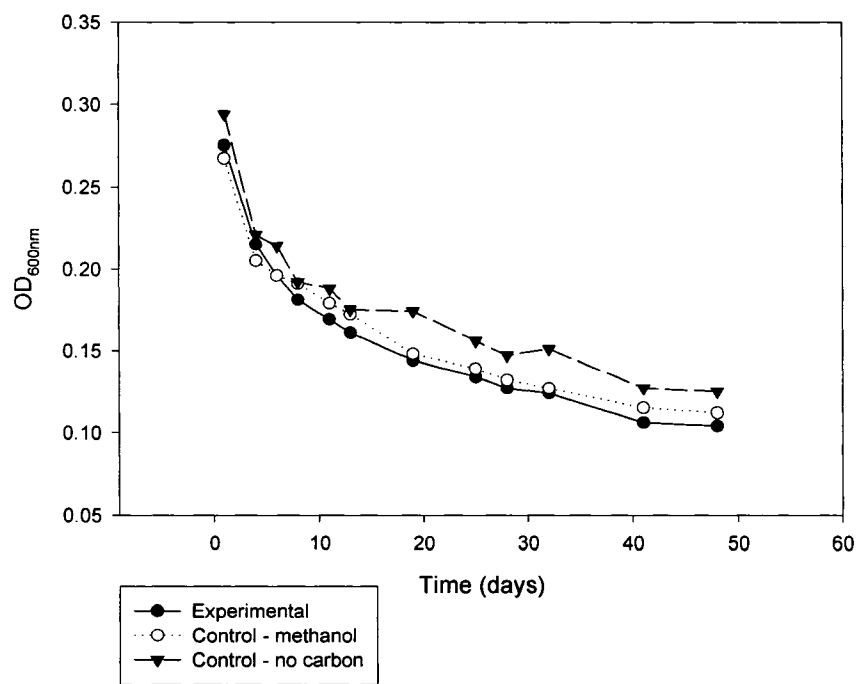


Fig. 3.7. Growth of SV3-2 (isolate A) spiked with Aroclor 1254 in Methanol.  
Note: Each data point is the average of two replicates.

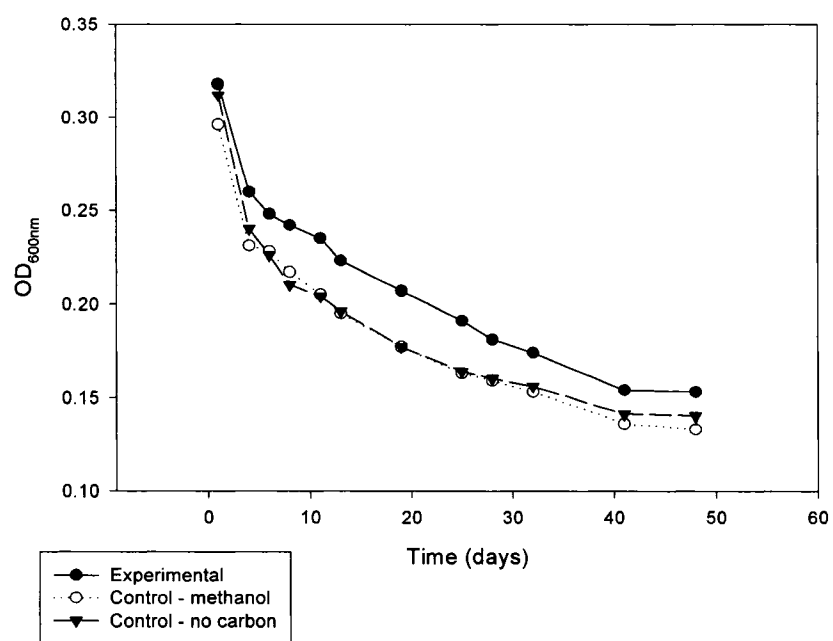


Fig. 3.8. Growth of SV3-2 (isolate B) spiked with Aroclor 1254 in Methanol.  
Note: Each data point is the average of two replicates.



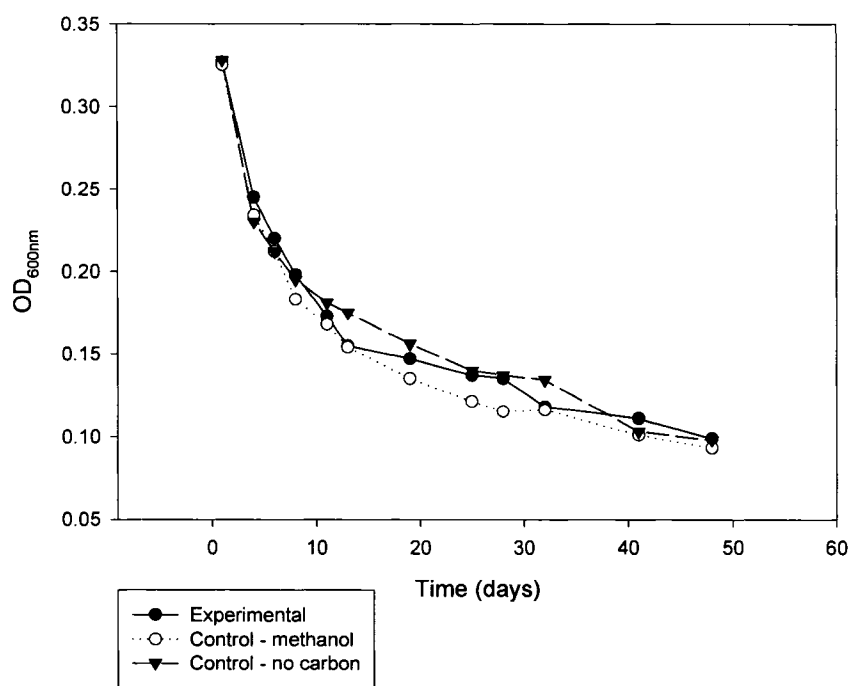


Fig. 3.9. Growth of SV3-2 (isolate C) spiked with Aroclor 1254 in Methanol.  
Note: Each data point is the average of two replicates.

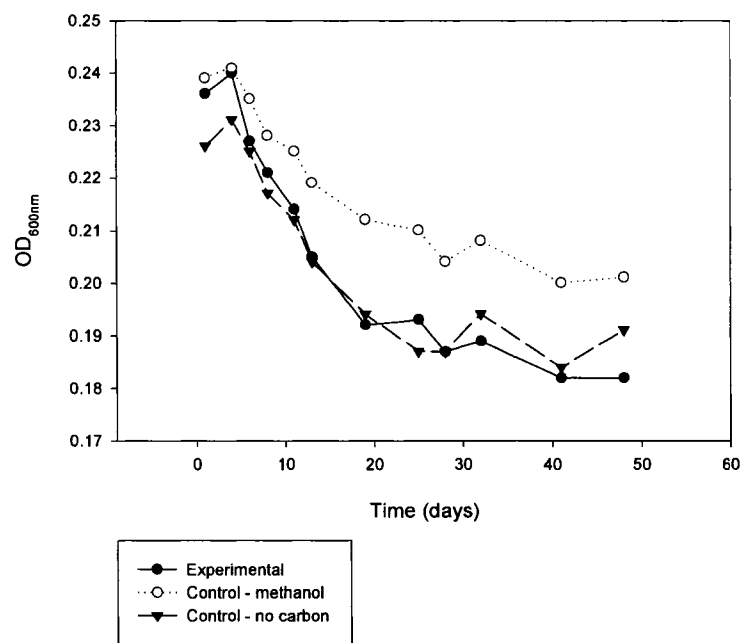


Fig. 3.10. Growth of SV3-2 (isolate D) soil-free culture spiked with Aroclor 1254 in Methanol.  
Note: Each data point is the average of two replicates.

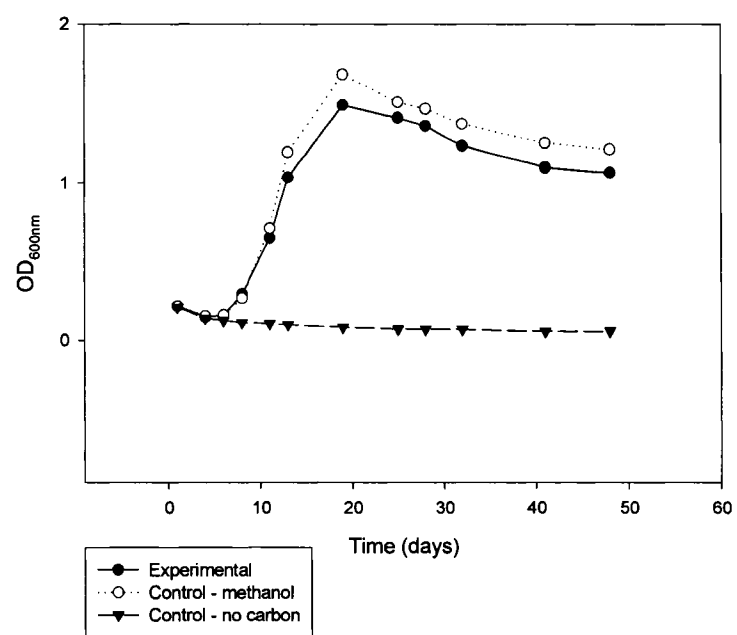


Fig. 3.11. Growth of SV3-2 soil-free consortium spiked with Aroclor 1254 in Methanol.  
Note: Each data point is the average of two replicates.

previous studies that methanol can be degraded by methanotrophic bacteria such as *Methylobacter*, *Methylococcus*, *Methylomonas* and *Pseudomonas* spp. utilizing the enzyme methanol dehydrogenase (Gibson, 1984). The growth in the Aroclor flask indicates that the consortium was not inhibited by the concentration of Aroclor present, and some PCB degradation may have occurred. Previous research has shown that methanogenic bacteria can also dechlorinate high molecular weight PCBs (Van Dort and Bedard, 1991). As the consortium grew on both the Aroclor mixture and the solvent alone, it cannot be stated with certainty that this consortium can degrade PCBs. Due to the ambiguity of the results, no GC analysis was performed on these samples.

### **3.3.3: Test tube experiments using soil-free microcosms: Growth of soil consortia on acetone and Aroclor 1254**

This set of experiments was set up to determine whether any of the consortia (both Argentia and Stephenville cultures) could degrade Aroclors. Due to the hydrophobic nature of Aroclor 1254 (it is an oil at room temperature), it was dissolved in acetone to create a concentrated solution. Early experiments using this mixture (Aroclor 1254 - 50 mg/mL in acetone) showed growth in controls as well as in experimental cultures. As a result, a series of test tube experiments were set up to test the ability of all consortia to grow on acetone as sole source of carbon. Thirteen of the 22 consortia tested were negative for growth on acetone. Three of these acetone-negative consortia, SV 3-1, 2-2

and 3-3 were then selected to test for the ability to grow on Aroclor 1254 in acetone. Previous research has shown that microbial dechlorination can be accomplished using a defined, sediment-free medium (Cutter *et al.*, 1998, Wu *et al.*, 2000). However, most research on PCB degradation has concentrated on the use of soil slurries, as they are more applicable to *in situ* studies (Tiedje *et al.*, 1993). Despite this fact, several studies have focused on biodegradation of PCBs and biphenyls using defined, sediment-free media. These include oxidation of biphenyl by a *Beijerinckia* spp. (Gibson *et al.*, 1973), degradation of PCBs by *Alcaligenes* sp JB1 (Commandeur *et al.*, 1996) and an assay to show the ability of several bacterial species, including *Pseudomonas putida* LB400 and *Alcaligenes eutrophus* H850, to degrade high molecular weight PCBs (Bedard *et al.*, 1986). In contrast to the present study, the aforementioned research has focused on the degradative abilities of individual species of bacteria. This research is based on the use of bacterial consortia, as it is widely known that complete mineralization of PCBs requires a bacterial consortium consisting of several species, each capable of degrading one of the compounds or their intermediates. Recent work has shown that locally isolated bacterial consortia are capable of degrading benzothiophene, carbazole and dibenzofuran under aerobic conditions in a defined medium (Meade, 1997).

The plots of optical density over time for these three consortia are shown in figures 3.12 to 3.14. The figures show that SV3-1 and 2-2 maintained a relatively stable growth pattern (figs 3.12 and 3.13). In contrast, SV3-3 grew much more readily in the Aroclor

mixture (fig. 3.14). Optical density remained relatively stable until day 22, then increased steadily until the end of the experiment at day 28. In addition, SV3-3 showed a slight increase in optical density in the C1 controls, which contained 0.1% acetone. This growth may be due to traces of biphenyl metabolites left in the culture when it was added acting as

a usable carbon source, or may be due to the bacteria feeding on dead cells. In contrast, acetone controls for the remaining two consortia, SV 3-1 and 2-2 (Figs. 3.12 and 3.13), show no increases in optical density over the length of the experiment. This would indicate that these consortia were unable to grow without a substantial source of carbon.

All samples were extracted in hexane at the end of each experiment for GC analysis. Samples were to be analyzed at DFO labs in St. John's. Unfortunately, the GC machine ceased functioning before the samples could be analyzed. To determine whether any PCB degradation had occurred in these samples, total bacterial counts, using dilution and spread-plating technique were performed on the experimental cultures on the first and final days of the experiment. As well, dry weights of the controls and experimental tubes were also determined, to see whether bacterial biomass had increased in the experimental tubes.

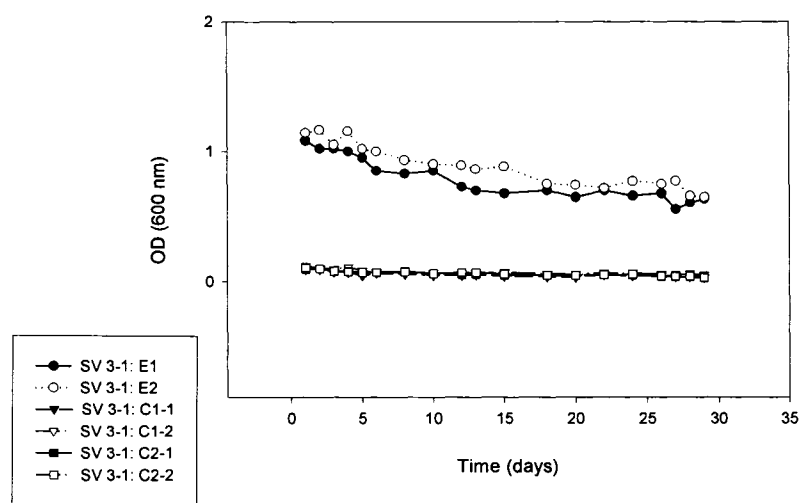
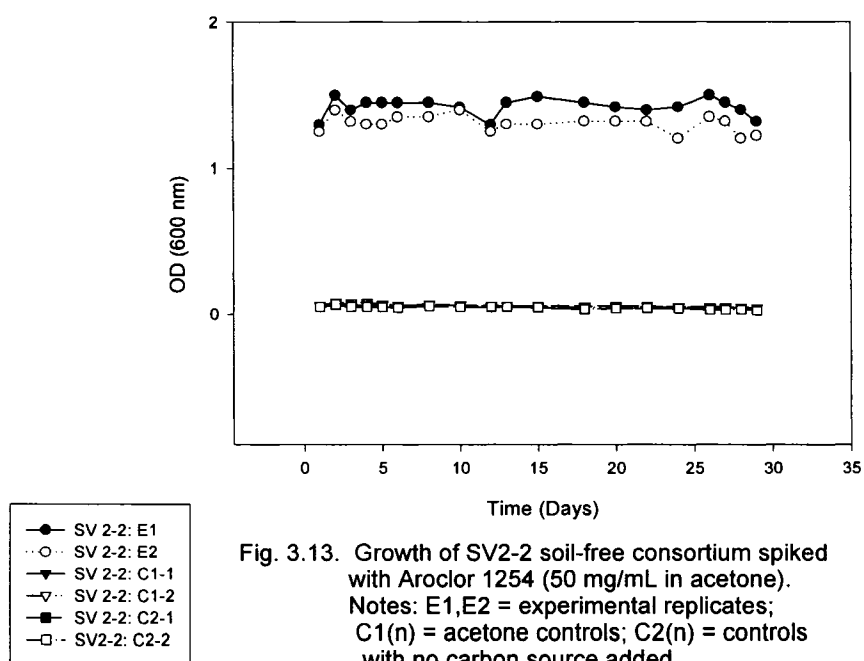


Fig. 3.12. Growth of SV3-1 soil-free consortium spiked with Aroclor 1254 (50 mg/mL in acetone).  
Notes: E1,E2 = experimental replicates;  
C1(n) = acetone controls; C2(n) = controls with no carbon source added.



**Fig. 3.13.** Growth of SV2-2 soil-free consortium spiked with Aroclor 1254 (50 mg/mL in acetone).  
 Notes: E1,E2 = experimental replicates;  
 C1(n) = acetone controls; C2(n) = controls with no carbon source added.



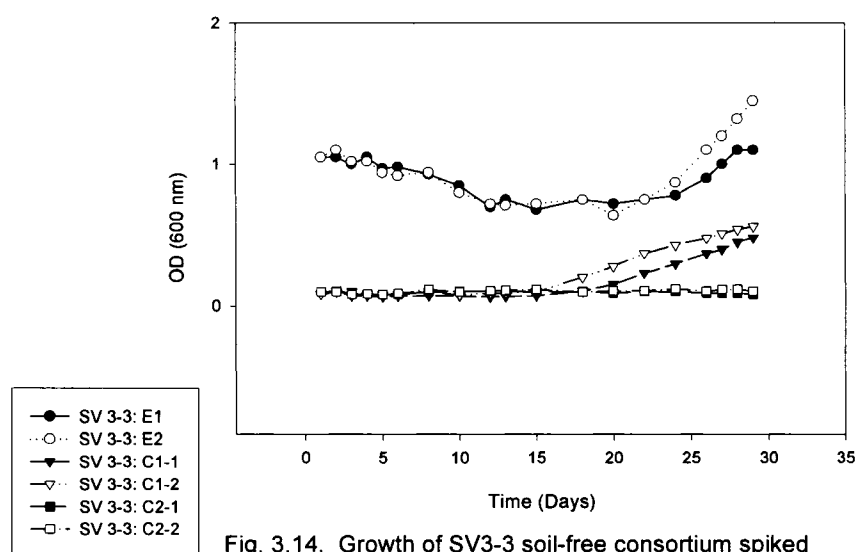


Fig. 3.14. Growth of SV3-3 soil-free consortium spiked with Aroclor 1254 (50 mg/mL in acetone).  
 Notes: E1,E2 = experimental replicates;  
 C1(n) = acetone controls; C2(n) = controls with no carbon source added.

The results of these analyses are shown in Table 3.5. Bacterial counts decreased for all cultures tested, with the smallest decrease observed in SV3-3. Observations during the experiment showed that SV3-3 produced a large pellicle of clumped cells, which did not disperse when the samples were vortexed prior to taking optical density readings. The pellicles were largest in the experimental tubes, indicating a visual confirmation of an increase in bacterial biomass. This result is supported by the dry weights shown in table 5. Each of the three consortia showed an increase in dry weight of cells in the experimental tubes as compared with controls, with the highest increase noted for SV3-3. The experimental results are encouraging for these consortia, especially sample SV3-3. The increases in dry weight in all three consortia indicate that these consortia were able to grow on the Aroclor 1254. Dry weight measurements are often used to show growth on the compound of interest (Meade, 1997). Cell numbers decreased for all consortia tested between day one and the final day of the experiment. The decreases observed in the bacterial counts are probably due to the cells beginning to die off after 28 days incubation. As the available carbon is used up, the bacteria start to die due to lack of a suitable energy source. The lack of a cometabolite also may have contributed to the decrease in cell numbers.

Table 3.5. Total bacterial counts (cfu/mL) and dry weights (mg) for selected Stephenville consortia incubated with Aroclor 1254 for 28 days.

Sample ID	Bacterial Count (initial)	Bacterial count (final)	Dry Weight of bacteria (expt)	Dry weight of bacteria (C1)	Dry weight of bacteria (C2)
SV3-1	$1.61 \times 10^8$	$4.38 \times 10^6$	79.35	77.75	64.95
SV2-2	$1.03 \times 10^8$	$9.9 \times 10^6$	78.50	76.65	73.30
SV3-3	$8.6 \times 10^7$	$2.65 \times 10^7$	72.80	68.75	70.60

Notes: Experimental tubes contained 0.1% Aroclor 1254; C = controls; C1 tubes contained 0.1% acetone; C2 tubes contained no additional carbon source. All tubes were prepared in duplicate - results shown are averages of two tubes for each dry weight value.

#### **3.3.4: Soil slurry experiments: Growth of consortia on biphenyl and Aroclor 1254**

In conjunction with SAIC Canada, the Department of Public Works and Government Services Canada (PWGSC), Royal Military College, and the University of British Columbia (UBC), a series of experiments were carried out from 2001-2003 to investigate treatment options for the PCB- contaminated Pinetree Radar Site, northwest of Stephenville. Investigators, led by Dr. W. Mohn of UBC were investigating anaerobic dechlorination of heavily chlorinated PCBs (Kuipers *et al.*, 1999) and were brought into the project to utilize the dechlorinating consortia isolated from this previous work to dechlorinate the Stephenville soil samples. Once this was achieved, the bacteria used for dechlorination were sent to RMC for scaleup using 500 kg bioreactors. The bacteria were inoculated into the bioreactors with soil slurries and incubated anaerobically for two months, then samples were sent to MUN for use in the last of five experiments carried out in this study.

PCBs are very resistant to degradation without first undergoing dechlorination, especially in soils and sediments contaminated with Aroclors 1254 and 1260, with 54% and 60% chlorine by weight, respectively. The anaerobic dechlorination of PCBs has been widely studied and is reviewed by Heider and Fuchs (1997). It occurs naturally in anaerobic sediments using nitrate, sulfate, or the PCB molecule itself as the primary electron acceptor (Alder *et al.*, 1993; Brown *et al.*, 1987; Tiedje *et al.*, 1993). Some researchers have also shown that some native bacterial consortia can preferentially remove

*ortho* chlorines (Berkaw *et al.*, 1996; van Dort and Bedard, 1991) while others dechlorinate *para* chlorines (Wu and Weigel, 1997) and still others *meta* chlorines (Kuipers *et al.*, 1999). Other researchers have demonstrated reductive dechlorination by bacteria in soil or sediment free media (Cutter *et al.*, 1998; Wu *et al.*, 2000). Most previous work has concentrated on the use of soil slurries to demonstrate dechlorination, as these are applicable to *in situ* studies (Bedard *et al.*, 1997; Brown *et al.*, 1987; Quensen III *et al.*, 1990; Tiedje *et al.*, 1993).

The benefits of reductive dechlorination are (1) it reduces the number of chlorines on the parent molecule, which can reduce the toxicity of the molecule, especially in the case of coplanar PCBs which resemble dioxins and furans (Furukawa, 1982) and (2) reduction of chlorines on the parent molecule makes it more susceptible to attack by aerobic microbes (Tiedje *et al.*, 1993; Wu *et al.*, 1998). Kuipers *et al.* (1999) studied reductive dechlorination of octa- and nonachlorobiphenyls in marine sediments by addition of enrichment cultures isolated from British Columbia, Canada, and showed evidence of complete reduction of the compounds to biphenyl. Typically, dechlorination takes up to 3 to 4 months incubation to achieve complete reduction of the compound to biphenyl. The bacteria capable of dechlorination of PCBs are not able to degrade the parent biphenyl molecule. Master *et al.* (2001) showed that reductive dechlorination did not lead to degradation of biphenyl by measuring the number of moles of biphenyl before and after treatment. The mole % remained relatively constant, although dechlorination did

occur.

The aim of these experiments was to investigate the ability of SV2-4 consortium, enriched by repeated transfer on biphenyl medium, to aerobically degrade PCBs, when inoculated into soil slurries. It has long been known that effective degradation of PCBs is usually achieved by use of bacterial consortia which contain from 6 to 12 species of bacteria. Each species is capable of attacking either the parent compound or one of its intermediates. As the primary goal in bioremediation is complete mineralization to CO<sub>2</sub> and H<sub>2</sub>O, the most extensive mineralization is achieved by a mixed community of microbes (Baker and Herson, 1994; Gibson, 1984; Norris *et al.*, 1994; Young and Cerniglia, 1995). The action of these microbial communities can be enhanced using biostimulation, which is addition of nutrients to stimulate *in situ* degradation of the compound by indigenous microbes (Harkness *et al.*, 1993), or by enriching a culture isolated from a contaminated site on a single substrate, such as biphenyl or chlorobenzoate and then inoculating the soil with the enriched culture, a process called bioaugmentation, to increase the rate of PCB biodegradation (Hickey *et al.*, 1993).

The experiments were carried out using three different soil samples, all originally collected from the Stephenville site. The first two experiments used low-PCB soil (20 mg/kg), the second two used high-PCB soil (>80 mg/kg), and the last used the dechlorinated soil slurry from RMC, as described above. The consortium was tested in soil slurries with MSM alone, spiked with biphenyl as cometabolite, or spiked with both

Aroclor 1254 and biphenyl.

In experiment 1, no PCB or biphenyl was added. When the enriched bacteria were incubated on a shaker in a soil slurry for 22 days, GC results show a decrease in PCB concentration ranging from 25-95% , or an average of 63% reduction for the three experimental replicates as compared with the control (Fig. 3.15). Bacterial counts (Fig. 3.16) show that the bacterial numbers decreased between day 1 and day 22 of the experiment. This was expected, as no supplemental source of carbon was added. However, the decrease in PCB concentration indicates that the added consortia was able to substantially degrade the PCB present. Without the addition of a cometabolite, or spiking with the Aroclor mixture, there was not enough carbon available to sustain the bacterial consortia for the full 22 days. Despite this fact, the bacterial consortium was able to decrease the concentration of PCB in the soil by up to 95% in 22 days. This result shows the benefit of using bioaugmentation to enhance the degradation of PCBs in soil. The enrichment of the consortium on biphenyl activated the enzymes necessary to effect PCB degradation (Hickey *et al.*, 1993).

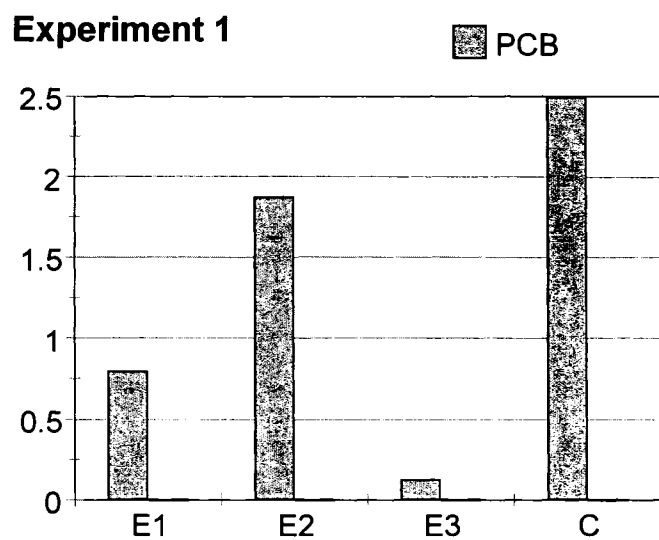


Fig.3.15. PCB concentrations ( $\mu\text{g/g}$ ) for soil slurries augmented with SV Consortium culture (unspiked). Notes: Biphenyl was not analyzed ; E = experimental; C = control.

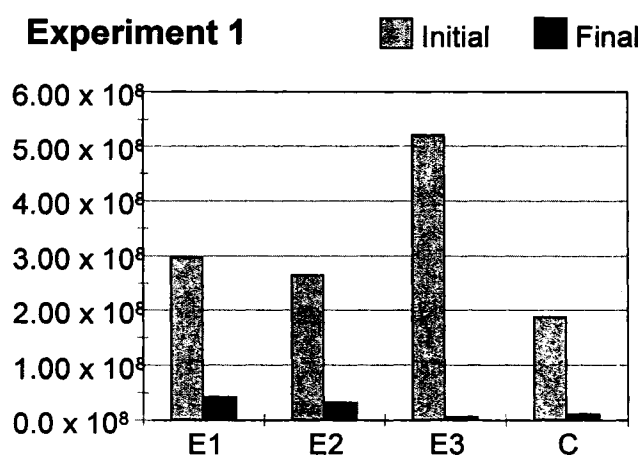


Fig. 3.16. Bacterial counts (cfu/mL) for soil slurries augmented with SV consortium culture. Notes: Initial counts taken on day 1, final counts on day 22; E = experimental, C = control.



In experiment 2, the experimental flasks and the control were supplemented with 0.01% biphenyl. The only difference between the experimental and the control is the addition of bacterial consortia culture to E1-E3, but not to the control. The results show a decrease in the PCB concentration for the experimental flasks ranging from 15-54% in the experimental replicates, averaging 32% reduction as compared with the control (Fig. 3.17). This result shows that the addition of the biphenyl-enriched bacterial consortia aided the native consortia in degrading the PCB present. Bioaugmentation with enrichment cultures grown on the parent compound has been shown to enhance biodegradation of PCBs in soil slurry experiments (Abramowicz, 1990). Bacterial numbers (Fig. 3.18) also decreased, but not as much as in experiment 1, due to the addition of the biphenyl as cosubstrate. However, the extent of PCB degradation was only half that observed in experiment 1. This may be due to the fact that the control was also spiked with 0.01% biphenyl. The addition of biphenyl as cosubstrate probably allowed some biodegradation of PCBs to occur via the native, non-enriched consortia present in the PCB-contaminated soil used in the slurries. Without an unspiked soil sample for comparison, this hypothesis cannot be confirmed.

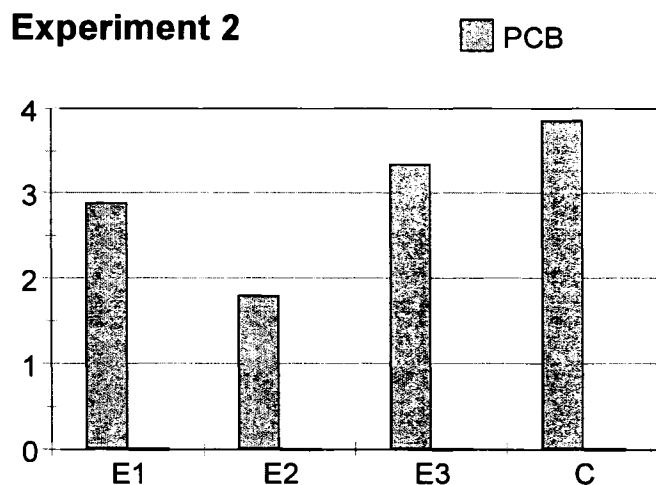


Fig. 3.17. Total PCB concentrations ( $\mu\text{g/g}$ ) for soil slurries augmented with SV2-4 consortium (experimental flasks) Notes: All flasks spiked with 0.1% biphenyl; E = experimental, C = control; biphenyl not shown as concentration was below detection limits.

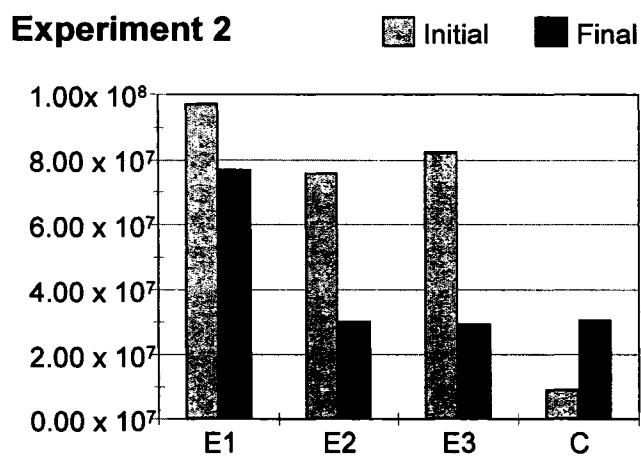


Fig. 3.18. Total bacterial counts (cfu/mL) for soil slurries augmented with SV2-4 consortium (experimental flasks). Notes: E = experimental, C = control; All flasks spiked with 0.01% biphenyl. (Initial:day 1, Final:day 22)

Experiment 3 used 2 mm soil spiked with 200  $\mu$ L AR1254 (E1-E4) and one gram of biphenyl (E3 and E4). Following 22 days incubation, PCB concentrations (Fig 3.19) for E1 and E2 (no biphenyl) were slightly lower than that for ES1, which contained only soil and AR1254, with a concomitant increase in biphenyl concentration, as compared with the control, C1. These results show that some PCB may have been degraded in these cultures. The results for E3 and E4 (soil plus AR1254 and biphenyl) show that both experimental cultures almost completely metabolized the added biphenyl (as compared with the soil plus AR1254 and biphenyl, ES2). As well, the PCB concentration decreased by almost half in E4 as compared with ES1 and ES2 (Fig.3.19). These results show that PCB was more efficiently degraded when biphenyl was added as cosubstrate. Further, as compared with experiment 2, the increase in the amount of biphenyl added as cosubstrate has the potential to effect greater biodegradation of PCBs, as the bacterial consortia were able to continue growing throughout the experiment. Figure 3.20 shows that bacterial numbers increased in E3 and E4, indicating that they were able to grow on the substrates provided. The increase in bacterial numbers for E1, which was not spiked with biphenyl, may be due to the presence of other hydrocarbons in the soil sample which could serve as a cosubstrate. Previous work has shown that the inhomogeneity of PCBs can cause large variability in their measurement, especially in environmental samples (Master *et al.*, 2001).

### Experiment 3

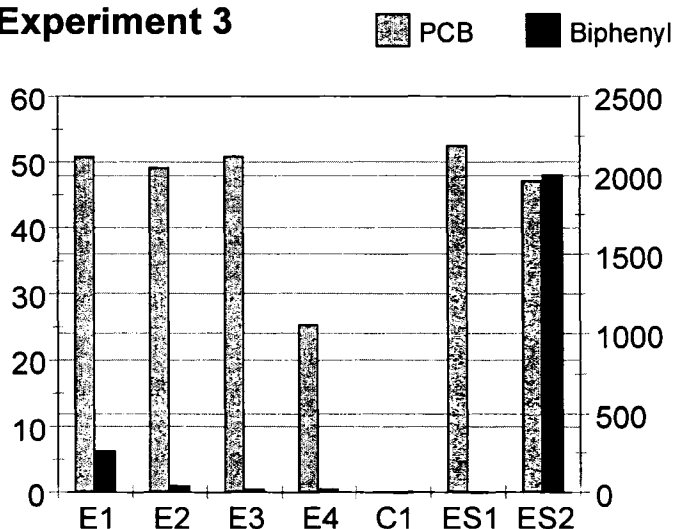


Fig. 3.19. PCB ( $\mu\text{g/g}$ ) and Biphenyl (ppm) concentrations for soil slurries augmented with SV2-4 consortium culture (experimental flasks). Notes: E = experimental, C = control; E1-E4 spiked with Aroclor 1254 (50 mg/mL in acetone); E3 and E4 Also spiked with 1g biphenyl; ES1 = soil + PCB, ES2 = soil + PCB + biphenyl)

### Experiment 3

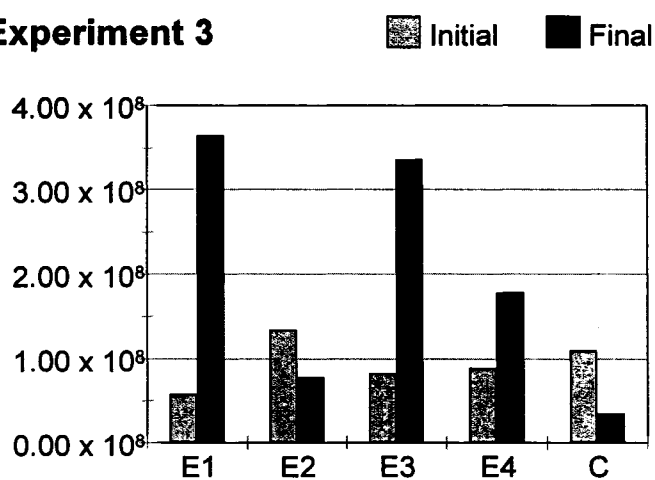


Fig. 3.20. Total bacterial counts (cfu/mL) for soil slurry experiment 3. Note: E1-E4 spiked with 200 $\mu\text{L}$  Aroclor 1254 (50 mg/mL in acetone); E3 and E4 also spiked with 1g biphenyl. E = experimental, C = control.

In experiment 4, a higher PCB (~ 80 mg/kg) soil sample was used. E1 and E2 were spiked with 0.5% biphenyl plus added consortia, while E3 and E4 had microbial consortia only added. The resulting PCB concentrations show higher values for E3 and E4 (no biphenyl) than for E1 and E2 (biphenyl added). All experimental cultures showed a dramatic decrease in PCB concentration as compared with the control (Fig.3.21). In all, there was a 35 to 90% reduction in the total PCB concentration in experimental flasks 1-4 as compared with the control. The average percent reduction in experimental flasks 1 and 2, where biphenyl was added was 80%. In contrast, experimental flasks 3 and 4, where no cosubstrate was added, had an average total PCB reduction of 50%. Results also show that bacterial numbers increased for E1 and E2, where biphenyl was added, but decreased in E3 and E4, where no cosubstrate was added (Fig. 3.22). These results further support that added biphenyl plus consortia effects greater rates of PCB degradation than added consortia alone. This result is supported by other studies, which have shown that bioaugmentation with PCB degrading bacteria and biostimulation with biphenyl enhances the rate of PCB degradation (Master *et al.*, 2001).

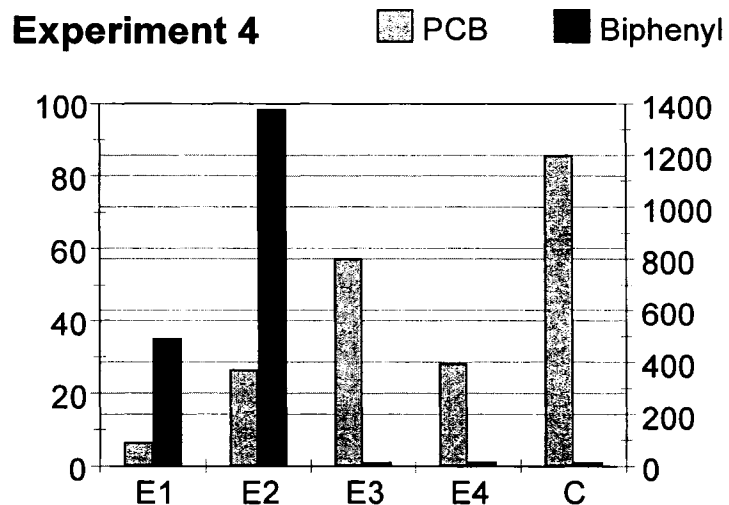


Fig. 3.21. Final PCB ( $\mu\text{g/g}$ ) and Biphenyl (ppm) concentrations for soil slurries augmented with SV2-4 consortium, experiment 4. Notes: E = experimental, C = control; E1 and E2 spiked with 1g biphenyl.

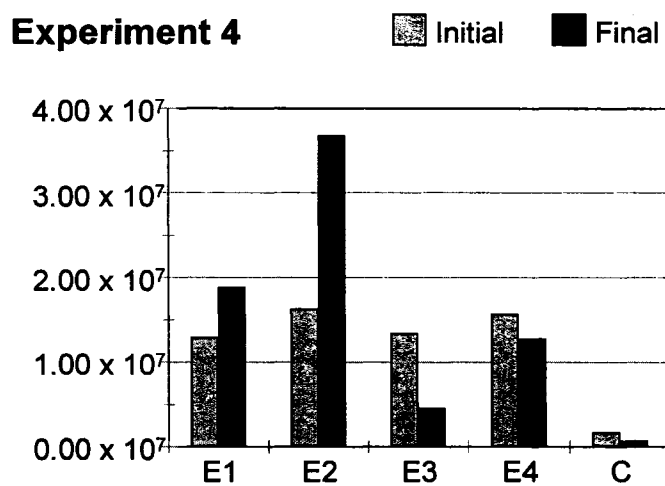


Fig. 3.22. Total bacterial counts (cfu/mL) for days 1 (initial) and 22 (final) for Soil slurries augmented with SV2-4 consortium. Notes: E = experimental, C = control; E1 and E2 spiked with 1 g biphenyl.

The last experiment was performed using dechlorinated soil obtained from RMC labs in Kingston, Ontario. This experiment was done to show the effect of the added bacterial consortia on the dechlorinated PCB present. In this experiment, E1 and E2 were spiked with biphenyl. Results show that PCB concentration decreased for both experimental setups to half of the control concentration (Fig. 3.23), regardless of the amount of biphenyl added. The decrease in PCB concentration, even in the samples not supplemented with biphenyl, indicates that the dechlorination of the PCBs reduced the number of chlorine constituents on the PCB molecules, thus allowing the consortia to effect degradation at similar rates to the unspiked samples. These results show that PCB degradation can be accomplished by sequential anaerobic/aerobic degradation steps. Bacterial numbers increased for E1 and E2, and decreased for E3 and E4, as compared with the control (Fig. 3.24). This result indicates that the use of biphenyl as cosubstrate kept the bacteria growing throughout the experiment. However, the results of this experiment show that dechlorinated soil may not need biostimulation with a cosubstrate to effect degradation, since the addition of the enriched consortia was enough to stimulate biodegradation of the lower molecular weight PCBs. The problem with dechlorination is that it requires a lengthy incubation time (up to three months) and the dechlorinating strains are incapable of degrading the parent biphenyl molecule (Kuipers *et al.*, 1999, Master *et al.*, 2001).

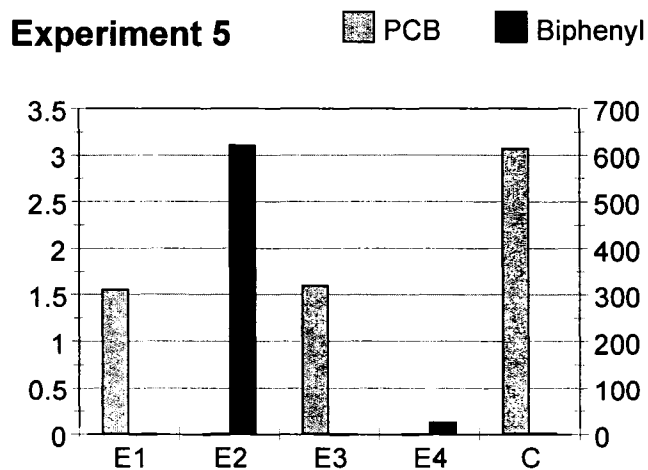


Fig. 3.23. PCB ( $\mu\text{g/g}$ ) and Biphenyl (ppm) concentrations for soil slurry experiment 5. (E1 and E2 spiked with biphenyl). Note: dechlorinated soil used; not enough Sample to analyze each flask for both PCB and biphenyl, so E1, E3 analyzed For PCB, E2 and E4 for biphenyl, C for both; E = experimental, C = Control.

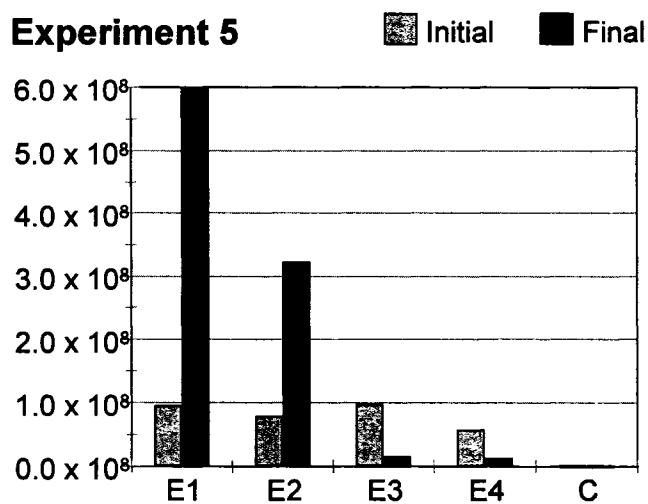


Fig. 3.24. Total bacterial counts (cfu/mL) for slurry experiment 5, using dechlorinated Soil augmented with SV2-4 consortium. Notes: E1 and E2 spiked with 0.5g biphenyl; E = experimental, C = control.



## **Chapter 4: Summary and conclusions**

### **4.1: Research summary**

All of the soil (Stephenville) and sediment (Argentia) samples contained bacteria that grew on biphenyl as sole source of carbon, showing the ubiquitous nature of biphenyl degraders in soils and sediment. It is probable that these organisms proliferate and ultimately dominate the soil microflora when contamination with polyaromatic hydrocarbons occurs. Many of the Argentia consortia also showed the ability to degrade other compounds, such as toluene, naphthalene, phenanthrene, and phloroglucinol. Several enzymes responsible for naphthalene degradation are also able to metabolize biphenyl and other polyaromatic compounds (Barriault *et al.*, 1998).

The sediment samples from Argentia showed a wide diversity of microbes present, with 18 strains characterized based on Gram reaction, shape, motility, and several biochemical tests which outlined their enzyme systems. It should be noted that these strains, initially delineated by differences in colony characteristics, were collected from all 10 samples, so may not be representative of each individual consortium. The consortia which were characterized in this study were those which showed growth on the compounds of interest. Most of the PAH and PCB degraders were Gram negative rods, and several were shown to be capable of anaerobic growth (for example, SV3-2 grew anaerobically in the TPPB experiment). This result is consistent with the growth of many of the Argentia soil isolates in the anaerobic region of Thioglycollate broth.

PCB degradation was clearly demonstrated by three of the consortia in this study. GC results were used to confirm the bacterial action on Aroclor 1254, a PCB mixture. From the data, it is apparent that the consortia were able to degrade some of the congeners present in the mixture. Due to the complexity of the mixture, and the number of homologues for several of the congeners, it was not possible to tell which of the congeners was being degraded, as well as the metabolic end products for each congener. For example, the Aroclor 1254 used in this study contains a large proportion of tetrachlorobiphenyls (42 possible isomers), pentachlorobiphenyls (46 possible isomers) and hexachlorobiphenyls (42 possible isomers) (<http://www.epa.gov/toxteam/pcbid/defs.htm>). It has been shown in previous studies that most aerobic consortia can degrade only the lower-chlorinated congeners (mainly mono-, di-, and trichlorobiphenyls), due to steric hindrance, especially in the case of coplanar PCBs (Baker and Herson, 1994). Thus, most studies have concentrated on use of single congeners, or defined mixtures of congeners (Bedard *et al.*, 1986). Research is currently underway to isolate and characterize single strains of bacteria, from local soils, which are capable of PCB biodegradation (Lambo and Patel, 2005a, in press). When using single strains, it is possible to determine the metabolic end-products of PCB metabolism using GC-MS. In this way, the abilities of the local bacteria to degrade PCBs can be characterized.

#### **4.2: Potential for on-site bioremediation**

Master and Mohn showed that PCB degradation rates were higher at temperatures above 20°C than at lower temperatures, even for species of bacteria isolated from Arctic and subarctic sites. This demonstrates that enzyme activity, and thus PCB degradation is temperature sensitive (Master and Mohn, 1998). For this reason, it was decided to run the PCB degradation experiments at 22°C rather than at 5°C. Most on-site applications would be undertaken in the summer, so the bacterial consortia would be more effective if grown at a higher temperature to allow maximum enzymatic activation. PCB degradation is a slow process due to the recalcitrance of the PCB molecule, so any factor, such as increased temperature, which could increase the rate of reaction would be beneficial to the removal of PCBs from northern environments. The consortium's ability to be psychrotolerant would allow the bacteria to survive in temperatures which may be suboptimal to some mesophilic species. Use of native consortia thus increases the chance of survivability *in situ*. Since many contaminated sites are in riverbeds, the lower ambient temperatures may slow down the naturally occurring biota from degrading the PCBs. Enrichment of native consortia by growth at higher temperatures may make these bacteria and their enzymes more thermostable and increase their degradative abilities.

This study shows that the catalytic activity of these psychrotolerant enzymes was maintained at temperatures up to 28°C. Thus, these bacterial consortia may be used in applications in temperate regions, such as soils and aquifers. Mohn *et al.* (1997) showed

that Arctic soil bacteria could mineralize biphenyl and PCBs at 7°C and 30°C. However, the rate of degradation was higher at 30°C. This research demonstrates the thermostable nature of some psychrotrophic, or psychrotolerant bacteria. Several of the isolates used in this study were observed to be capable of growth at refrigeration temperatures. Recent work has confirmed that psychrotrophic bacteria capable of degrading PCBs are present in Newfoundland soils (Lambo and Patel, 2005b, in press).

In this study, the use of biphenyl as cosubstrate was shown to enhance PCB degradation in soil slurry experiments. This result is supported by recent studies, which also show that soils contaminated with PCBs showed higher rates of mineralization of biphenyl than soils not contaminated (Mohn *et al.*, 1997). This research shows that previous contamination with hydrocarbons selects for organisms capable of their degradation.

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