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Major professor

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ENHANCEMENT OF SITE SPECIFIC ANAEROBIC REDUCTIVE DECHLORINATION OF POLYCHLORINATED BIPHENYLS

By

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ABSTRACT

ENHANCEMENT OF SITE SPECIFIC ANAEROBIC REDUCTIVE DECHLORINATION OF POLYCHLORINATED BIPHENYLS

By

Matthew John Zwiernik

Polychlorinated biphenyls (PCBs) are widespread, priority pollutants which persist in the environment and tend to bioaccumulate. Toxicological data has shown that PCBs elicit a spectrum of toxic responses in both humans and laboratory animals. These characteristics have implicated PCBs in the decline of fish eating birds and mammals. Although they are considered recalcitrant microorganisms can degrade PCBs. The bioremediation of PCBs has been conceptualized as a sequential process involving the anaerobic reductive dechlorination of PCBs followed by aerobic mineralization. This has not been realized because the full potential of anaerobic reductive dechlorination of PCBs is rarely achieved. This dissertation describes investigations designed to identify and overcome site specific limitations to the maximum extent of anaerobic PCB dechlorination.

Because PCBs are of industrial origin they are usually associated with related environmental pollutants. Residual petroleum hydrocarbons and

other non-polar contaminants were found to reduce both the rate and extent of PCB dechlorination. This response was identical to that which would be predicted based solely on the reduction of PCB solution concentrations due to an innocuous sorptive phase. This suggests that petroleum hydrocarbons reduce the bioavailability of PCBs to dechlorinating microorganisms.

Heavy metals are the most commonly observed co-contaminant associated with PCBs. Anaerobic reductive dechlorination of PCBs in laboratory assays were adversely affected by zinc solution concentrations less than or equal to those found at many PCB contaminated sites. We therefor tested two means of alleviating metal toxicity: precipitation (adding $FeSO_4$) and chelation (adding citrate or EDTA). Metal toxicity was reversed by additions of EDTA or citrate; however, in slurries amended with $FeSO_4$ dechlorination was enhanced. Subsequent experiments designed to elucidate the mechanism of enhancement suggest that sulfate stimulates the growth of sulfate reducing organisms responsible for PCB dechlorination, while Fe^{2+} reduces sulfide bioavailability and hence toxicity. Ferrous sulfate is an inexpensive, innocuous compound which could be utilized to overcome factors limiting both the extent of *in-situ* dechlorination in metal and non-metal contaminated sediments as well as the implementation of sequential anaerobic/aerobic biotreatment systems.

To my Parents; John Anthony Zwiernik, Jr. and Susan Marie Zwiernik, who have given me every opportunity to achieve my goals. To my brother Michael and sister Julie who have shaped my life more than they could know.

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INTRODUCTION

Polychlorinated biphenyls (PCBs) are widespread, priority pollutants which persist in the environment and tend to bioaccumulate. It is estimated that approximately 1.4 billion pounds of PCBs have been produced worldwide and that several hundred million pounds have been released into the environment since 1929.[1] Although the use of PCBs has been restricted since the 1970's they are ubiquitous environmental contaminants.[2] Once introduced into the environment PCBs tend to persist due to their thermal, chemical and biological stability. PCBs are hydrophobic in nature and therefore tend to associate with organically rich material. Environmentally exposed organic phases such as river sediments or biological tissue lead to accumulation, bioaccumulation, and eventually bioconcentration.[3] Toxicological data on PCBs have shown that PCBs elicit a spectrum of toxic responses in both humans and laboratory animals. These include; lethality, reproductive and developmental toxicity, immunosupressive effects, hepatotoxicity, neurotoxicity, and carcinogensis.[4-7] In 1976 the United States Environmental Protection Agency listed PCBs as a priority

pollutant stirring extensive interest in the safe disposal and/or biotransformation of these compounds.

Commercial PCBs were manufactured and used as complex mixtures consisting of 60 to 90 (of a possible 209) PCB congeners, differing in position and number of chlorines on the biphenyl structure.[2] Chlorines may be attached to one or both rings and may vary from one to ten (Figure 1). These chlorines not only give PCBs their thermal and chemical stability, they also impart a biological stability as well. This is because common microbial oxygenase enzymes used in the aerobic degradation of similar compounds are obstructed from the aromatic rings of the biphenyl by the chlorine substituents.[8] It is only since the recent discovery of the microbial reductive dechlorination of PCBs that microbial destruction has been considered an important environmental fate.[9-11]

The aerobic degradation of highly chlorinated PCBs generally does not occur. However mono- and di-chlorinated biphenyls are mineralized rather rapidly in well aerated systems because biphenyl is highly reduced allowing aerobic organisms to use it as a source of carbon, hydrogen, and electrons. In contrast, highly chlorinated biphenyls are less reduced and also lack available hydrogens. Therefore the more highly chlorinated biphenyls can only serve as a carbon source after the chloro-substituents which block

enzymes from attacking the ring are removed. Removal of the chloro-substituents requires immense amounts of energy in an aerobic environment making utilization of highly chlorinated PCBs as a single substrate energetically unfavorable. As a result there are four general relationships between PCB structure and aerobic biodegradation.[12] 1)The less chlorinated the biphenyl the faster the degradation takes place. 2)Dioxygenation takes place on the ring with the least chlorine substituents. 3)PCBs with chlorine substituents on both rings are more recalcitrant than isomers containing an unchlorinated ring. 4)PCBs with more than 5 chlorine substituents are generally not degraded in aerobic systems.

Anaerobic reductive dechlorination of PCBs involves the removal of chlorine atoms directly from the biphenyl ring and replacement with hydrogen. In the anaerobic environment the growth of microorganisms is generally limited by the availability of electron acceptors. Microorganisms can utilize relatively oxidized PCBs as electron acceptors by employing the process of reductive dechlorination. The resulting mixture of PCB congeners is electrmagnetically reduced and contains less chlorine. Two important environmental consequences result. First, the steric hindrance encountered by common oxygenase enzymes is decreased resulting in a mixture that is more energetically favorable and susceptible and to aerobic

mineralization (conversion of PCBs to CO_2). Secondly, the resulting congener mixture is generally less toxic.[13-15]

Practical bioremediation schemes utilizing sequential anaerobic/aerobic treatments of PCBs have not been realized to date. While the aerobic degradation of di, mono, and unchlorinated biphenyls in the environment is rapid, the anaerobic dechlorination required to get them there is generally slow, inconsistent, and incomplete. While *in-situ* reductive dechlorination has now been reported in anaerobic sediments at numerous locations, Hudson River (NY), Silver Lake (MA), Sheboygan River (WI), Waukegon Harbor (IL), New Bedford Harbor (MA), Hoosie River (MA) and the River Raisin (MI), the extent varies widely among sites, ranging from 0 to >90 percent.[16] Explanations for this variation include lack of appropriate organisms and/or environmental conditions.

DISSERTATION OBJECTIVES

The overall objective of this research is to consistently enhance microbial PCB reductive dechlorination to make it a useful bioremediation technology. This includes; 1)identifying environmental factors that limit PCB dechlorination in anaerobic sediments, 2)developing treatment methods

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to overcome those factors, 3)consistently maximize the rate and extent of PCB dechlorination observed, 4)and identifying the organism or organisms responsible for PCB reductive dechlorination.

BACKGROUND INFORMATION ON PCBS

PCB Nomenclature

The term polychlorinated biphenyl (PCB) is used to refer to the entire class or any one subset of one or more compounds having the formula $C_{12}H_{10-n}Cl_n$ (where n=1-10; i.e., mono-chlorobiphenyl through deca-chlorobiphenyl), with the general structure represented in Figure 1A. This results in the possibility of 209 different PCBs which are said to be congeners. When PCBs are subdivided by degree of chlorination, the term homologue is used. PCBs of a given homologue with different chlorine substitution positions are called isomers.

Full chemical names of PCBs have proven unwieldy resulting in numerous shorthand nomenclatures. Throughout this text we will identify chlorines on ring A (ring containing the most chlorines) by their numbered position (Figure 1B). Chlorines on ring B will be identified by the position

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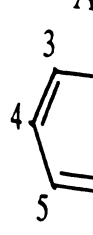
number followed by a prime symbol (2'), and the abbreviation CB will be used for chlorobiphenyl. Chlorine positions will be described in ascending order; example 2,2'4,4'6-CB. Chlorine positions can also be described according to their relative location on the biphenyl molecule. Chlorines located at positions 2 and 6, 3 and 5, and 4 are described as *ortho*, *meta*, and *para* chlorines, respectively (Figure 1C).

Complex commercial mixtures used in these studies were manufactured by Monsanto and sold under the registered trade-mark of Aroclor. The mixture Aroclor 1242 for example means that the mixture contained 12 carbons (biphenyl) and was 42 percent chlorine. This system of nomenclature also holds true for Aroclor 1248, 1254, 1260 and 1262.



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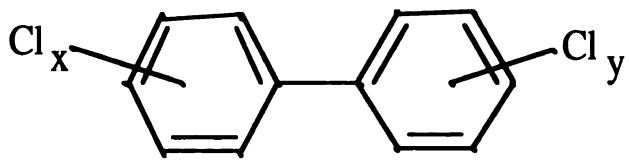

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209 congeners are theoretically possible
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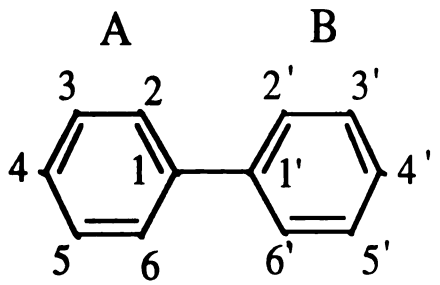

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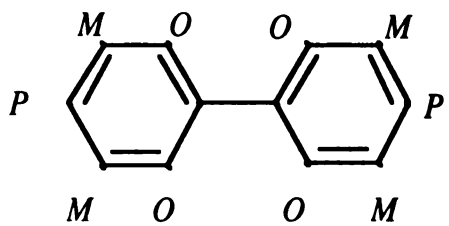

(C)

P-para *M-meta* *O-ortho*

Position Numbering

Relative Position Designations

Figure 1. Polychlorinatedbiphenyl (PCB) nomenclature. (A)The general structure of PCBs. (B)The numbering of positions ont the biphenyl ring. (C)Relative substitution position on the biphenyl ring.

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Physical and Chemical Properties

PCB physical properties include a high log K_{ow}, low water solubility, low vapor pressure and high dielectric constants. The chemical properties include flame retardance and low chemical reactivity (resistant to acids, bases, and hydrolysis and oxidation).[17]

PCB Uses

Commercial PCB mixtures were used in a wide variety of applications, including dielectric fluids in capacitors and transformers, heat transfer fluids, paints, lubricating and cutting oils, hydraulic fluids, pesticide additives, copy paper, carbonless paper, adhesives and plastics.[1]

PCB Toxicity

PCBs are now known to elicit a spectrum of toxic responses in both humans and laboratory animals including lethality, reproductive and developmental toxicity, porphyry, body weight loss, dermal toxicity, immunosupressive effects, hepatotoxicity, neurotoxicity, thymic atrophy, and carcinogensis.[4,6,7] Reproductive failure linked to PCBs has been

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observed in several mammalian species and has been implicated as instrumental in the declining populations of fish eating birds and mammals.[4,18-20] PCBs have been implicated as environmental endocrine disrupters. Increases in the uterine weight and uterine glycogen were observed in female rats exposed to commercial PCBs.[21,22] Increased testicular weight was observed in rats exposed to Aroclor 1254 before weaning and in mice exposed to 2,2',4,4',5,5'-hexachlorobiphenyl during gestation.[23,24] *In vitro*, PCBs have been shown to directly inhibit the fertilization of mouse gametes.[25]

Present Status and Location of PCBs

PCBs can be considered ubiquitous pollutants. They have been found in nearly all marine plant and animal specimens, fish, mammals, birds (especially fish-eating birds), bird eggs,[26] and of course, humans. All U.S. residents have measurable PCBs in their adipose tissue.[27] Background levels are generally considered to be parts per million (ppm) in sediments, parts per billion (ppb) in soils and food, and sub-parts per trillion in water.[17] By virtue of their high octanol-water partitioning coefficient (K_{ow}) PCBs tend to accumulate in the non-polar lipid and fatty tissues of living

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organisms.[2] As with the well publicized case of DDT,[28] PCBs biomagnify through the food chain (Table 1). Respective concentration factors of 10^3, 10^5 and 10^8 have been reported from Lake Ontario water to sediments, catfish, and Herring gulls.[29] This brings to the forefront the extreme importance of even low level sediment contamination on the exposure of PCB to all parts of the food chain.

The National Research Council states that the major continental sink for PCBs is fresh water sediments. Of the 1.25 billion pounds of PCB estimated to have been produced in the United States about 25 million pounds remains accessible to the mobile environmental reservoir.[30] The environmental transport of PCBs to this fresh water sediment (sink) is complex and global. Both non-point and point sources are responsible. Non-point sources include atmospheric deposition by rain, snow, dry fall out, and vapor phase. Point sources consist primarily of underground leakage by abandoned industrial waste confinement and dump sites.

Table 1. Estimated concentrations and distribution of PCBs in the environment.

Matrix	*Location*	*Concentration Range*	*Mass*
Air	Rural	0.1-2 ng/m^3	
	Urban	0.5-30 ng/m^3	
	Great Lakes	0.1-5 ng/m^3	
Water	Marine	0.3-10 ng/L	
	Great Lakes	1-150 ng/L	
Soil	Rural	<1 ng/g	
	Urban	<1-2 ng/g	
Sediments	Marine	< 1 ng/g	~1x10^9 g
	Fresh water	10-250 µg/g	~ 4x10^9 g
Tissue	Fish	0.1-190 µg/g	
	Fish eating birds	100-14,000 µg/g	
	Fish eating mammals	1-45 µg/g	0.3x10^6 g
	Human adipose	0.3-10 µg/g	

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It is estimated that 8.8 million pounds of PCBs presently reside in fresh water sediments.[31] These compounds are most often associated with other contaminants of industrial origin. The International Joint Commission (formed by the U.S. and Canada) has designated 31 sediment sites as areas of concern due to environmental contamination within the U.S. and joint waters of the Great Lakes basin alone. Of these 31 sites 29 contain PCBs as a primary or secondary contaminant.[32] One single 23 mile stretch of the Hudson River has received ~1 million pounds of PCBs between 1966 and 1974 most of which now lies in the river sediment.[33] The EPA presently has on record 646 sites which are contaminated with PCBs. Of these ~70% are or include fresh water sediment.[34]

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CHAPTER 1

The Effects of Petroleum and Associated Non-polar Co-contaminants on the Bioavailability and Reductive Dechlorination of Aroclor 1242

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ABSTRACT

Because of their widespread use together in industrial applications residual petroleum hydrocarbons and other non-polar contaminants are often found in conjunction with PCBs at contamination sites. Intrinsic reductive dechlorination of PCBs at these sites is often limited or non-existent. Sediments of one such site, Silver Lake (MA) do not support PCB dechlorination either *in-situ* or in laboratory assays despite some historical evidence of minimal dechlorination. Dechlorination assays using a model system (known to support PCB dechlorination) amended with either pure petroleum hydrocarbons or the non-polar contaminants extracted by 1,1,2-trichlorotrifloroethane (CFC) from Silver Lake sediments resulted in a reduction in both the rate and extent of PCB dechlorination. This response was identical in both slope and intercept to that which would be predicted based solely on the reduction of PCB solution concentrations due to an innocuous sorptive phase (added non-polar compound). This research suggests that petroleum hydrocarbon co-contaminants in the absence of any associated toxic components reduces the bioavailability of PCBs to dechlorinating microorganisms.

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INTRODUCTION

Polychlorinated biphenyls are ubiquitous environmental contaminants of concern because of their toxicity, persistence, and tendency to bioaccumulate. Polychlorinated biphenyls were often used in conjunction with petroleum hydrocarbons. This is because each can impart desirable characteristics, e.g. flame retardance, heat stability and high temperature viscosity for PCBs and fluidity, friction reduction, and heat transfer for petroleum hydrocarbons, needed for industrial applications.[1] Improper disposal of these mixtures (dielectric fluids, lubrication oils, cutting oils, hydraulic fluids, and heat transfer fluids) results in the presence of these compounds as co-contaminants at many sites. If biological remediation of PCBs is to become a viable option for their destruction, the effects of petroleum hydrocarbons on PCB reductive dechlorination must be understood.

Petroleum hydrocarbons and chlorinated biphenyls are both mixtures of nonionic organic compounds (NOCs) with high octanol-water partition coefficients (K_{ow}) and low water solubilities (S_w). At relatively low levels of concentration (sub ppm) the constituents of these mixtures partition into soil and sediment organic matter where they become immobilized.[2] At higher concentrations, both petroleum hydrocarbon mixtures and

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commercial PCB mixtures may form separate (water immicible) phases that become associated with soils and sediments.[3,4]

Petroleum hydrocarbons and chlorinated hydrocarbons are generally considered recalcitrant in anaerobic environments. Metabolic steps in the biodegradation of these compounds follow two major strategies: oxidation and/or reduction. In anaerobic environments PCBs are reductively dechlorinated but not oxidized.[5] Hydrocarbons are already chemically reduced and hence generally are not subject to significant reductive transformations.[6] Numerous studies have demonstrated biodegradation of aromatic hydrocarbons under strict anaerobic conditions.[7] However the primary mode of action still follows an oxidative strategy. Toluene, benzene, and a few alkanes have been shown to be oxidatively biodegraded under strict anaerobic conditions by sulfidogenic and methanogenic cultures.[8-10] In the absence of molecular oxygen, water derived oxygen serves as a reactant, and carbon dioxide or sulfate as electron acceptors. These few substrates are oxidized to hydroxylated aromatics or fatty acids and then further metabolized by ring cleavage and beta-oxidation.[11]

It has been suggested that hydrocarbons in association with PCBs may prevent or limit the process of anaerobic reductive dechlorination.[12,13] Physiological as well as environmental factors have been implicated. Light

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aliphatic hydrocarbons (C3-C8) have a higher water solubility than the heavy aliphatic hydrocarbons which may increase their bioavailability and hence toxicity to bacteria. In addition light aliphatic hydrocarbons may have a solvation effect on cellular lipids and membranes, altering their permeability or destroying the cellular integrity.[14] Other co-contaminants such as methylated mercury partition into the hydrocarbon mixture and may also be inhibitory or toxic to bacteria which are capable of dechlorinating PCBs.[15] Petroleum hydrocarbon co-contaminants supply a major source of carbon resulting in increased numbers of less diverse microorganisms.[16-18] Under otherwise nonlimiting conditions these co-contaminants provide a selective advantage to hydrocarbon utilizing bacteria. The resulting population shift produces a less diverse bacterial community which is less likely to possess the ability to reductively dechlorinate PCBs.

The presence of bulk phase hydrocarbon in sediment may substantially increase the coefficients (K_p) of the congeners and this may limit the bioavailability of PCBs to the dechlorinating organisms.[4,5] The bulk hydrocarbon phase has been shown to be an effective partition medium for poorly water soluble organic contaminants.[19] In general, sorption of contaminants by soils, and sediments reduces their bioavailability to microorganisms.[20,21] The presence of an additional partition phase in the

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form of bulk phase hydrocarbon may further reduce bioavailability, and in this fashion limit the rate and/or extent of PCB dechlorination.

The objective of this study was to determine whether, and to what extent, petroleum hydrocarbons inhibit the reductive dechlorination of PCBs in anaerobic sediment slurries. PCB dechlorination was evaluated utilizing clean sediments amended with a combination of petroleum hydrocarbons and PCBs, in parallel with sediments from a environmental site which contain these compounds and which presently do not support the reductive dechlorination of PCBs.

MATERIALS AND METHODS

Three experiments were designed to investigate the effects of petroleum hydrocarbons on PCB dechlorination as well as the specific effects of Silver Lake oils and greases and their associated co-contaminants. PCBs found in Silver Lake (SL) sediments have historically undergone limited *in-situ* anaerobic reductive dechlorination. Unlike other sediments the SL sediments do not support dechlorination in laboratory assays.[22]

In the first experiment the possible inhibitory effects of SL petroleum hydrocarbons and their associated non-polar co-contaminants were tested

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by removing these compounds from the SL sediment and then testing the ability of the extracted sediments to support anaerobic reductive dechlorination. First however we had to establish that the solvent extraction process itself did not adversely affect the ability of sediments to support dechlorination. To accomplish this we compared the ability of Red Cedar River (Lansing, MI) sediments which are known to support PCB dechlorination, to their solvent extracted counterparts. The Red Cedar River (RC) sediments used in this preliminary experiment, as well as the SL sediments used subsequently, were processed in identical manners. Samples (20 g) of air dried sediment were solvent extracted with either 1,1,2-trichlorotrifloroethane (CFC) or dichloromethane (DCM) in a Soxhlet apparatus for 12 hours. Sediment samples were then air dried for 24 hours. Solvent was then removed from the non-polar fractions of the SL sediment extracts using a rotary evaporator, and the non-polar co-contaminant content of the sediment was determined gravimetrically.

The ability of the RC and SL sediments, before and after solvent extraction, to support anaerobic reductive dechlorination was tested. Experimental vessels consisted of 60 ml serum bottles which contained 10 g of sediment. The bottles were then evacuated and refilled with N_2 in a glove box lock, then flushed with N_2-CO_2 (80:20,vol/vol) with a Hungate

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apparatus. The bottles were first tested for their ability to maintain strict anaerobic conditions. For this, inoculum containing known PCB dechlorinating organisms was prepared by eluting them from HR sediments as previously described.[23] A 10 ml portion of the HR inocculum was amended with 10 µl ethanol and added to each bottle. The bottles were sealed with butyl stoppers and aluminum crimp caps, and incubated in the dark at 37°C for 10 days. After ten days, headspace gas was analyzed for CH_4 and bottles which contained methane (indicating strict anaerobic conditions) were autoclaved for 90 minutes on two consecutive days. Methane production was determined by gas chromatography using a thermal conductivity detector.

The bottles were then re-inoculated to assay for PCB dechlorination activity. Twenty four hours after removal from the autoclave 10 ml inoculum eluted from PCB contaminated HR sediments were added in addition to 10 ml sterile RAMM.[24] Using sterile anaerobic technique the butyl stoppers were removed the bottles. The bottles were then flushed with purified, filter-sterilized N_2-CO_2 (80:20,vol/vol) and 80 µl of the PCB congener 2,34 trichlorobiphenyl in acetone was added to give a final PCB concentration of 250 µg/g sediment. Teflon lined stoppers and aluminum crimp caps were used to reseal the experimental vessels. The biological

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controls (negative controls) were autoclaved 90 min. twice, with a 24 hour interval of incubation at room temperature in-between. All the samples were then shaken by hand for 1 min. and subsequently incubated stationary in the dark at 25°C. Sampling for PCB dechlorination activity and methane production took place at 6 week intervals. Methane production was determined by gas chromatography using a thermal conductivity detector. Headspace gas was analyzed for the determination of methane production after shaking the culture and before sampling the slurry for PCB analysis. A 2 ml slurry subsample was removed from each serum bottle using a sterile disposable 5 ml pipette tip with the bottom 1.5 mM removed to increase the bore diameter. Simultaneously the bottle was being flushed with purified, filter-sterilized N_2-CO_2 (80:20,vol/vol). Each bottle was then resealed with a new Teflon lined stopper and aluminum crimp cap. Dechlorination activity was determined by PCB analysis and data summation as described previously.[25]

A second experiment was conducted to evaluate the effect of the SL petroleum hydrocarbons (and associated non-polar co-contaminants) on PCB dechlorination in sediments known to support PCB dechlorination. SL "non-polar" extract was added to HR sediment at a concentration of 6.2% dry weight, which was the approximate weight at which it occured in the

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SL sediment. Extracts were sequestered from the Silver Lake Sediments by Soxhlet extraction. The CFC extract was analyzed for hydrocarbon and metal content via chromatography/NMR and inductively coupled plasma emission spectroscopy respectively. Acetone was added to the CFC and DCM extracts which were then separately added to non-PCB-contaminated HR sediments. Acetone was removed from the sediment-extract combinations by rotary evaporation. This process also served to homogenize the mixtures. The assays were spiked with 250 μg/g sediment of Aroclor 1242 and inoculated as described above except that the slurries contained 2 g of the appropriate HR or HR non-polar extract amended sediments in 28 ml Balsh tubes (Bellco Glass Inc., Vineland, NJ.).[23] Treatments included unamended HR sediment, HR sediment amended with CFC or DCM extract, and unamended-autoclaved HR sediments (negative controls). A final treatment of HR sediment subjected to the acetone addition and homogenization process but lacking the DCM or CFC extract was added to ensure the sediment manipulation process did not adversely effect dechlorination activity. Sampling for PCB dechlorination activity and methane production took place at 4 week intervals. The entire contents of tubes were extracted and analyzed as previously described.[23]

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The non-polar extract of SL sediments may contain compounds other than petroleum hydrocarbons which may be toxic or inhibitory to the PCB dechlorinating microorganisms.

A third experiment was developed to evaluate the effects of pure petroleum hydrocarbons alone on the microbial dechlorination of PCBs. Various amounts (0%,0.25%,1%,4% wt/wt) of a pure petroleum mixture dissolved in acetone were added to HR sediments known to support PCB dechlorination. As in the second experiment these sediments were then tested for their ability to support dechlorination activity. All assays were set up and sampled in the same manner as the second experiment except 1 g of HR sediment was added to each tube instead of 2 g and the sediments were amended with various amounts of vacuum pump oil (0%,0.25%,1%,4%) instead of SL extract. Vacuum pump oil was used because it is low in sulfur (the presence of sulfur may stimulate sulfur reducers) and does not contain any additives such as detergents or corrosion inhibitors which could influence the experimental results. Autoclaved assays served as negative controls while unamended assays served as a positive control. A treatment of acetone addition, homogenization and evaporation was used to test the effects of the oil addition process. PCB analysis and data summation were done as previously described.[23]

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RESULTS

Experiment 1

The removal of the non-polar co-contaminants from the SL sediment did not impart it the ability to support the reductive dechlorination of PCBs. This is indicated by the fact that for added 2,34 trichlorobiphenyl the average number of *meta* plus *para* chlorines per biphenyl did not decrease significantly from 2 during 12 weeks of incubation in either the non-extracted sediment or the same sediment extracted with CH_2Cl_2 or CFC to remove the non-polar co-contaminants. Significant dechlorination of 2,34-trichlorobiphenyl did occur in Red Cedar River sediments; the average number of *meta* plus *para* chlorines declined from 2 to 1.1 (Table 1). However solvent extraction of Red Cedar River sediments impeded their ability to support anaerobic PCB dechlorination. Extraction with 1,1,2-trichlorotrifloroethane (CFC) resulted in a relatively small decrease in the extent of dechlorination (from 2.0 to 1.34 *meta* plus *para* chlorines) as compared to nonextracted sediments. While RC sediments extracted with Dichloromethane (DCM) were able to support little or no PCB dechlorination.

Table 1. Dechlorination of 2,34-trichlorobiphenyl in sediment slurries. Data are reported as the number of *meta* plus *para* chlorines per biphenyl after 0, 6, and 12 weeks of incubation (mean of triplicate samples ± standard deviation). Chlorine data is based on the added congener 2',3,4-CB and its potential break down products. [a]

	Incubation Time (weeks)					
Treatment	*0*		*6*		*12*	
Silver Lake Sed.						
not extracted	2.12	±0.01	2.07	±0.05	2.16	±0.09
CH_2Cl_3 extracted	2.00	±0.06	1.99	±0.01	2.02	±0.19
CFC extracted	2.04	±0.05	1.92	±0.03	2.04	±0.08
autoclaved	2.10	±0.02	2.11	±0.01	2.12	±0.06
Red Cedar Sed.						
not extracted	2.00	±0.00	1.35	±1.10	1.10	±0.04
CH_2Cl_3 extracted	2.00	±0.00	1.98	±0.02	1.81	±0.22
CFC extracted	2.00	±0.06	1.62	±0.05	1.34	±0.52
autoclaved	2.00	±0.00	2.00	±0.00	2.00	±0.00

[a]Values are reported as average number of *meta* plus *para* chlorines per biphenyl because dechlorination typically occurs from these positions but not from the *ortho* positions.[5]

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Regardless of PCB dechlorination activity, methane was detected in the headspace of all non-autoclaved treatments indicating the activity of methanogens. Methane production was highest in the unextracted SL and RD sediments (Figure 1). Methanagenic activity was detected in both the SL and RC extracted sediment treatments but was significantly less than their unextracted counterparts.

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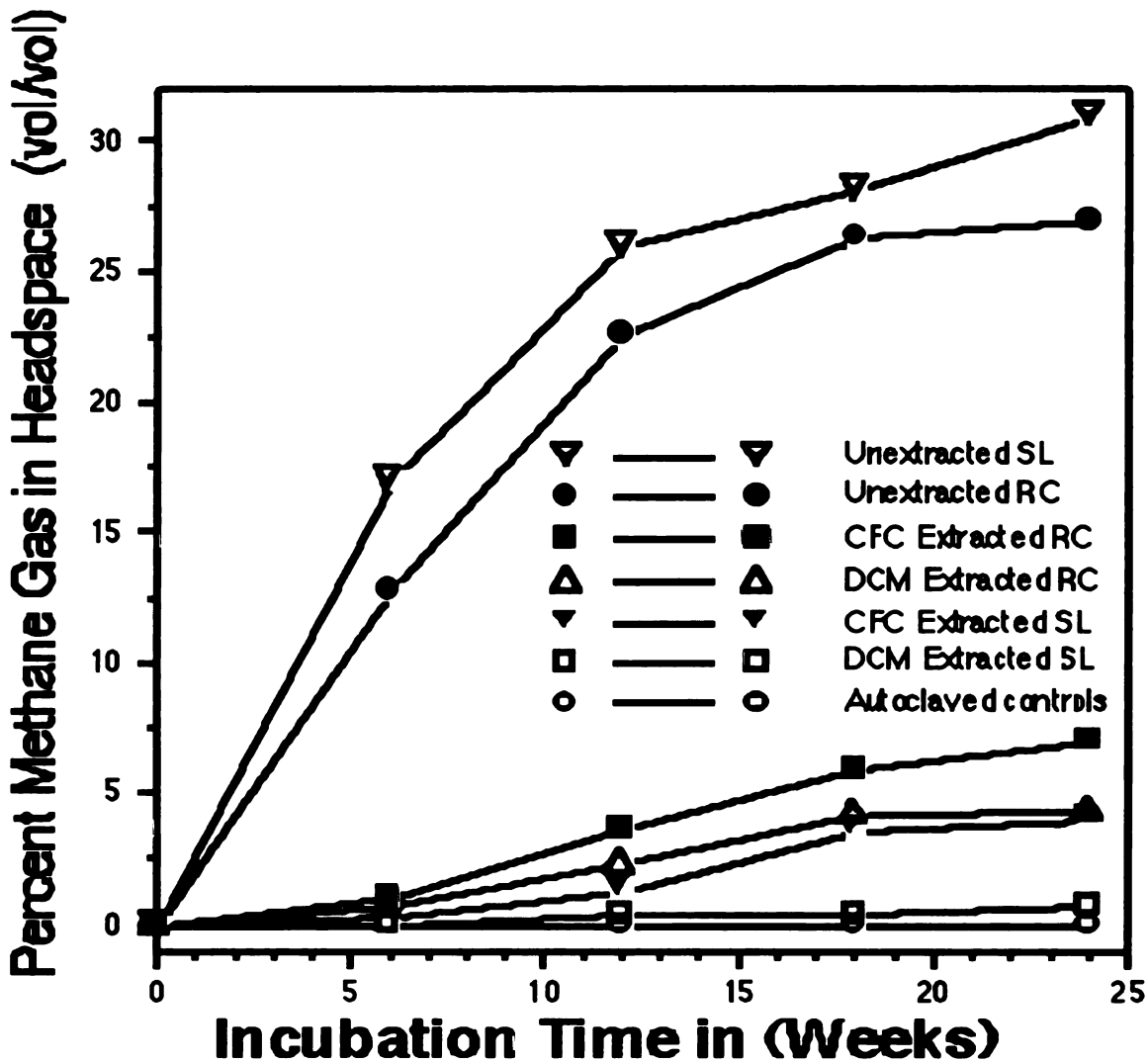

Figure 1. Methane production in dechlorination assays utilizing PCB and petroleum contaminated Silver Lake (SL) sediment and non-contaminated Red Cedar River (RC) sediments before and after extraction with dichloromethane (DCM) or Freon (CFC).

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Experiment 2.

Hudson River sediments known to support PCB dechlorination were amended with DCM and CFC extracts from SL sediments and then tested for retention of their ability to support PCB dechlorination activity. Both the SL CFC and DCM extracts inhibited dechlorination of Aroclor 1242 (Figure 2). No dechlorination was observed in HR sediments amended with DCM extract (data not shown) and only minor dechlorination occurred when assays were amended with CFC extract (Figure 2). PCB dechlorination was observed in HR river sediment not amended with SL extract (positive controls). The procedure used to add and homogenize the extract including the use of acetone had no effect on dechlorination (data not shown). Methanogenic activity was detected in all live assays. Analysis of the SL non-polar CFC extract indicated that it was comprised of 90.5% hydrocarbons, 8.9% polar compounds, and 0.8% asphaltenes.

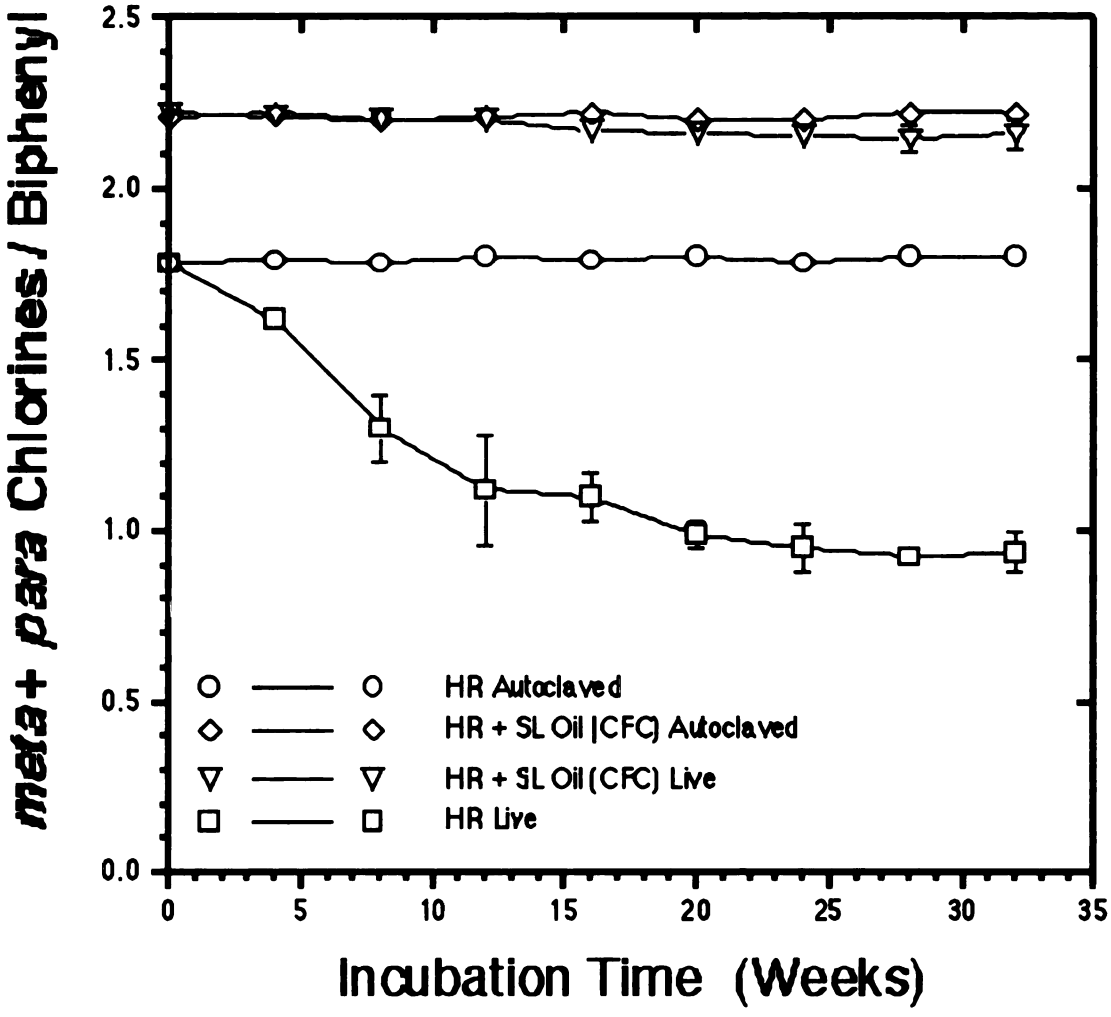

Figure 2. Dechlorination of Aroclor 1242 by HR microorganisms added to sterile anaerobic slurries of HR sediment amended with non-polar co-contaminants (6.2% wt/wt) extracted from Silver Lake sediment using CFC. The higher initial *m* + *p* chlorines in the SL oil amended treatments are the result of PCB congeners coextracted from SL sediments by CFC.

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Experiment 3.

The effect of pure petroleum hydrocarbons on PCB dechlorination was determined by adding various amounts of a vacuum oil (0%,0.25%,1%,4% wt/wt) to laboratory HR sediments known to support PCB dechlorination. Diminution in the dechlorination of Aroclor 1242 was dependent on the amount of oil added (Figure 3). The effect was greatest for this highest amount of oil addition and was still observed albeit to a lesser extent, at an oil content of 0.25%. The addition of 4% decreased the maximal rate as well as the extent of dechlorination observed by about half. Exposure of HR sediment to acetone, as well as the homogenization process, had no significant effect on dechlorination rates or extents in the absence of oil.

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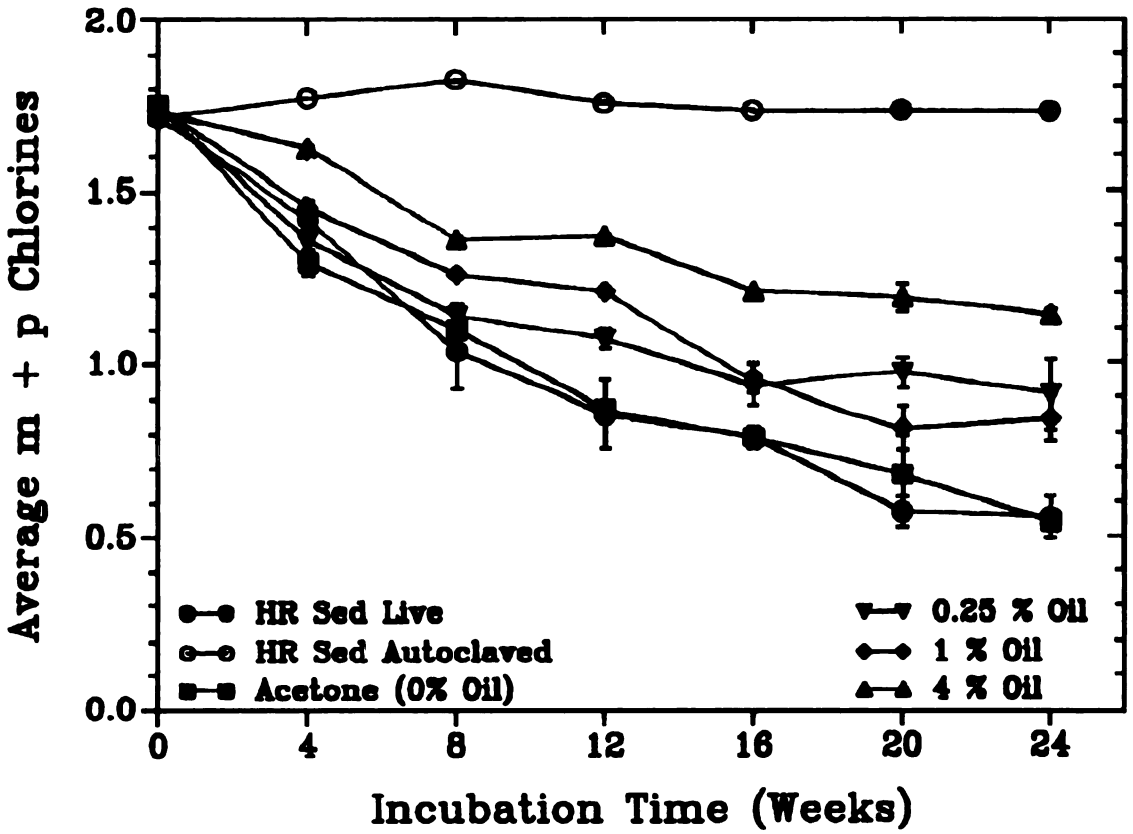

Figure 3. Dechlorination of Aroclor 1242 by Hudson River microorganisms added to sterile slurries of Hudson River sediments amended with 0.25 to 4.0% wt/wt of vacuum oil.

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DISCUSSION

Both pure petroleum hydrocarbons as well as the oil, grease and associated contaminants extracted from Silver Lake sediments by organic solvents inhibit anaerobic PCB dechlorination. Solvent extractions designed to remove the petroleum hydrocarbons and associated co-contaminants found in SL sediment did not impart the ability to dechlorinate PCBs. This may be due to the extensive heavy metal contamination also present in these sediments that would not be removed by extraction with DCM or CFC. When non-contaminated sediments known to support dechlorination of PCBs were extracted with the same solvents, PCB dechlorination was inhibited (Table 1). The inhibition was nearly complete when DCM was used, but only partial when CFC was used as the extractant. Thus, in the case of CFC extracted SL sediments the total lack of PCB dechlorination was not likely due to CFC exposure, but rather to some other inhibitor present in the sediments but not extracted by CFC. The inhibitory effect of DCM extraction on PCB dechlorination in sediments which otherwise support this activity may be due to the removal of compounds essential to the dechlorinating community or process.

The addition of non-polar extracts from SL sediments, as well as pure petroleum hydrocarbons, to clean sediments known to support

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dechlorination activity inhibited PCB dechlorination. The rates of dechlorination of Aroclor 1242 were slowed and the extent simultaneously lowered in sediments with pure petroleum added, and the effects were greater at higher rates of addition. The addition of the SL CFC extracted non-polar fraction at 6.2% (vol/vol) was also deleterious to dechlorination activity.

Careful examination of the results presented herein and results from two other previous studies[26,27] reveal that the PCB dechlorination rates can be related to their predicted solution concentrations. In each of these studies the solution concentration of PCBs was altered either directly by addition of PCBs to the system or indirectly by shifting the sorption equilibrium via alteration of the sorptive phases present in the sediments.

The rates of dechlorination of PCB's may depend in part on their availability to PCB dechlorinating microorganisms. Several previous studies have indicated in soil- or sediment-water systems only compounds in the aqueous phase are available to microorganisms.[20,21] Partitioning of PCBs into sediment or soil organic matter controls the aqueous phase concentration and hence availability of PCBs to bacteria. Mechanistically, natural organic matter appears to function as a partition medium for the sorption of non-polar organic compounds.[25,28] The extent of sorption is

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inversely related to the solute water solubility, and directly related to the soil or organic matter content. The partition mechanism manifests linear sorption isotherm described by the simple linear equation x/m=KC, where x/m is the solute concentration in the bulk sorptive phase, C is the solute concentration in water, and K is the distribution coefficient. This distribution coefficient can be normalized based on the fraction of natural organic matter present to define a new value $K_{oc}=K/f_{oc}$ where f_{oc} is the fractional organic matter content of the sediment. Chiou *et al.* demonstrated that K_{oc} values obtained for a non-ionic organic compound on different sediments were relatively constant and unique to the compound in question.[29] This indicates that natural sediment organic matter behaves similarly as a partitioning medium regardless of its origin. Knowing the PCB K_{om} value and the sediment f_{om}, aqueous phase concentrations of PCBs can be increased in a predictable manner by adding PCBs to the system.

The presence of anthropogenic organic phases in soils or sediments, in addition to natural organic matter, will also alter the solution concentrations of PCBs. Boyd and Sun demonstrated that residual petroleum hydrocarbon phases in soils and sediments act as partition phases for NOCs, and that these phases are ~10 times more effective on a unit weight basis than natural organic matter.[19] They found that the sorption coefficients (K_s) for

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NOCs such as pentachlorophenol and toluene could be accurately estimated for systems containing both natural organic matter and residual petroleum hydrocarbons. The overall sorption coefficient defining the soil- or sediment-water distribution could be accurately predicted from the fractional oil (f_{oil}) and organic matter (f_{oc}) content and the corresponding oil-water (K_{oil}) and organic matter-water (K_{oc}) partition coefficients:

$$K=f_{oc}K_{oc}+f_{oil}K_{oil} \qquad [1]$$

Because both K_{ow} and K_{oil} are based on partitioning between water and bulk phase hydrocarbons, Boyd and Sun[19] found that readily available K_{ow} values could be used as an approximation for K_{oil}:

$$K=F_{oc}K_{oc} + f_{oil}K_{ow} \qquad [2]$$

Using this system, distribution coefficients (K) were accurately predicted for a variety of NOCs in both soils and sediments containing residual oils and natural organic matter.

The first laboratory study of PCB dechlorination indicated that the rate and extent of dechlorination was directly dependent on the total PCB

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concentration.[30] Similarly, a survey showed that 93% of environmental sediment samples with PCB concentrations of 100 μg/g or greater were extensively dechlorinated, while only 63% of samples containing PCB concentrations of 5-10 μg/g had undergone similar transformation.[31] One possible explanation for the concentration effect is that higher overall PCB concentrations will manifest higher solution PCB concentrations, and it is this latter pool that is available for dechlorination. Re-examination of the studies done by Rhee *et al.*[26] and Abramawitz *et al.*[27] also support the relationship between solution concentration and dechlorination rate. In these studies solution concentrations of PCBs were manipulated by addition of PCBs to the system. Incremental additions of NOCs such as PCBs to a sediment solution system result in a simultaneous linear increase in both the sorbed and solution phase concentrations as defined by the sorption coefficient K=(x/m)/C. The studies of Rhee *et al.*[26] and Abramowicz *et al.*[27] show that increasing the concentrations of total PCBs added to laboratory assays increased the maximal dechlorination rates. In each experiment this relationship increased linearly between PCB concentration of 20 to 800 μg/g sediment.[5]

Incrementally additions of petroleum hydrocarbons to sediment, such as those described herein, would result in an increase in K (equation [1])

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and hence a decrease in the PCB solution concentrations. Thus, aqueous phase PCB concentrations can be altered in a predictable fashion by addition of PCBs or petroleum hydrocarbon components.

To analyze the relation between aqueous phase PCB concentration and rates of PCB dechlorination, data from the study described here where pure petroleum was added to sediments, was pooled with the Abramowicz *et al.*[27] and Rhee *et al.*[26] studies. Based on the multiple term partitioning equation [2] we estimated the solution concentrations of PCBs in the various experimental systems based on knowledge of f_{oc}, f_{oil}, K_{oc}, K_{ow} (Table 2).

Table 2. Sediment characteristics, Aroclor mixture and partition coefficients used to estimate solution PCB concentrations in various studies.

Study	PCB content (μg/g)	Organic carbon (%)	Petroleum hydrocarbon fraction (%)	Log K_{oc}	Log K_{ow}
Zwiernik	500	3.7	0	3.36[a]	4.5[c]
Zwiernik	500	3.7	0.25	3.36[a]	4.5[c]
Zwiernik	500	3.7	1	3.36[a]	4.5[c]
Zwiernik	500	3.7	4	3.36[a]	4.5[c]
Abramowicz	20-3,000	3.7	0.0626	3.76[b]	5.1[d]
Rhee	20-800	5.14	0	3.36[a]	4.5[c]

[a] ref.32
[b] Adjusted for PCB mixture of Aroclor 1242, 1248, 1260 (7:2:1)
[c] ref.33
[d] Adjusted for PCB mixture of Aroclor 1242, 1248, 1260 (7:2:1)

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A plot of the estimated solution PCB concentrations versus the maximal dechlorination rates resulted in a linear relationship for all three sets of data (Figure 4). Correlation coefficients for the regression lines were 0.9929, 0.9997, and 0.9974 for data from Rhee *et al.*[26], Abramowicz *et al.*[27], and the current study, respectively. Multiple linear regression comparisons indicated that slopes of the regression lines for the three independent sets of data were not significantly different ($P \leq 0.05$). Thus regardless of the method used to manipulate solution concentrations the corresponding maximum dechlorination rates remained the same. This indicates a high probability of similar cause and effect, namely that aqueous phase PCB concentrations control bioavailability and hence dechlorination rates. Because the individual regression lines are not statistically different, a single regression analysis of all data was performed yielding the equation:

PCB dechlorination rate (ng-atoms Cl^-/g sediment/week) = 21.61 Estimate of aqueous PCB concentration (μg/l) + 4.4 [3]

The maxium dechlorination rate observed in the HR sediments amended with SL CFC extract was also consistent with the values predicted from the relations between PCB solution concentrations and dechlorination rate

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depicted in Figure 4. CFC extract added at 6.2% (v/v) to sediments with Aroclor 1242 at 500 μg/kg results in estimated PCB solution concentration of 0.44 μg/L. This solution concentration corresponds to a predicted maxium dechlorination rate of 13.6 ±17.6 atoms Cl^-/g sediment/week based on equation [3]. The maximum dechlorination rate observed in the CFC extract amended assays was 4.6 ± 2.4 ng atoms Cl^-/g sediment. The general agreement between predicted and measured rates suggests that sediments amended with CFC SL extract are not affected differently from those amended with pure petroleum hydrocarbons. In addition these dechlorination rates were not different from those predicted for the experiments where estimated solution concentrations were altered by the total amount of PCBs added, again suggesting that the reduction in dechlorination rate for each of these assays was due to PCB bioavailability alone.

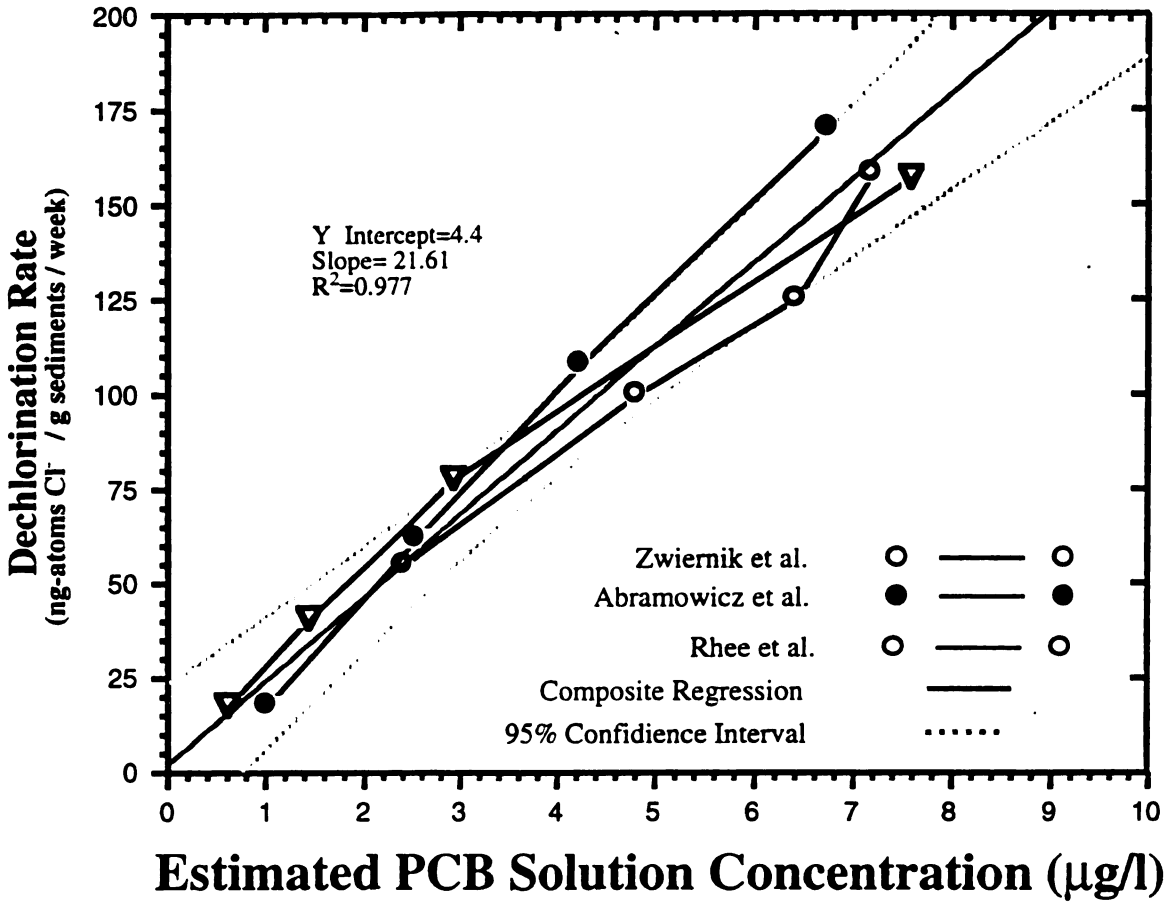

Figure 4. Maxium dechlorination rate vs estimated solution concentrations of PCBs in three separate experiments. Multiple linear regression analysis indicates that the data for the oil addition experiment (Zwiernik et al.) is not significantly different (P±0.05) from either the Abramowicz et al. of Rhee et al. experiments. Therefore all data was included in a single regression analysis.

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The correlation between the rate of PCB dechlorination and solution concentration may be a useful tool for estimating PCB biodegradation in sediments which may also be contaminated with petroleum. For the three independent studies examined, both the common linearity and consistent ratio (slopes of regression lines) suggests a common mechanism. The diversity of the experiments examined including use of sediments from different locations, those with and without anthropogenic bulk petroleum hydrocarbon phases, suggests this predictive relationship may be effective over an extended range of conditions. In these studies natural organic matter contents ranged from 1.7% in clean HR sediments to 9% in lake sediments. Petroleum hydrocarbon co-contaminants ranged in both residence times (10 days to >20 years) and hydrocarbon contents (0 to 6.2%) and consisted of residual waste oil mixtures to pure petroleum hydrocarbons. In addition these studies utilized trace total PCB concentrations ranging from 20 to >800 μg/gm sediment, as well as aqueous phase PCB concentrations ranging over approximately one order of magnitude. However it is premature to broadly extrapolate these relationships to other sites. In each of the cases studied the origin of the dechlorinating microbes was similar (HR) and the PCB congener mixture was primarily Aroclor 1242. Other commercial PCB mixtures (e.g. Aroclor

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1254 and 1260) and perhaps other PCB dechlorinating populations may manifest different relationships between dechlorination rates and aqueous PCB concentrations. However, since there are only a few commonly encountered PCB mixtures and PCB dechlorination processes,[5] a set of predictive equations for the important combinations seems attainable. The ability to predict aqueous PCB concentrations exists based on the work of Sun and Boyd.[19] The presence of toxic co-contaminants, as for example heavy metals, would preclude the use of such predictive equations.

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CHAPTER 2

The Inhibitory Effects of Heavy Metals on Anaerobic Microbial Dechlorination of Aroclor 1242 in Sediments

ABSTRACT

Many sediments such as those found in Silver Lake (MA) contain metal co-contaminants which may be responsible for the obstructing *in-situ* anaerobic reductive dechlorination of PCBs. Anaerobic reductive dechlorination of PCBs in a model system (known to support dechlorination) were adversely affected by zinc solution concentrations of 12 ppm. Dechlorination was arrested at zinc solution concentrations of 23 ppm. These concentrations are less than or equal to the zinc concentrations found in Silver Lake interstitial water. Additions of metal salts to a model system at rates designed to match the solution concentrations of copper (1 ppm), chromium (0.2 ppm), and lead (1.9 ppm) in Silver Lake interstitial water did not effect overall extent of dechlorination however initial dechlorination rates were stimulated for highest additions of both copper ($CuCl_2$) and chromium ($K_2Cr_2O_7$). In parallel experiments metals were extracted from SL sediments in order to elucidate their affects on reductive dechlorination in a existent system. Metals were removed using the common remedial practice of soil washing. Removal of metals did not enable these sediments to support dechlorination. In fact the metal removal practices significantly hampered the ability of previously active dechlorination assays to support reductive dechlorination.

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INTRODUCTION

Heavy metals are the most commonly observed co-contaminant associated with PCBs yet their effects on anaerobic biodegradation of these compounds is unknown.[1] PCBs are of industrial orgin and often found with related environmental pollutants in contaminated soils and sediments. PCBs were often used as coolants in the metal casting and plating industries. Improper disposal or accidental spills of mixed or multiple single waste streams associated with these industrial processes, has resulted in sediments that are contaminated with both PCBs and heavy metals. The International Joint Commission (formed by the U.S. and Canada) has designated 31 sites as areas of concern due to environmental contamination within the U.S. and joint waters of the great lakes basin. Of these 31 sites, 29 contain PCBs and each contains heavy metals as co-contaminants.[2] If biological remediation of PCBs is to become a viable option for their destruction, the effects of metals on PCB biodegradation must be understood.

Bioremediation technologies for PCBs have often been conceptualized as a sequential process involving anaerobic dechlorination followed by an aerobic mineralization.[3] This process has not been realized, in part, because

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anaerobic dechlorination is often slow, inconsistent, and incomplete at waste sites that are candidates for biotreatment. Surveys of sites contaminated with PCBs and other co-contaminants suggest that microorganisms capable of PCB dechlorination are usually present yet dechlorination does not occur or is severely limited.[4] This indicates that the organisms are somehow restricted by the environment within which they live.

Even though some heavy metals (eg. Fe, Cu, Ni, Zn) are essential for microbial growth as micronutrients, excessive amounts can be toxic to bacteria. Chronic pollution of environmentally significant metals may shift or terminate the natural proponderance of the terminal flow of carbon. The results can be alteration or termination of desired catabolic activities. The inhibition of aerobic microbial decomposition of synthetic organic compounds due to metals is well documented.[5,6] Similarly well studied are the effects of metals on specific groups of anaerobic organisms (eg. methanogens, sulfate reducers) during sewage sludge digestion.[7,8] The inhibitory effects are often related to disruption of physiological processes, such as sulfate reduction, methanogensis,[9,10] acetate incorporation, and glucose uptake.[11] At sufficiently great concentrations, metals may inhibit enzyme function. In this capacity they act primarily as nonspecific,

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reversible, noncompetitive inhibitors. This can be charicterized by a proportional decrease in the maxium removal (K_{max}) and growth (u_m) rates with metal concentration, while K_m (saturation concentration) remains unchanged. Because this type of inhibition is non-specific the effect of multiple metals is additive and inhibition is not completely reversible by high substrate concentration. Less frequently, metals act as competitive inhibitors. This type of inhibition is dependent on the relative concentrations of the metals and their affinities for the affected enzyme.

Sediments of Silver Lake (MA) are known to be contaminated with both commercial PCBs and heavy metals. The PCBs present in these sediments have undergone only limited *in-situ* dechlorination, in contrast to PCBs in other sediments (eg. Hudson River) which have been extensively dechlorinated. The objective of this research is to understand the effects of heavy metals on PCB dechlorination and to identify if heavy metals are limiting the dechlorination PCB in SL sediments.

MATERIALS AND METHODS

Two experiments were designed to isolate the effects of metals on anaerobic PCB dechlorination. The first experiment was designed to induce

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toxicity in order to identify inhibitory levels of specific metal ions using a model system. This was done by adding four different individual metal salts to sediment slurries inoculated with a PCB degrading consortium; this system has the established capability to anaerobically dechlorinate PCBs in the laboratory.[12] In the second experiment, metals were extracted from Silver Lake (SL) sediments in an attempt to alleviate the inhibitory effects of metals on the anaerobic reductive dechlorination of PCBs. The standard remedial practice of soil washing was used to remove metals from the PCB contaminated SL sediment. These sediments were then tested for their ability to support PCB dechlorination in laboratory assays.

Standard Dechlorination Assays

The standard dechlorination assays used have been described previously.[12,13] Non-PCB-contaminatd sediments in this case Hudson River (HR) sediment (2 g), and 2ml reduced anaerobic minimal media or RAMM[14] are contained within, oxygen free (N_2:CO_2; 8:2 v/v purged), 15 X 150 mm Balch tubes. Tubes are sealed with buytal stoppers and aluminum crimp caps. An inoculum (1 ml) obtained by shake eluting microorganisms from PCB free HR sediment[12] is added to the sediment slurries which are then incubated at 32°C until CH_4 is detected in the head space (~10 days)

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and then autoclaved. This preincubation procedure insures anaerobic conditions. Using sterile techniques, microorganisms shake eluted from PCB contaminated HR sediment (1 ml, referred to as H7 inoculum) and 500 μg of Aroclor 1242 in acetone (5μl) are added to the Balsh tubes, sealed with a Teflon lined butyl stopper and incubated at 25 C until sampling. This system has previously been shown to consistently dechlorinate Aroclor 1242 by removing chlorines from the *meta* positions, resulting in accumulations of primarily *ortho* and *para* chlorinated congeners.

The HR sediment slurries were amended with 10, 25, 50, 250 and 500 mg metal/l of $ZnCl_2$ or $CuCl_2$, or with 15, 30, 75, 150 and 750 mg metal/l of $PbCl_2$ or $K_2Cr_2O_7$ (Multiply solution conc. by 3 for μg metal/g dry sediment). These metals were among those listed as priority pollutants by the U.S. Environmental Protection Agency as well as the most abundant in SL sediment.[15] Concentrations of metal salt needed to match solution concentrations of metals observed in Silver lake interstitial water were estimated using the metal speciation program MINTEQA2.[16] Sediment slurries were amended with 1ml of an individual metal salt solution following pre-incubation and then processed as described above. Autoclaved slurries served as negative controls, and treatments without metals served as positive controls. Triplicate samples for each metal

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concentration were taken 7 times at four week intervals, solvent extracted and analyzed for PCBs. Headspace gas of each sample was analyzed via gas chromatography with a thermal conductivity detector. Additional triplicate cultures were taken 4 times at 8 week intervals and analyzed for metal solution concentrations. Sediment slurries were centrifuged at 6000xg and the supernatant filtered through 0.45 μm membrane filter under anaerobic conditions. The filtrate was acidified with HCl and analyzed for the 13 most prevalent metals by plasma emission spectrophotometry.

PCB dechlorination activity was determined as described previously.[12] Briefly, the sediment sample was solvent extracted first with 10 ml acetone and twice with 10 ml of a 9:1 hexane:acetone solution. The internal standard (octachloronaphthalene) was added to bring the final volume concentration to 1.6 ug/l. A separatory funnel containing solvent extract was back extracted with approximately 50 ml 2% aqueous NaCl to remove acetone. The remaining hexane was then shaken with 2 to 4 ml of concentrated sulfuric acid. The acid was drained, and the hexane extract rinsed twice with an addition 50 ml of the NaCl solution. Residual water was removed from the hexane extract with anhydrous Na_2SO_4. The sample was then passed through a 30 ml champagne funnel (Supelco Inc.) with its stem packed with Florisil and acid rinsed copper powder (in a 4:1 ratio) to

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remove polar contaminants and residual elemental sulfur. Quantitative analysis of PCBs was accomplished using capillary gas chromatography with electron capture detection and optional mass analysis.[12] Rates and extent of dechlorination was determined by comparing changes in the average number of *meta* plus *para* chlorines per biphenyl in the congener mixture as a whole over time.

Silver Lake System

An experiment was designed to evaluate the effects of removing heavy metals from the SL sediment on PCB dechlorination, both in the presence and absence of non-polar co-contaminants. Pre-incubated SL sediments were treated to remove either the non-polar co-contaminants, heavy metals, or both. Non-polar co-contaminants were removed by Soxhlet extraction of SL sediment for 12 hours with either 1,1,2-trichlorotrifloroethane (CFC) or dichloromethane (DCM). Metals were extracted with 1N HCl or EDTA. The metal extractions were performed using a 2:1 volume to mass ratio of extractant to sediment, and shaken on a horizontal shaker for 12 hours. The extracted sediments were then utilized in PCB dechlorination assays. Parallel treatments utilizing non-contaminated Red Cedar River sediments were used to demonstrate the dechlorination activity of the HR inoculum

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(positive controls). Assays with autoclaved Red Cedar or Silver Lake sediments served biological (negative) controls. Dechlorination assays were similar to those described above except that 2 g of the extracted sediments were substituted for the PCB-free HR sediment. Sample pH was adjusted to 6.8 after pre-incubation if necessary by adding 0.1 N NaOH. A single PCB congener, 2',3,4-chlorobiphenyl (CB) (5 µl in acetone to a final concentration of 250 µg/g), was substituted for Aroclor 1242. This congener was used because it is readily dechlorinated and because it allowed for the quantification of dechlorination in the presence of background PCBs. Samples were extracted after 0, 6, and 12 weeks of incubation at 25 °C as described above, and analyzed for 2',3,4-CB and its potential dechlorination products.

RESULTS

Dechlorination of Aroclor 1242 was assessed in sediments amended with various concentrations of four individual heavy metals: Cr, Cu, Pb, and Zn. Dechlorination in sediments not amended with metals (positive controls) resulted in the average loss of 1.2 *meta* plus *para* chlorines per biphenyl (Figure 1). Zinc was the only metal to show an adverse effect on PCB dechlorination at the concentrations examined (Figure 2). Zn additions

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of 500 ppm completely inhibited PCB dechlorination for the duration of the experiment. Zn additions of 250 ppm increased the variability of dechlorination over time while reducing the extent of dechlorination by 42%. Copper or Cr additions at 50 or 250 ppm appeared to enhance the initial rate but not the ultimate extent of Aroclor 1242 dechlorination (Figure 3). No initial stimulation occured at the higher rate of Copper addition (500 ppm) rather only an increase in variability. Additions of Cu and Cr at lower concentrations than 50 ppm had no effect on dechlorination as compared to the positive controls (data not shown). The addition of Pb at concentrations between 5 and 250 ppm had no effect on dechlorination (data not shown). Dechlorination did not occur in the autoclaved controls. Headspace gas analysis indicated no significant differences in methane content over time among treatments in which dechlorination rates were not significantly different from the positive controls (Figure 4). In the treatment where dechlorination did differ from the positive controls there were also differences in methane production (Figure 4). There was a significant lag in methane production in sediments treated with 500 ppm Zn, and total methane porduced was diminished throughout the incubation. In treatments where PCB dechlorination was initially stimulated (high additions

of Cr and Cu) there was a concurrent inhibition of methane production as compared to the positive controls.

Standardization of methane production for the Cr and Cu (50 and 250 ppm) treatments as a percent change from the positive controls, revealed a direct correlation between the stimulation of dechlorination and the inhibition of methane production (Figure 5). Regression analysis suggests that these relationships are linear for each individual treatment (Cu 50 ppm R^2=0.9651, Cu 250 ppm R^2=0.8926, Cr 50 ppm R^2=0.9775, Cr 250 ppm R^2=0.9864). Multiple linear regression analysis determined that the two Cr treatments were not significantly different (at a 95% confidence level) resulting combined correlation coefficient of 0.94819. This suggests that the inhibition of methanogensis and the concurrent stimulation of dechlorination are most likely due to the same mechanism for at least these two treatments (Cr 50 and Cr 250). The aqueous concentrations of the 13 most prevalent metals in the sediment slurries are reported in Table 1. Iron was the only metal detected at levels that exceeded the levels found in the SL sediments; this occurred in all sediments amended with metals throughout the duration of the experiment. Of the four metals individually added, Zn was the only one found at concentrations equal to that found in SL sediment slurries at

the end of the 32 week incubation; this occurred only at the highest rate of addition (500 ppm Zn).

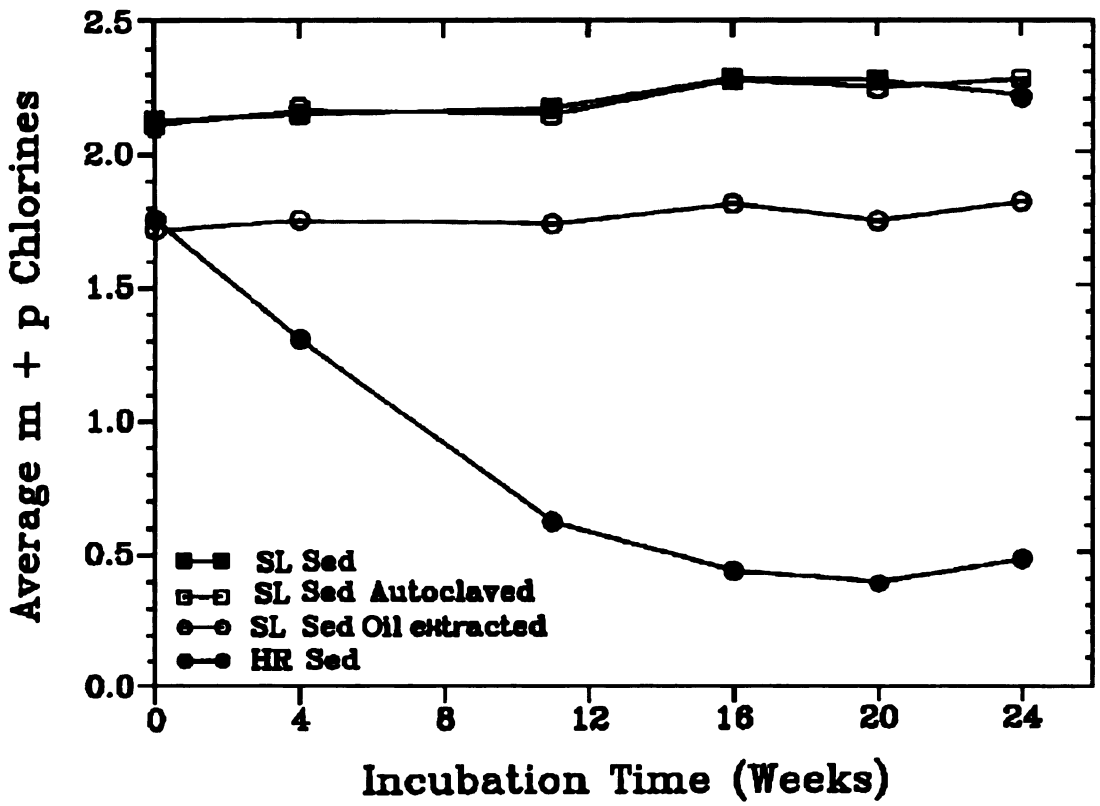

Figure 1. Dechlorination of Aroclor 1242 by Hudson River microorganisms added to sterile slurries of Silver Lake Sediments, solvent extracted Silver Lake sediments, Hudson River sediments (SDAs) and autoclaved controls.

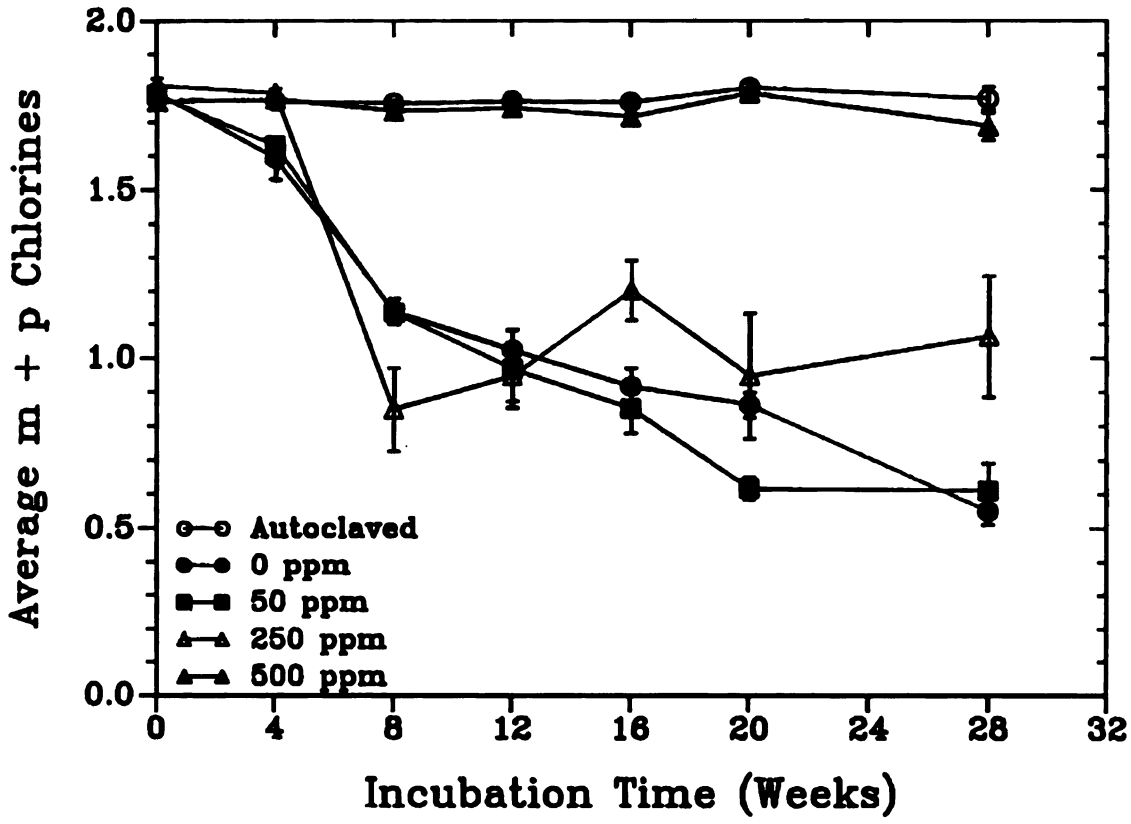

Figure 2. Dechlorination of Arochlor 1242 in upstream Hudson River sediments amended with different concentrations of $ZnCl_2$. Error bars indicate the standard deviation of triplicate samples.

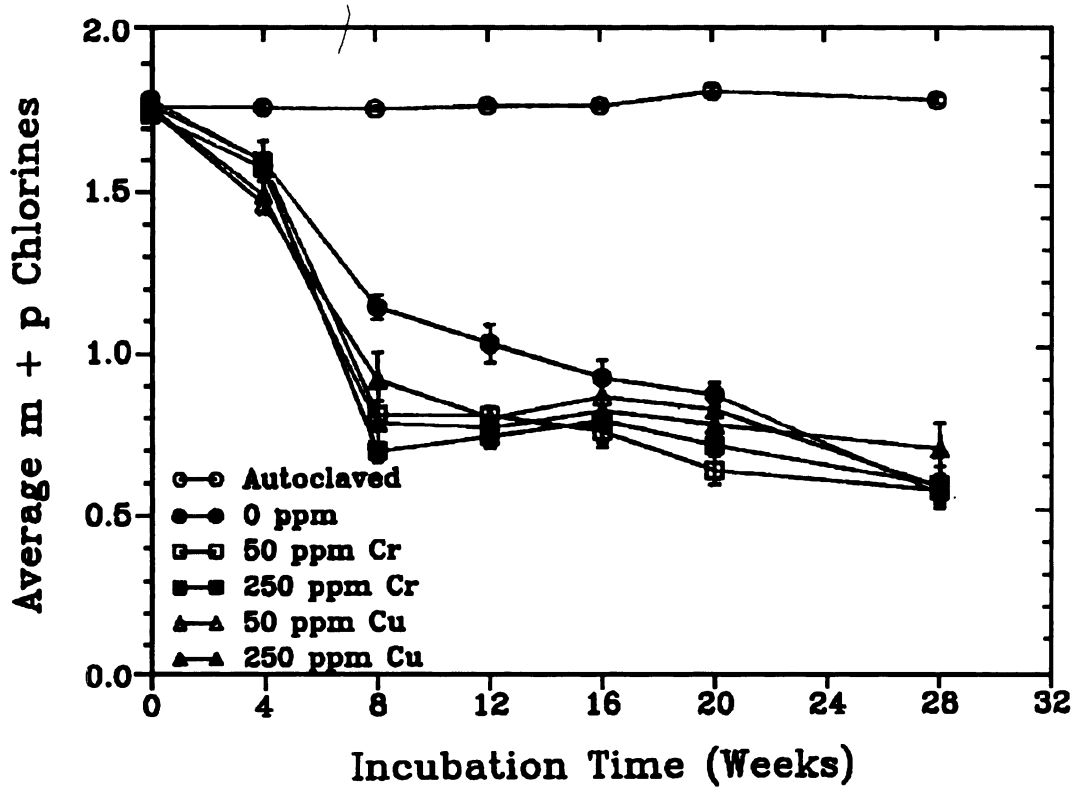

Figure 3. Effects of Cu and Cr additions on the dechlorination of Aroclor 1242 in Hudson River sediments. Error bars indicate the standard deviation of triplicate samples.

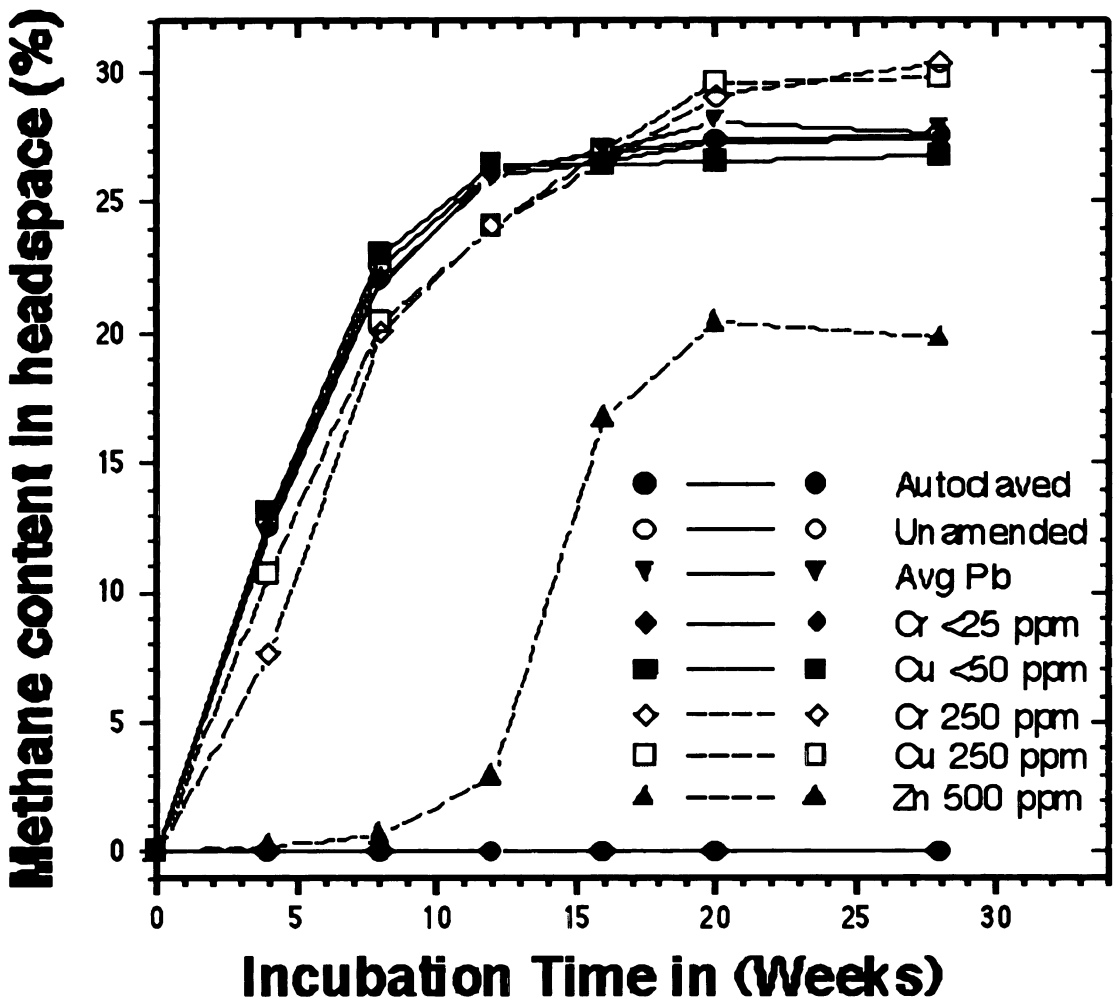

Figure 4. Methane gas content in the assay headspace analyzed at four week intervals prior to PCB extraction. There was no significant differences in methane content over time between treatments in which dechlorination rates were not significantly different from the positive controls.

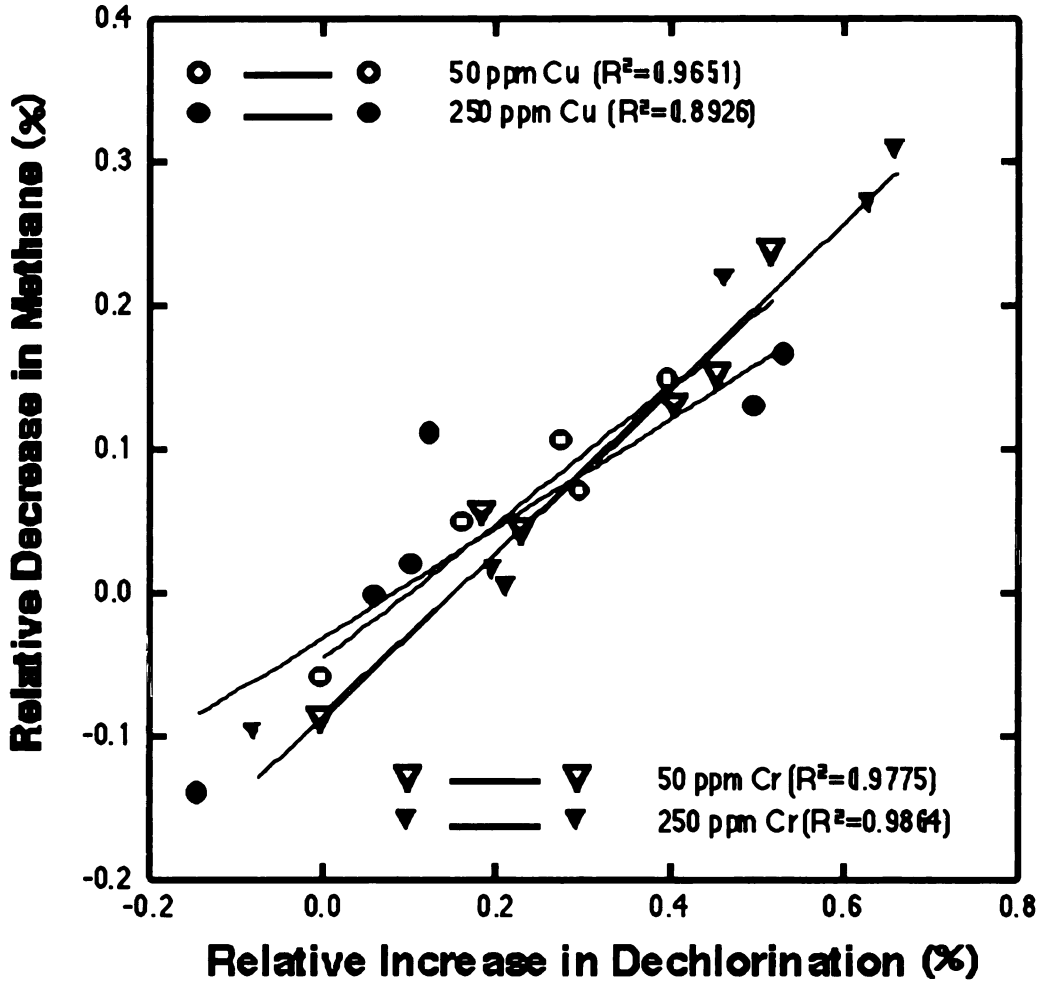

Figure 5. Difference in sample headspace methane and corresponding PCB *meta* plus *para* chlorine content as compared to the positive controls.

Table 1. Sediment slurry solution concentrations (ppm) of selected metals via. DCP analysis for metal amended Standard Dechlorination Assays (SDA), sampled Silver lake sediments (SL) and the tenth day of dechlorination assays using air dried SL sediments.

Treatment	Cr	Cu	Fe	Pb	Zn
Silver Lake *in-situ*	0.20	1.04	1.3	1.93	23.7
incubated[a]	6.7	2.3	49.3	4.3	412.2
Hudson River initial	ND	0.07	1.19	0.04	0.70
250 ppm Cr.					
week 0	8.4	0.16	32.3	0.14	2.6
8	0.7	0.11	31.6	0.09	2.0
16	0.13	0.03	16.0	0.12	0.9
24	0.06	0.09	15.0	0.09	0.9
32	ND	0.07	10.7	0.12	1.0
500 ppm Cu					
week 0	0.07	29.3	61.7	0.11	4.1
8	0.06	7.8	67.4	0.14	1.0
16	ND	1.1	34.9	0.07	0.31
24	ND	0.5	21.4	ND	0.77
32	ND	0.6	18.2	0.13	0.62
250 ppm Pb					
week 0	0.07	0.19	32.8	47.6	6.4
8	0.09	0.20	41.4	8.2	6.7
16	ND	0.06	22.4	2.1	2.7
24	ND	0.17	9.6	0.3	0.9
32	ND	0.09	14.3	0.16	2.7
500 ppm Zn					
week 0	1.3	0.33	20.4	2.7	63.7
8	0.09	0.24	26.8	2.2	46.3
16	ND	0.18	13.2	0.06	23.4
24	ND	0.20	8.2	1.0	22.7
32	ND	0.11	8.4	0.54	22.6

[a] Sampled after 10 day incubation under methanogenic conditions

Dechlorination activity Silver Lake sediments extracted to remove metals and/or non-polar co-contaminants was non-existent. The only treatment to show significant dechlorination activity was the positive control (Red Cedar River sediments) for which the average number of *meta* plus *para* chlorines declined from 2.00 to 1.10 (Table 2). This was also the only treatment in which changes in headspace methane content occurred. The pH was determined for each sample fell within the 6.3 to 6.9 range throughout the incubation. Analysis of the aqueous phase showed metal concentrations at or below those observed in non-metal amended Hudson River sediment slurries for all samples.

Table 2. The average number of *meta* plus *para* chlorines per biphenyl for triplicate samples of each treatment after 0, 6, and 12 weeks of incubation. Chlorine data is based on the added congener 2',3,4-CB and its dechlorination products. Standard deviations are shown in parentheses.

	Incubation Time (weeks)		
Treatment	0	6	12
Silver Lake			
live	1.82 (0.01)	1.77 (0.05)	1.79 (0.09)
autoclaved	1.80 (0.02)	1.81 (0.01)	1.81 (0.01)
extracted			
CH_2Cl_3	1.61 (0.26)	1.89 (0.01)	1.67 (0.19)
CH_2CL_3+ 1N HCl	1.83 (0.00)	1.86 (0.00)	1.81 (0.02)
CH_2CL_3 + 1M EDTA	1.87 (0.01)	1.90 (0.03)	1.86 (0.03)
CFC	1.67 (0.30)	1.92 (0.03)	1.76 (0.08)
CFC + 1N HCL	1.89 (0.04)	1.94 (0.01)	1.86 (0.01)
CFC + 1M EDTA	1.61 (0.31)	1.88 (0.02)	1.87 (0.02)
1N HCL	1.83 (0.01)	1.88 (0.01)	1.83 (0.01)
1M EDTA	1.82 (0.03)	1.84 (0.01)	1.85 (0.02)
Red Cedar			
live	2.00 (0.00)	1.35 (1.10)	1.10 (0.04)
autoclaved	2.00 (0.00)	2.00 (0.00)	2.00 (0.00)

DISCUSSION

Sediments contaminated with PCBs often contain co-contaminants of which the most prevelent are heavy metals. Analysis of PCB contaminanted sediments from several locations including the Hudson River, New Bedford Harbor, River Rasin, Saginaw River and Silver Lake reveal the presence of these components. Sediments form Silver Lake contain elevated concentrations of chromium, copper, nickel, lead and zinc. Correlation of PCB dechlorination with sediment characteristics suggests that excessive quantities of heavy metals my inhibit dechlorination activity (Table 3). Historically PCBs present in Silver Lake sediment have undergone extremely limited reductive dechlorination. No dechlorination has been documented in the last decade. Futhermore, SL sediment does not support anaerobic reductive dechlorination in laboratory assays where organisms with the demonstrated ability to dechlorinate PCBs are added.

Table 3. Survey of sediment with heavy metal co-contaminants and their ability to support PCB dechlorination under assay conditions.

Sediment origin	Cl atoms removed (μg)	Metals (μg/g)				
		Cr	Cu	Ni	Pb	Zn
Hudson R. Clean	2.66	11	8	7	29	62
Hudson R. SF	2.83	10	5	6	27	61
Hudson R. H7	1.64	538	45	24	274	210
Lagoon A	0.0	36	316	170	1,186	224
New Bedford Harbor	0.34	68	549	28	624	1,523
River Raisin D	0.76	2,792	303	138	134	550
River Rasin E	0.0	124	286	119	185	435
Saginaw Bay	0.0	3,714	366	327	223	799
Silver Lake	0.07	593	2,741	325	1,519	3,832

Generally metal ions in solution are considered bioavailable and therefore potentially inhibitory to microorganisms. Analysis of interstatial water taken from intact SL sediments showed that Zn (23 ppm), Pb (1.9 ppm), Cu (1ppm) and Cr (0.2 ppm) were present in the highest solution concentrations of all metals tested. When previously air dried SL sedimentswere incubated for 10 days under methanogenic conditions, much higher Zn concentrations(429 ppm)were observed. Concentrations of Pb (3ppm), Cu (2 ppm) increased slightly while the Cr concentration (0.06 ppm) decreased. Similarly, zinc was the only individual metal added to HR sediment slurries that resulted in solution concentrations similar to those observed in SL interstatial water. Of the six metals present in SL sediments at the highest total concentrations, zinc was also the only metal added to HR sediment slurries that inhibited reductive dechlorination. These results suggest that the high level of Zn present in SL sediments, and its tendency to exist in soluble forms, may be responsible for the inhibition of PCB dechlorination in these sediments.

Precipitation by sulfide is generally considered the most important factor limiting the solubility of metals in anaerobic environments.[17] In anaerobic fresh water sediments, low concentrations of SO_4^{-2} are typically found due to its conversion to sulfide by sulfate reducing bacteria. Once

sulfate is consumed methanogensis predominates as the main terminal oxidative process.[18,19] The inhibitory effects of most heavy metals on microorganisms in anaerobic environments should be mitigated to the extent that metal sulfide precipitation occurs. Severe metal loading beyond this capacity will result in elevated solution concentrations of metals and may inhibit microbial activity.

Solution concentrations of the heavy metals in HR sediments amended with exogenous metals are consistent with the solubility products of the corresponding metal sulfides. The descending order of solution concentration was Fe >Zn >Pb >Cu, consistent with the solubilities of the metal sulfides.[20] All treatments with additions of metal salts over 50 ppm resulted in elevated solution levels of Fe^{2+} due to competition reactions. Indegenous FeS present in the HR sediments has a higher solubility product ($K_{sp}=10^{-18}$) than any of the sulfides of the metals added. Once added to HR sediment slurries, the exogenous metals will combine with sulfide present in sediment FeS. This results in the release of Fe^{2+} which is relatively non-toxic at levels approaching several hundred mg L^{-1}. Zinc sulfide has the second highest solubility product ($K_{sp}=10^{-22}$) among the predominant metals present in our sediment slurries making it the least effective exogenous metal for forming the corresponding metal sulfide. The presence of large

amounts of Zn in both the SL sediments and Zn amended HR sediments therefore resulted in the elevated levels observed. This would appear to account for the inhibitory effect of Zn on PCB dechlorination in the Zn amended sediments, and perhaps for the lack of *in-situ* dechlorination ability of SL sediments.

Inhibition of one physiological group of bacteria often results in the stimulation of another competing group. This may account for the initial stimulation of PCB dechlorination observed in HR sediments amended with Cr and Cu. This is consistent with previous research which has documented initial inhibition (lag phase) followed by a period of stimulation of methanogensis in the presence of Zn^{2+}, Cu^{2+}, and Cr^{6-} in anaerobic systems.[9,21] Likewise, initial differential inhibition of methanogenisis followed by a selection and enrichment of metal tolerant methanogenic bacterial populations was noted for marine sediments subjected to chronic metal pollution.[22] We observed a similar trend in methanogenic activity here for the 250 ppm Zn and Cu treatments, and the 50 and 250 ppm Cr treatments. Studies conducted by both Rhee *et al.*[23] and Ye *et al.*[24] found methanogenesis was not essential during the anaerobic reductive dechlorination of PCBs. The direct correlation of the initial inhibition of methanogenic activity and the stimulation of PCB dechlorination observed

here, along with the known substrate competition effects and the exemption of methane production for PCB dechlorination, suggest that the microorganisms competing with methanogens may be responsible for the initial stimulation of PCB dechlorination.

The addition of selected metals may also stimulate dechlorination in a more direct fashion. Metal additions were shown to enhance the levels of soluble Fe in treatments which displayed an initial stimulation of PCB reductive dechlorination. Increased Fe in solution reflects low solution phase sulfides, a toxic metobolic bi-product, as well as increased levels of bioavailable Fe. Some halo-respiring sulfate reducers are known to be sensetive to sulfide toxicity.[25] Additionally, enzymes, required for proper energy metabolism of organisms are effected by the availability of metallic co-factors.[26] Iron is one such co-factor used in numerous anaerobic enzymatic pathways of both sulfate reducers and to a lesser extent methanogens.[27]

No PCB dechlorination occurred in any of the treatments which contained Silver Lake sediments regardless of whether metals and/or non-polar co-contaminants were removed. Identical extraction of Red Cedar River sediments resulted in some detrimental effects on dechlorination. Thus the inability of metal extracted sediments to support anaerobic

reductive dechlorination does not necessarily preclude that excess metals are responsible for limitations in the *in-situ* reductive dechlorination of PCBs. Furthermore the metal addition experiments demonstrate that solution concentrations of Zn similar to those found in SL sediments completely inhibits anaerobic reductive dechlorination of PCBs in HR sediment that otherwise support dechlorination. These results implicate metal contamination, especially Zn, as being responsible for the limitations of PCB reductive dechlorination in SL sediments.

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CHAPTER 3

Metal Toxicity Abatement in Anaerobic PCB Dechlorinating Sediments

ABSTRACT

A sequential anaerobic/aerobic biotreatment scheme for PCB contaminated sediments has been proposed. However, a majority of investigated PCB contaminated sediments contain heavy metal co-contaminants which can inhibit PCB dechlorination. This potentially limits the use of microbial dechlorination as part of a biotreatment process. We therefore tested two means of alleviating metal toxicity: precipitation (adding $FeSO_4$) and chelation (adding citrate or EDTA). Aroclor 1242 was dechlorinated in a model system consisting of anaerobic sediment slurries inoculated with PCB dechlorinating microorganisms. Additions of the metal salt $ZnCl_2$ to the model system prevented dechlorination while $PbCl_2$ decreased the rate and extent of dechlorination. These effects were reversed by subsequent additions of EDTA or $FeSO_4$ to the Zn treated model system and $FeSO_4$ eliminated inhibition by Pb. PCB dechlorination is inhibited in Silver Lake (SL) sediments, most likely due to high levels of metals. The PCB 2',3,4 trichlorobiphenyl was dechlorinated in SL sediment slurries amended with citrate or $FeSO_4$, but not EDTA, nor in unamended SL sediment slurries. For both the model system and SL system the inhibition of dechlorination experienced due to heavy metals was not only reversed in slurries amended with $FeSO_4$ but was actually enhanced over

that of the positive controls. Thus both chelation and precipitation are promising methods for alleviating inhibition of PCB dechlorination due to metal toxicity.

INTRODUCTION

Anaerobic reductive dechlorination plays a critical role for both the natural attenuation as well as the proposed active bioremediation of polychlorobiphenyls (PCBs).[1] Because PCBs are of industrial origin, their occurrence as an environmental contaminant is often in conjunction with heavy metals. The reductive dechlorination of PCBs is extremely important because the resulting less chlorinated PCB mixture is less toxic,[2,3] has a lower bioacumulation factor,[4] and is readily biodegradeable aerobically.[5] Data suggest that the presence of metals often obstruct or impede the anaerobic reductive dechlorination of PCBs.[6] Overcoming the limitation heavy metal toxicity imposes on anaerobic PCB dechlorination is imperative if biological remediation of these compounds is to become a viable option in a large number of contaminated sediments.

The commercial biotreatment of PCBs has yet to be realized despite advantages over alternative technologies in both cost savings and the

reduction of human exposure. This system is based on a two step process in which aerobic microorganisms oxidize PCB congeners that have previously undergone extensive anaerobic dechlorination.[1] This process results in the complete destruction of the PCB without the generation of toxic emissions and/or by-products. The widespread presence of microorganisms capable of these processes has been documented in surveys of PCB contaminated sediments.[1,7-9] The aerobic mineralization of extensively dechlorinated PCB congeners is generally quick, consistent and complete. Therefor the successful biotreatment of PCBs hinges on the ability of anaerobic organisms to reductively dechlorinate the biphenyl molecule.

Anaerobic reductive dechlorination of PCBs is a process whereby chlorines are removed directly from the biphenyl ring and replaced by hydrogen. While this process infers little reduction in the mass of the PCBs it can potentially reduce the PCB mixture from heavily chlorinated to mono and di-chlorinated congeners. The resulting congener mixture is not only rendered aerobically degradable,[5] but also less toxic,[2,3] and less likely to bioaccumulate.[4] This means that the extensive anaerobic reductive dechlorination of PCBs is not only paramount to success of the two step total degradation of this compound, but may itself effect a sufficient

detoxification and decrease in bioacumulation potential to reach risk based target cleanup criteria. Unfortunately, despite the aforementioned widespread presence of anaerobes capable of this process the full potential of anaerobic reductive dechlorination of PCBs is rarely observed.

Surveys of PCB contaminated sites suggests that heavy metals often limit the intrinsic reductive dechlorination of PCBs.[10] Laboratory assays have shown that heavy metals can be detrimental to PCB dechlorination even at solution concentrations significantly lower than those observed at these contamination sites.[6] The sediments of Silver Lake (MA) contain high concentrations of metals in both the sediment and solution phases and do not support PCB dechlorination.[6] Laboratory assays previously known to support dechlorination were rendered incapable of dechlorination when solution concentrations of Zinc were elevated to levels parallel to those observed in the Silver Lake system. Even assays with the solution concentrations of Zinc elevated to 50% of those observed in the Silver Lake System exhibited a marked effect on the dechlorination of added PCBs.[6]

Because of their physical and chemical characteristics PCBs were often used in the metal casting and plating industries. Disposal of the resulting waste stream into waterways has resulted in metals being the most common PCB co-contaminent. The International Joint Commission (formed by the

U.S. and Canada) has designated 31 sites as areas of concern due to environmental contamination within the U.S. and joint waters of the Great Lakes basin. Of these 31 sites, 29 contain PCBs and each posses heavy metals as co-contaminants.[11] On a national level the EPA lists 70 percent of investigated PCB contaminated sites as being co-contaminated with heavy metals.[12]

The toxicity of a specific metal to a sediment organism is dependent on the species of metal present, the bioavailability of the metal, and the sensitivity of the organism.[13] Metals in solution are considered bioavailable and are therefore of greatest concern.[14] Metal toxicity is alleviated by either removing the metals from the entire system, the solution phase, or by rendering them unavailable to the organism. Total metal removal through leaching and or extraction can be cost prohibitive and requires the disturbance of the sediment, thereby remobilizing PCBs and risking extensive exposure to the biota. In addition these processes often remove metals and other nutrients to levels lower than those required for the sediment microorganisms to live.[6] Alternatively simply reducing the solution concentrations and or bioavailability of these compounds may remit the ability of *in-situ* anaerobic microorganisms to effectively dechlorinate PCBs in a safe cost effective manner.

In most anaerobic sediments the solution concentrations of heavy metals and hence heavy metal bioavailability is controlled by their interaction with authigenic sulfide. This is done through the formation of insoluble heavy metal-sulfide minerals or their co-precipitation with or adsorption on iron sulfide minerals.[15] Sulfides are produced microbially from the reduction of sulfates and/or from the degradation of sulfur containing organic compounds. The addition of sulfate has long been used to increase anaerobic digestor performance in the presence of excess heavy metals.[16] More recently sulfate reduction has been used for the removal of metals from industrial and mine effulents,[17] waste water treatment,[18] and drinking water supplies.[19]

Sulfate added as ferrous sulfate reduces anaerobic metal toxicity under a broad range of conditions.[18] As described above, the sulfate in this compound is microbially reduced to sulfide which then forms insoluble complexes with heavy metals. The Fe^{2+} in turn is able to remove any excess sulfide from solution (FeS) while simultaneously co-precipitating any residual metals. In addition, the reduced precipitate FeS has a significantly higher solubility than most other toxic heavy metals. Therefore the more toxic and less soluble heavy metals will engage in competition reactions in which they combine with the sulfide in FeS, releasing Fe^{2+} which is

relatively non-toxic up to several hundred mg/liter. Even in cases where sulfide exceeds the binding capacity of both heavy metals and FeS, Fe^{2+} is able to form the exceptionally stable insoluble precipitant FeS_2 (Pyrite), reducing soluble sulfide even further additions through a number of mechanisms. Ferrous sulfate can protect organisms which are sensitive to sulfide while simultaneously making the sulfide available to precipitate and co-precipitate toxic metals. We therefore tested the ability of $FeSO_4$ additions to emancipate PCB dechlorination activity in metal contaminated sediments.

Organic ligand chelators have also been shown to protect organisms from the toxic effects of heavy metals by reducing their bioavailability.[18,20-22] These compounds can be man made, such as the disodium salt of ethylenediaminetetraacetic acid (EDTA) and trimercapto-s-triazine, or can be produced by the organisms themselves, such as citrate and oxalic acid. The two chelators examined in this experiment were chosen because of their range in binding affinities toward multi-valent metal cations (EDTA > citrate) and their applicability to environmental use. Commercially available and already in use in a broad range of applications such as detergents, food additives, and medical therapies, EDTA has proven itself as an effective, relatively non-toxic compound in this capacity.[23] Citrate or citric acid is a

natural compound produced by yeasts, fungi and plants and has also been shown to reduce the bioavailability of metals.[18] Like EDTA, this compound is non-toxic, inexpensive, and has sanctioned environmental introduction; however it has a much lower heavy metal binding affinity.

In this study we examined two non-invasive methods of reducing the detrimental effects of heavy metals on the anaerobic reductive dechlorination of PCBs. Biological sulfate reduction enhanced through the addition of $FeSO_4$ was utilized to form insoluble precipitates of solution phase heavy metals in an attempt to reduce their bioavailability. Alternately two separate metal chelating agents, EDTA and citrate, each with significantly different binding affinities were individually tested for their ability to detoxify heavy metals through a reduction in bioavailability.

The two methods (three treatments) were superimposed on two separate types of dechlorination assays, each designed to simulate the anaerobic sediment environment. The first assay type was the simplified or "model system" in which dechlorination assays previously known to support PCB dechlorination were spiked with inhibitory levels of either Zn or Pb salts. This approach allowed us to asses the effectiveness of EDTA and citrate in alleviating Zn and Pb toxicity in the absence of other contaminants. The second assay type tested the effectiveness of the

treatments in a complex and more realistic "demonstration system". These assays consisted of Sliver Lake (SL) sediments which contain multiple contaminants including excessive amounts of petroleum hydrocarbons, numerous metals, and PCBs. Laboratory assays containing these sediments do not support the reductive dechlorination of PCBs.

MATERIALS AND METHODS

Sediment Collection

Sediments were sampled from three sites. The first two were from the upper Hudson River near Hudson Falls, NY. Non-contaminated ("clean") sediments used in the "Model System" dechlorination assays were collected just upstream from Aroclor 1242 contamination at river mile 205. The PCB dechlorination consortium added to all dechlorination assays was eluted from Aroclor 1242 contaminated sediment collected at river mile 193.5. Sediments used in the "demonstration system" dechlorination assays were obtained from Silver Lake (MA). All sediments were collected via post-hole digger to a depth of approximately 25 cm and transported to the laboratory

in completely filled and tightly sealed Teflon® lined paint cans to minimize exposure to oxygen.

Preincubations

Incubations were performed in order to insure the existence and integrity of anaerobic conditions in the assay vessels prior to the actual dechlorination assay (Balsh Tubes 15 X 150 mm). For this, each tube containing 2 gm of the appropriate air-dried, sifted sediment received 1 ml of inoculum eluted from "clean" upstream HR sediments[24] and was then monitored for methane production. After methane was detected in the headspace (~10 days for model system assays, 16 days for demonstration assays) the tubes were autoclaved for 2 h on 2 consecutive days.

Model System Dechlorination Assays

Dechlorination assays consisted of 2 g "clean" Hudson River sediment and 2 ml reduced anaerobic minimal media (RAMM).[25] Following the pre-incubation procedure, these tubes were amended with (1 ml) of one of the following three solutions: either $ZnCl_2$ or $PbCl_2$ to solution concentrations of 500 μg/ml to induce metal toxicity, or an equal volume of sterile purified water for tubes which were to be used as positive controls. All additions

were made using sterile anaerobic techniques. After 24 hours the assays were manipulated as described in the treatment section below.

Demonstration System Dechlorination Assays

Assays consisted of 2 g (SL) sediments and 3 ml RAMM. Because of the complex mixture of weathered PCBs already present in the sediment, 50 μg/g of the single congener (2',3,4-CB) was added to the sediment slurries as an indicator of dechlorination activity rather than Aroclor 1242.

Treatments

Three compounds were tested for their effectiveness at alleviating the inhibition of PCB dechlorination due to heavy metal toxicity. Treatments included $FeSO_4$, EDTA and citrate. Initial treatment solution concentrations were 9.6 mM, 13.4 mM and 15.9 mM respectively for the model system and slightly higher in the demonstration system assays (10 mM, 14.3 mM, and 17.6 mM) due to the presence of multiple metals. Amendments (1 ml) were added as filter sterilized, degassed solutions and allowed to sit overnight. Treatments were superimposed on both the "model system" and "demonstration system" assays. After 12 hours each tube was inoculated with 2 ml of a microbial consortium eluted from PCB contaminated HR

sediment as previously described.[24] A 10% solution of Aroclor 1242 (Monsanto Co., St Louis MO) in acetone was added to all model system assay tubes (250 μm per g air dried sediment). A 10% solution of 2',3,4-CB (AccuStandard, New Haven CT) in acetone was added to all demonstration system assay tubes (50 μm 2',3,4-CB per g air dried sediment). During the addition of PCBs, the assay tubes were flushed with filter-sterilized O_2-free N_2-CO_2 (80:20 vol/vol) using a Hungate apparatus. Assay tubes were crimp sealed with sterile Teflon® coated rubber stoppers, vigorously vortexed, then incubated stationary in the dark at 22°C. Autoclaved slurries served as negative or sterile controls.

Sample Extraction and Analysis

Triplicate samples were sacrificed at 4 week intervals, solvent extracted, and analyzed for PCBs using capillary gas chromatography with electron capture detection as previously described.[24] The course of PCB dechlorination was followed by plotting the average number of *meta* plus *para* chlorines for each treatment versus incubation time. Dechlorination patterns were evaluated by assessing changes in specific congener concentrations over time.

RESULTS AND DISCUSSION

Treatment systems based on both chelation or precipitation were able to negate the inhibitory effects of heavy metals on the process of PCB dechlorination. PCB dechlorination did not occurred in autoclaved controls. Likewise PCB dechlorination did not occur in untreated Zn spiked model system assays (Figure 1) or untreated demonstration assays. Untreated Pb spiked model system assays did dechlorinate Aroclor 1242; however the activity was inhibited as compared to the positive controls (without Pb) (Figure 2). The inhibitory effects of Pb or Zn observed in untreated metal spiked model system were reduced in the EDTA treated versions of these assays. The dechlorination activity in the Zn + EDTA assays were nearly identical to the positive controls (without Zn), because of abatement of the induced metal toxicity.[23] All model system assays treated with $FeSO_4$ also displayed dechlorination; however unexpectedly the dechlorination activity in these assays was greatest among all treatments. In these treatments twice as many *meta* and *para* chlorines were removed from Aroclor 1242 resulting in a significant increase in the overall extent of dechlorination as compared to the positive controls (without Pb or Zn). Conversely, model system assays treated with citrate significantly inhibited the dechlorination

of Aroclor 1242 in the Pb spiked versions while dechlorinaton did not occur in versions spiked with Zn.

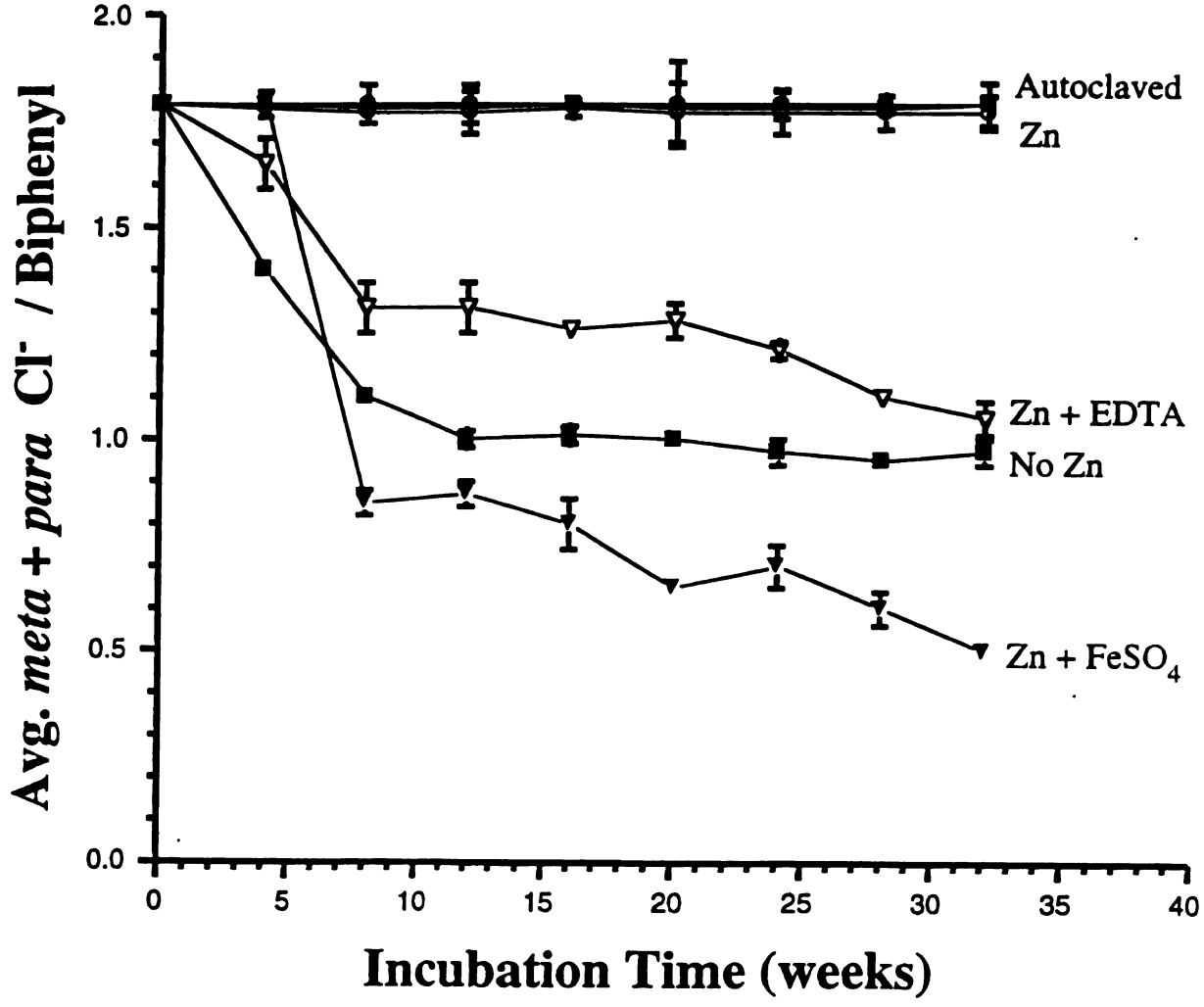

Figure 1. The effect of chelation (amendments of Citrate or EDTA) or precipitation (amendments of $FeSO_4$) the on dechlorination of Aroclor 1242 in zinc spiked model system assays. Error bars indicate the standard error of triplicate samples.

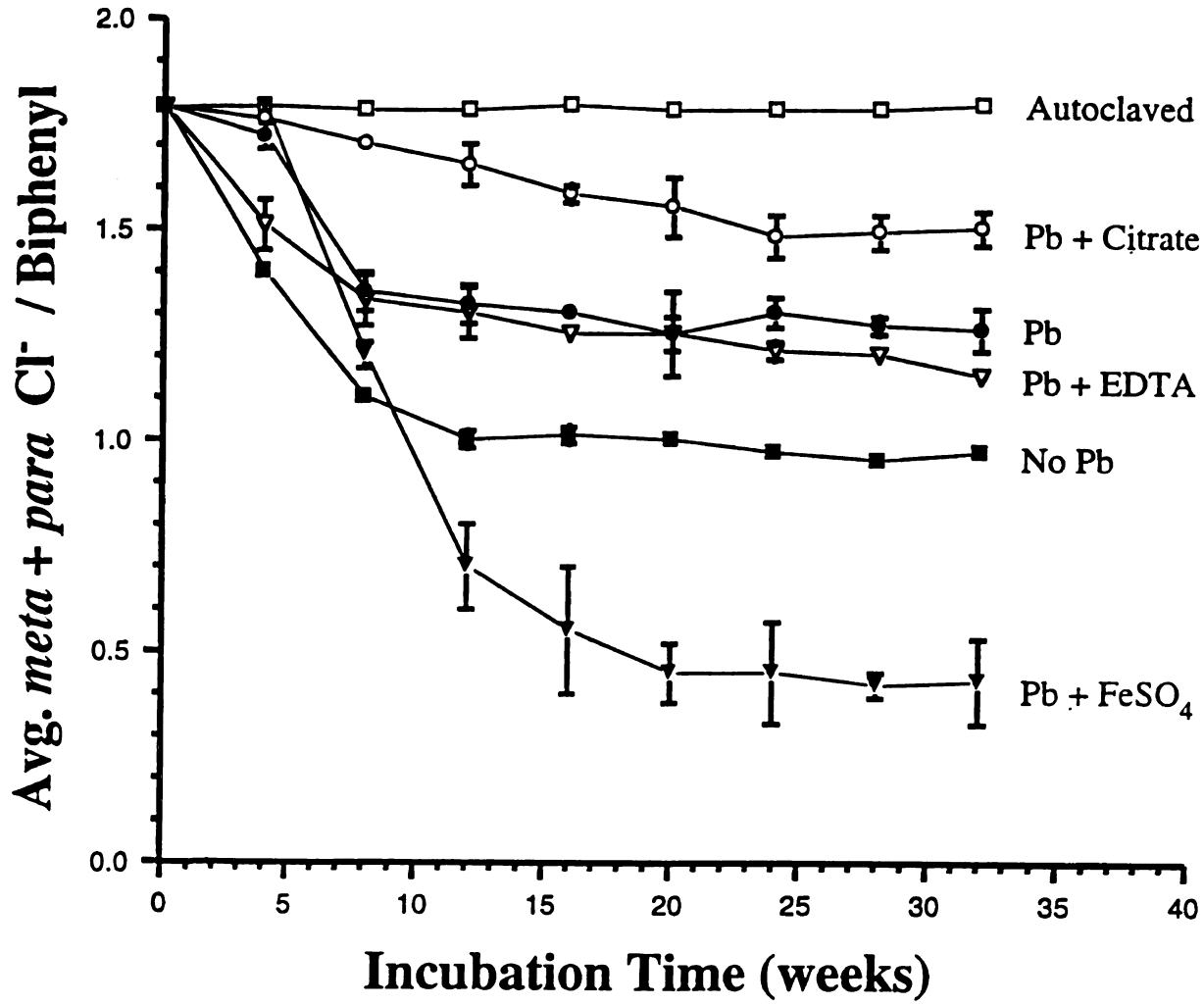

Figure 2. The effect of chelation (amendments of Citrate or EDTA) or precipitation (amendments of $FeSO_4$) the on dechlorination of Aroclor 1242 in lead spiked model system assays. Error bars indicate the standard error of triplicate samples.

Demonstration system (SL) assays amended with either $FeSO_4$ or citrate were able to support the dechlorination of the added PCB congener 2',3,4-CB (Figure 3). Not unlike the model system, the added PCB was most effectively dechlorinated in the $FeSO_4$ amended assays. Chelation was also effective at abating metal toxicity in the demonstration system assays. However unlike the model system, EDTA was not effective at reducing acute metal toxicity and citrate was (Figure 3). The ineffectiveness of the EDTA amendment was likely due to collateral cell toxicity not related to its effectiveness as a metal chelator. EDTA has been shown to affect cellular lipids and membranes, altering their permeability and decreasing cell viability.[26] While only a slightly higher concentration of EDTA was used in the demonstration assays these assays contained numerous co-contaminants whose increased internal exposure would be detrimental to cell function. Untreated demonstration assays, did not dechlorinate the added PCB congener suggesting that metals in the Silver Lake sediments were detrimental to the dechlorination activity. Dechlorination of the native PCBs was below detection limits in all demonstration system assays because of low bioavailability.[26]

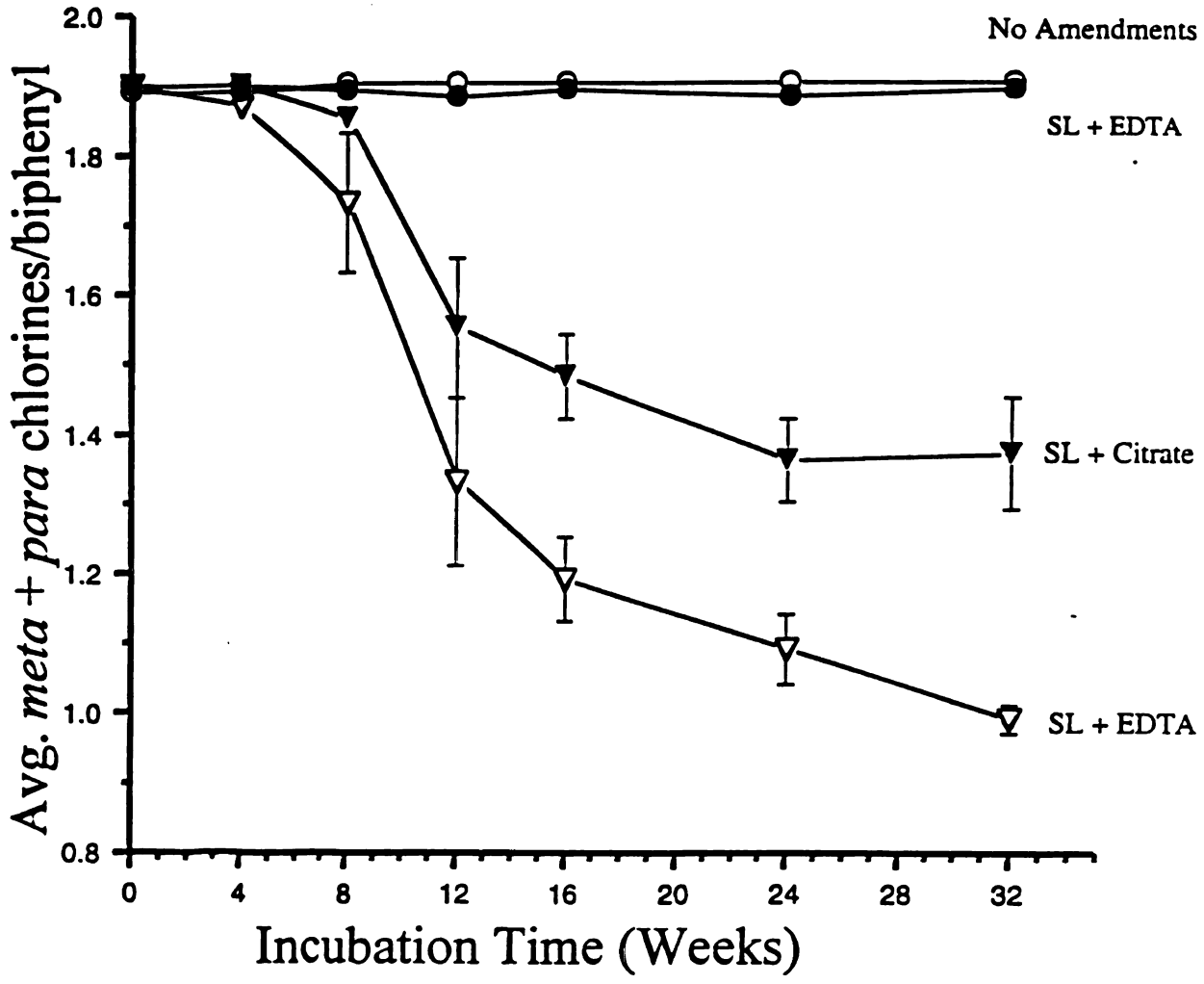

Figure 3. The effect of chelation (amendments of citrate or EDTA) or precipitation (amendments of $FeSO_4$) the on dechlorination of 2',3,4-CB in demonstration system (SL) assays. Error bars indicate the standard error of triplicate samples.

After 32 weeks of incubation the solution concentrations of all measured metals were reduced in unamended and $FeSO_4$ amended assays (Table 1). $FeSO_4$ amended assays resulted in non-detectable or non-quantifiable levels of all metals tested except zinc and iron. Solution concentrations of these two metals declined from initial concentrations of 23 and 24 ppm respectively to ~1.2 ppm for zinc and from 480 and 520 ppm to 160 and 86 ppm iron in $FeSO_4$ amended SL and Zn spiked assays, respectively. The incubation of unamended assays, including model system assays spiked with Zn or Pb, resulted in only minor reductions of detectable metals. Initial solution concentrations of Fe were elevated in all metal spiked unamended model system assays. This was most likely the result of competition reactions in which Zn or Pb replaced Fe in iron sulfide complexes. Assays amended with either of the metal chelating agents (EDTA, citrate) sustained high solution phase metals concentrations throughout the duration of the experiment.

One of the most interesting results of this experiment was the stimulation of dechlorination in $FeSO_4$ amended assays as compared to the positive controls. In both systems the greater extent of dechlorination was due to the more effective removal of chlorines from the *para* positions of the biphenyl molecule. This was most evident in the model system in which

meta and *para* dechlorination togather reduced the average number of chlorines per biphenyl from 1.2 to 0.4.

Presently six different PCB dechlorination process have been observed, each with specific congener specificitys and resulting congener profiles.[9] It has been suggested that these distinctions are the result of differences in the dechlorinating microorganisms active at various sites. The process most often observed in assays using microorganisms originating from the Hudson river is the removal of chlorines from the *meta* positions or process M. In the model system, EDTA amended Zn and Pb spiked samples, as well as positive controls, all displayed this activity which results in the accumulation of numerous *ortho* and *para* chlorinated PCB congeners. The resulting congener profile (after 32 weeks of incubation) can be seen in the histogram representation of their GC chromatograms (Figure 4, Histogram B). Characteristic changes in the congener mixture for process M include reductions in the more heavily chlorinated congeners (higher peak number) and accumulation of peaks 7 (2',4-CB), 11 (2',2,4-CB), 19 (2,4',4, and 2',2,4,6-CB), and 26 (2',2,4',4-CB) as compared to typical Aroclor 1242 pattern (Figure 4, histogram A). The congener profile resulting from model system assays amended with $FeSO_4$ was drastically different (Figure 4, Histogram C). In these assays the extensive *meta* dechlorination (process

M) was combined with the loss of virtually all *para* chlorines (process Q) which resulted in accumulations of principally *ortho* substituted mono- and di-chlorinated congeners (peaks 1 (2-CB) and 4 (2',2-CB)). Thus the greater extent of dechlorination observed in the $FeSO_4$ amended model assays occurred because process M and Q were active, while only M occurred in the other dechlorinating treatments. This suggests that the addition of $FeSO_4$ may somehow select for the microorganisms responsible for process Q dechlorination.

Treatments based on each of the two non-invasive methods were able to abate the metal toxicity experienced in each of the two systems. The addition of $FeSO_4$ stimulated the reductive dechlorination of PCB beyond that experienced in non-metal contaminated assays. Although more applied testing is required, this greatly increases the potential of non-invasive, *in-situ* and intrinsic remediation of PCBs in heavy metal contaminated sediments.

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CHAPTER 4

$FeSO_4$ Amendments Stimulate Extensive Anaerobic PCB Dechlorination

ABSTRACT

Anaerobic microbial reductive dechlorination of PCBs is important because it removes the chlorine substituents that block aerobic metabolism, and it reduces PCB toxicity. Although this process occurs widely in nature, its extent is often limited to dechlorination of some of the chlorines in the *meta* positions of the biphenyl. In this report we demonstrate the ability to consistently achieve nearly complete *meta* plus *para* dechlorination of Aroclor 1242. This involves the additions of $FeSO_4$ to PCB contaminated sediments, and results in ~90 mole % of the total PCBs being converted to aerobically degradable *ortho*-substituted mono- and di-chlorinated congeners. We propose that Iron sulfate provides two mutually beneficial effects leading to its stimulation of anaerobic PCB dechlorination. Sulfate stimulates growth of sulfate reducing organisms responsible for PCB dechlorination, while Fe^{2+} reduces sulfide bioavailability and hence toxicity by forming the insoluable precipitate FeS. Ferrous sulfate is an inexpensive, innocuous compound which could be utilized to overcome factors limiting both the extent of *in-situ* dechlorination as well as the implementation of sequential anaerobic/aerobic biotreatment systems. In addition it is expected that the toxicities of Aroclors, and hence the risk they pose, will be substantially reduced at sites where PCBs have been extensively dechlorinated.

INTRODUCTION

It is estimated that ~600 million kilograms of polychlorinated biphenyls (PCBs) have been produced worldwide and that several million kilograms have been released into the environment.[1] Commercial PCBs were manufactured and used as complex mixtures of chlorine substituted biphenyl molecules, typically consisting of 60 to 90 of a possible 209 PCB congeners. Several commercial PCB mixtures (e.g. Aroclors) exist, each with a specific chlorine content and congener profile.[2] These mixtures are distributed throughout the global ecosystem at relatively low concentrations, but can be found at much higher concentrations at specific locations, often in sediments.[3] PCBs are generally considered persistent environmental contaminants primarily because chlorine substituents prevent common microbial oxygenase enzymes from attacking the aromatic rings of biphenyl. The reductive dechlorination of PCBs by anaerobic bacteria has recently been established as an important environmental fate of these otherwise recalcitrant compounds.[4-6] This process replaces chlorines on the biphenyl ring with hydrogen, reducing the average number of chlorines per biphenyl in the resulting product. Reductive dechlorination of PCBs is important because the dechlorinated products are more susceptible to aerobic metabolism including ring opening and mineralization. Furthermore, reductive dechlorination reduces the toxicity of PCBs. We have recently

established that the inhibitory effects of PCBs on mouse gamete fertilization and their Ah receptor mediated activity ("dioxin-like" toxicity) are reduced or eliminated by anaerobic microbial dechlorination.[7,8]

In-situ reductive dechlorination has been documented in anaerobic sediments at numerous locations including the Hudson River (NY), Silver Lake (MA), Sheboygan River (WI), Waukegon Harbor (IL), New Bedford Harbor (MA), Hoosic River (MA), River Raisin (MI), and the Housatonic River (MA).[9] Although the intrinsic anaerobic reductive dechlorination of PCBs is well documented, the extent of dechlorination may vary considerably among sites, ranging from <10 to >90 percent removal of *meta* plus *para* chlorines; removal of *ortho* chlorines is not generally observed. Based on chromatographic profiles of dechlorinated product mixtures, several dechlorination processes have been described.[9] They may occur singularly or in combination in the environment. Apparently these processes result from congener specificities of distinct species or strains of dechlorinating microorganisms active at each site.[9-11] The singular processes designated M and Q are the most extensive *meta* and *para* dechlorination respectively. This is because neither requires that a chlorine be adjacent to the position dechlorinated. All other dechlorination processes have this requirement and hence result in a lower extent of dechlorination.

Process M removes chlorines from the *meta* (3,3′,5,5′) positions of biphenyl and appears to be the most widely distributed in anaerobic sediments. This is also the process most commonly exhibited by the unamended Hudson River (HR) inoculum used in our present investigation. The resulting dechlorinated PCB product mixture consists of an accumulation of numerous *ortho* and *para* chlorinated congeners. Two enrichment cultures obtained from the same parent microbial consortium (i.e. HR) provides some information on the organisms responsible for process M. Ye *et al.*[11] observed process M dechlorination by pasturized cultures, and concluded that the organisms responsible for this activity were sulfate reducing, spore formers. A second enrichment culture capable of of process M dechlorination was established through large additions of the single congener 2,3,6-CB.[12] Using antibiotics as specific inhibitors it was further concluded that these organisms were most likely gram-positive.

Process Q removes *para* (4,4′) chlorines from the biphenyl ring and is rarely observed *in-situ* and difficult to obtain in laboratory incubations. This activity has only been reported for organisms originating from PCB contaminated HR sediments.[9,13] Recently, Williams[12] developed the only known enrichment culture exclusively displaying process Q activity. From this culture he determined that, like process M activity, non-methanogenic, gram positive organisms were essential for process Q dechlorination.

The most extensive dechlorination activity, designated process C, is process M and Q acting in conjunction. This results in PCB congeners substituted solely in the *ortho* positions. These congeners are less toxic,[7,8] have lower bioaccumulation factors,[14] and are readily susceptible to rapid aerobic mineralization.[15] Unfortunately, the full dechlorination potential of process C is often unrealized in PCB contaminated sediments. In fact, this activity has only been documented *in-situ* in PCB contaminated sediments of the Hudson River.[4,16]

Reliable achievement of process C is desirable for both the development of sequential anaerobic/aerobic PCB biotreatment technologies as well as minimal-input *in-situ* bioremediation of PCB contaminated sites.[15,17,18] In recent experiments investigating the efficacy of adding various reagents to alleviate inhibition of the PCB dechlorination by heavy metals, we discovered that process C dechlorination of Aroclor 1242 could be achieved by amending HR amended sediment slurries with $FeSO_4$. The objective of this study was to document the stimulation of anaerobic PCB dechlorination by $FeSO_4$, and to elucidate the underlying mechanisms involved.

METHODS AND MATERIALS

Sediment Collection

Sediment was sampled from two different sites on the upper Hudson River (HR) near Hudson Falls, NY. Non-PCB contaminated ("clean") sediments used in the dechlorination assays, were collected just upstream from the origin of the PCB contamination at river mile 205. Sediment contaminated with Aroclor 1242 was obtained downstream at river mile 193.5. The PCB dechlorinating microbial consortium utilized herein was eluted from these sediments. Sediments were collected via a post-hole digger to a depth of approximately 25 cm and transported to the laboratory in completely filled and tightly sealed Teflon® lined paint cans to minimize exposure to oxygen.

Dechlorination Assays

Laboratory assays similar to those used previously were designed to simulate the anaerobic sediment environment.[5,6] Anaerobic sediment slurries consisted of 2 g of air-dried clean upstream Hudson River sediment and 3 ml reduced anaerobic minimal media (RAMM).[19] Slurries were contained within O_2 free 15 X 150 mm glass Balch tubes sealed with Teflon® coated butyl rubber stoppers (The West Co. Phoenixville, PA). A

pre-incubation procedure was used to insure anaerobic conditions prior to initiation of the actual dechlorination assay. For this, each tube received 1 ml of inoculum eluted from clean up-stream HR sediments and was then monitored for methane production. When methane was detected in the head space(~10 days), the tubes were autoclaved at 121°C for 2 h on two consecutive days. Various amendments (described below) were added to each pre-incubated tube via sterile anaerobic technique. After 24 h each tube was inoculated with 2 ml of a microbial consortium eluted from PCB contaminated HR sediment obtained as previously described.[6] A 10% solution of Aroclor 1242 (Monsanto Co., St Louis MO.) in acetone was then added to each tube to give a final PCB concentration of 250 μg/g air dried sediment. During this procedure the tubes were flushed with filter-sterilized O_2-free N_2/CO_2 (80:20, vol/vol) using a Hungate apparatus. The assays tubes were crimp sealed with sterile Teflon® cotated rubber stoppers, vigorously vortexed, then incubated statically in the dark at 22 ^{0}C.

Treatments

Various amendments were added to dechlorination assays in order to elucidate the mechanistic basis for the stimulatory effect of $FeSO_4$ on PCB dechlorination. These include an unamended control, an autoclaved plus $FeSO_4$ (10 mM) control, and the following treatments: $FeSO_4$ (10 mM and 20 mM), Na_2SO_4 (10 mM), $FeCl_2$ (10 mM), $FeSO_4$ (10 mM) plus

Na_2MoO_4 (3.7 mM), and Na_2SO_4 (10 mM) plus $PbCl_2$ (10 mM). The treatments were preformed in triplicate. The amendments (1 ml) were added as sterilized, degassed solutions.

Headspace Methane Content

Prior to PCB extraction the headspace gas of each assay was analyzed for methane content utilizing a gas chromatograph coupled to a thermal conductivity detector (Carle Instruments Inc.).

Sample Extraction and Analysis

Triplicate samples of each treatment were sacrificed at predetermined time intervals. The entire contents were solvent extracted, purified and analyzed for congener specific PCB content as previously described.[6]

Sulfate and Sulfide Analysis

Additional triplicate samples of each treatment weresacrificed at predetermined time intervals (except weeks 6 and 12 were duplicate samples were sacrificed). Assay vessels were centrifuged and transfered to an anaerobic glove box where the supernatant was removed and filitered (45 μm filter) A 1ml portion of the supernatant was transfered to a sample vial and analyzed for sulfate content via. ion exchange chromatography (Dionex,

model 2000I); 4mls were processed for colorometric analysis of sulfide as described by Cline.[20]

RESULTS AND DISCUSSION

The dechlorination of Aroclor 1242 by HR microorganisms was stimulated by the addition of $FeSO_4$. Figure 1 depicts this graphically by plotting change in the average number of *meta* plus *para* chlorines per biphenyl over time. In the $FeSO_4$ amended sediments (10 mM or 20 mM) the average number of *meta* plus *para* chlorines per biphenyl was reduced from 1.78±0.02 in the parent Aroclor to 0.30±0.01 in the dechlorinated product mixture. In the unamended controls, a more limited dechlorination occurred, resulting in an average number of *meta* plus *para* chlorines per biphenyl 0.80±0.04. As generally observed for the dechlorination of Aroclors there was no evidence for the removal of *ortho* chlorines. Dechlorination did not occur in autoclaved biological controls amended with $FeSO_4$.

The impact of $FeSO_4$ amendment on PCB dechlorination can be better understood in the context of the different dechlorination processes. Microbial dechlorination of individual congeners may vary greatly, even within the same sediment, depending on the PCB mixture, time of incubation, environmental conditions, and microbial populations. Process M

is most commonly exhibited by the Hudson River (HR) inoculum used in our investigation, and is characterized by the removal of *meta* chlorines.[11] As expected process M occurred in our unamended positive controls resulting in the accumulation of numerous *ortho* and *para* chlorinated congeners (Figure 2, histogram B), namely 2,4-CB (peak 7), 2',2,4-CB (peak 11), 2,4',4-CB/2',2,4,6-CB (peak 19) and 2',2,5',5-CB (peak 24). The loss of virtually all *para* chlorines (process Q) in addition to the loss of *meta* chlorines (process M) occurred when $FeSO_4$ was used in conjunction with Hudson River inoculum (Figure 2, histogram D).

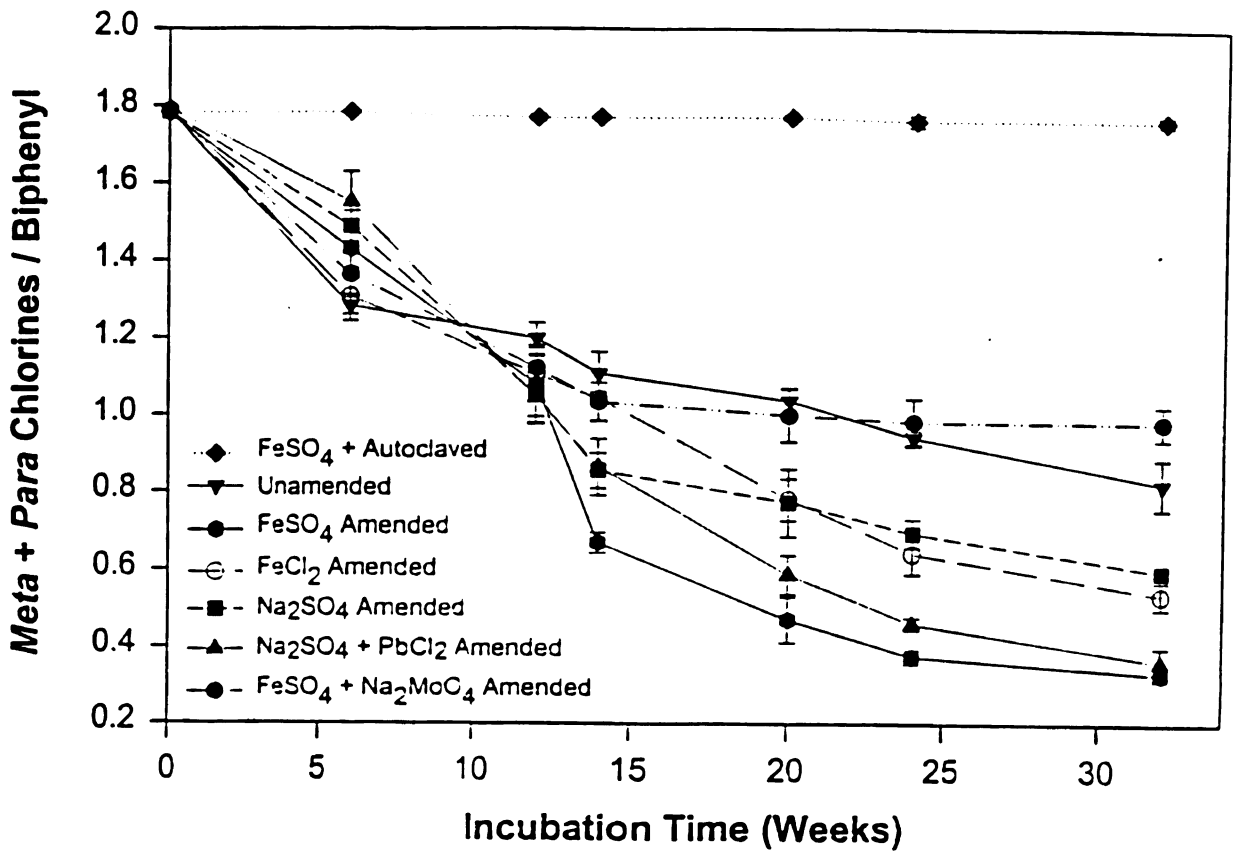

Figure 1. Effects of $FeSO_4$ amendments on anaerobic microbial dechlorination of Aroclor 1242. Rates and extents of dechlorination were determined by comparing changes in the average number of *meta* + *para* chlorines per biphenyl (no *ortho* dechlorination was observed). Error bars indicate standard error of triplicate samples. Unamended samples served as positive controls to establish indigenous dechlorination activity; autoclaved

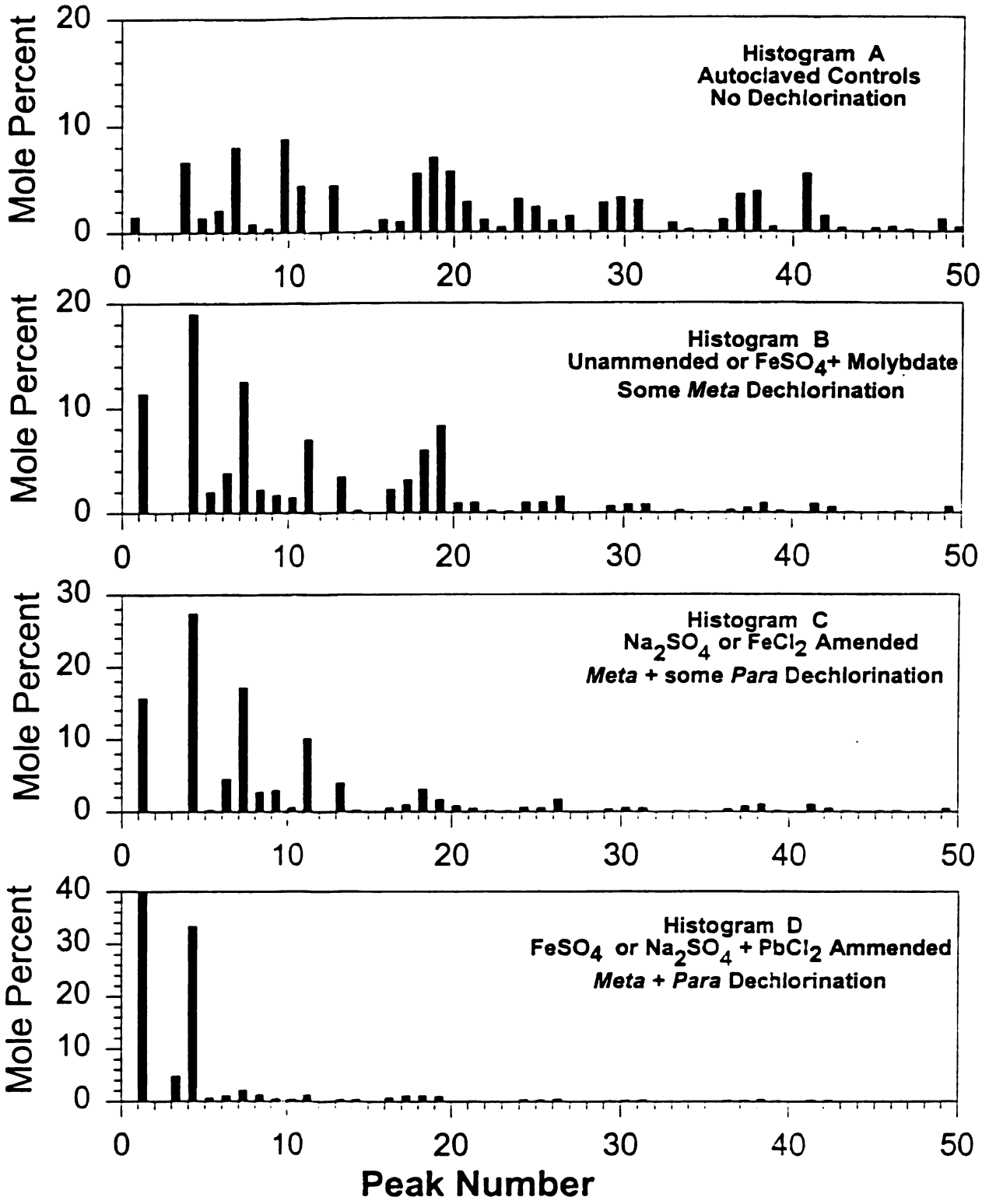

Figure 2. Changes in PCB congener profiles resulting from dechlorination (at 32 weeks) as seen through histogram representations of GC chromatograms. In general peak numbers correlate to chlorine content, with lower numbered peaks representing lesser chlorinated congeners. Histogram A represents unaltered Aroclor 1242.

The combination of these two activities (process C) resulted in ~90 mole% of the total PCBs being converted to *ortho*-substituted mono- and di-chlorinated congeners, i.e., 2-CB (peak 1) and 2',2-CB/26-CB (peak 4). Thus, the greater extent of dechlorination observed in the $FeSO_4$ amended treatments occurred because processes M and Q were both active, but only M occurred in the unamended treatment. Quensen and Bedard described pattern C as process M and Q occurring in succession, each likely due to the activity of an individual bacterial species or strain.[9]

Pattern C is distinguished by the accumulation of 2 CB and 2',2 CB/2,6-CB and was originally described in 1987 for PCBs extracted from sediment samples taken from the upper Hudson River.[4,13,16] We also observed this when sediments containing Aroclor 1242 were incubated in the laboratory with organisms freshly eluted from the same location.[9] However, all our subsequent attempts to obtain process C activity using organisms eluted from the same sediment after cold storage or from fresh samples have been unsuccessful. Additionally, more limited types of dechlorination are more commonly observed *in-situ* and in laboratory experiments using organisms from various locations including New Bedford Harbor, Hudson River, Woods Pond, Silver Lake, and the River Raisin.[9] It was not until the current study that process C activity could be reproducibly obtained through the use of $FeSO_4$ additions.

Other researchers have had some success in stimulating more limited *para* dechlorination processes (P and LP) but not in the presence of process M. Bedard *et al.*,[9,21] demonstrated the priming of *para* dechlorination of PCBs in Housatonic River and Wood's Pond sediment by the addition of single PCB or polybrominated biphenyl (BB) congeners (e.g. 2',3,5',4-CB, 2',3,4',4-CB, 2,4,5-CB, 2,5-BB, 2,6-BB, 2,3',5-BB). Similarly they have shown that addition of the single congener 2,3,4,5,6-CB resulted in both partial *meta* and *para* dechlorination, process N and LP respectively.[22] Unfortunately, both of these enhancements require the additional introduction of high concentrations of PCB or PBB congeners (~750 ppm) so their practical utility is questionable. Furthermore, to obtain the maximum extent of dechlorination it is critical that both process M and Q are operative. The significance of our observation is that we have identified an innocuous compound that can be used at a reasonable concentration (10 mM or *ca.* 10.6 lbs. $FeSO_4$/ton sediment) to enhance the overall extent of dechlorination by activating the most extensive *para* dechlorination process (process Q) without inhibiting process M. No other feasible approaches for enhancing microbial PCB dechlorination have been described.

We propose that $FeSO_4$ provides two mutually beneficial effects. First, it provides sulfate as an electron acceptor, which stimulates the growth of sulfate-reducing bacteria which are responsible for the *para*

dechlorination activity (process Q). Secondly, Fe^{2+} removes sulfide formed during sulfate reduction by forming the insoluble precipitate FeS, reducing sulfide bioavailability and hence toxicity. Once sulfate is consumed, an increased number of sulfate reducers utilize PCBs as an alternate electron acceptor, leading to extensive *meta* and *para* dechlorination.

Desulfomonile tiedjei, a sulfate reducer that is able to dechlorinate chlorobenzoates, provides a good model for conceptualizing the results reported here. Sulfoxy ions stimulate growth of this organism, but inhibit its dechlorination of 3-chlorobenzoate.[23] However if the sulfoxy ions become limited, the organism can reductively dechlorinate chlorinated benzoates.[19,24] It seems plausible that the microorganisms responsible for *para*-dechlorination of PCBs described here, and *Desulfomonile tiedjei,* are both sulfate reducers, whose growth is stimulated by SO_4^{2-} additions. Then, following depletion of SO_4^{2-}, they utilize chloroaromatic compounds as electron acceptors resulting in dechlorination. Numerous researchers have tried unsuccessfully to stimulate dechlorination by adding various electron acceptors (SO_4^{2-}, NO^-_3, CO_2, and ferric oxyhydroxide).[10,25,26] However, if the primary electron acceptor must be limiting before dechlorination will occur, as for *Desulfomonile tiedjei*, then large or repeated additions of electron acceptors should inhibit dechlorination, as has been the case in previous studies.[10] Also, accumulation of reduced

substrates such as sulfide can be toxic to these sulfate reducing organisms[27]. *Desulfomonile tiedjei* is known to be particularly sensitive to sulfide toxicity.[28]

A series of treatments were designed to separate the effects of Fe^{2+}, sulfate and sulfide, and to test our hypothesis regarding the stimulatory effect of $FeSO_4$. Experimental controls included no amendment (deionized H_2O), and $FeSO_4$ amended sterile and non-sterile controls. A treatment of $FeSO_4$ plus Na_2MoO_4 was used to provide solution concentrations of $FeSO_4$ while simultaneously blocking sulfate reduction. This was designed to establish the involvement of sulfate reducers in the stimulation. An amendment of Na_2SO_4 provided an equal amount of sulfate as the $FeSO_4$ amendment but did not provide Fe^{2+} as a means to bind sulfide; a $FeCl_2$ treatment provided Fe^{2+} but not sulfate. A treatment consisting of Na_2SO_4 and $PbCl_2$ provided sulfate as an electron acceptor as well as an alternate metal (Pb^{2+} rather than Fe^{2+}) to bind sulfide but not provide excess Fe^{2+}.

There was no evidence of *para* dechlorination in the unamended controls, but rather only partial *meta* dechlorination (Figure 1). Additions of $FeSO_4$ or $NaSO_4$ plus $PbCl_2$ resulted in the activation of *para* dechlorination to nearly identical extents and patterns of dechlorination, greatest among all treatments (Figure 1). The form of bivalent metal made no difference in the stimulatory effect observed in these two treatments. Both Pb^{2+} and Fe^{2+}

will form insoluble metal sulfides due to their extremely low solubility products (K_{sp}=1x10^{-19} and 7x10^{-29} for FeS and PbS).[29] The removal of sulfide was observed visually in the $FeSO_4$ and $PbCl_2/Na_2SO_4$ treatments which produced a black precipitate commencing at week six, but not in the other two $NaSO_4$ treatments. Measurements of soluble sulfide showed considerably lower sulfide concentrations when sulfate was added with Fe^{2+} or Pb^{2+} as compared to its addition as Na_2SO_4 alone (Figure 3). The addition of Fe^{2+} (as $FeCl_2$) or SO_4^{2-} (as $NaSO_4$) alone, did not manifest the extensive dechlorination observed in the $FeSO_4$ or $PbCl_2/Na_2SO_4$ treatments. These results are consistent with the concept of sulfate additions stimulating growth of the dechlorinating bacteria, and Fe^{2+} (or Pb^{2+}) reducing sulfide toxicity.

We propose that the stimulatory effect of on *para* dechlorination resulted from an increase in the population of sulfate reducing bacteria. The $FeSO_4$ plus Na_2MoO_4 treatment resulted in an extent of dechlorination similar to the unamended controls. Thus, when sulfate is not added (unamended control) *para* dechlorination does not occur, nor does it occur when a sulfate reduction inhibitor (Na_2MoO_4) is added in conjunction with an otherwise stimulatory sulfate source (i.e., $FeSO_4$). While the available sulfate provided in each of these treatments is not a specific inhibitor of any physiological group, the addition of sulfate usually stimulates sulfate-reducing bacteria and concomitantly inhibits methanogenic bacteria due to

bioenergetic advantages.[30] Here the shift in the terminal electron acceptor to sulfate is evidenced by the lack of methane production in treatments where sulfate is added, and the production of methane in the $FeSO_4$ plus Na_2MoO_4 treatment where sulfate reduction is blocked (Figure 4). Furthermore when sulfate is added in the absence of Na_2MoO_4 (i.e as Na_2SO_4, $FeSO_4$ or $Na_2SO_4/PbCl_2$) it is depleted rapidly during the first two to four weeks of incubation (Figure 3). Each of these observations is consistent with our proposal that sulfate additions stimulated the growth of the dechlorinating bacteria.

Numerous studies have reported that the presence of available sulfate inhibits PCB dechlorination.[10,25,26,31] Here, in treatments where sulfate reducers were supplied with sulfate ($FeSO_4$, and $Na_2SO_4/PbCl_2$), dechlorination was initially inhibited (through week 4) as compared to the no amendment control (Figure 1). Dechlorination in these treatments commenced between weeks 4 and 6, corresponding exactly to the depletion of sulfate (Figure 3). We suspect that the initial inhibition of dechlorination was due to a shift in the electron acceptor from PCBs to sulfate; the higher free energy available from sulfate reduction initially stimulated the growth of the dechlorinating microorganisms while simultaneously suspending PCB dechlorination. Consistent with this is the observation that after the initial inhibition, the dechlorination rate in the $FeSO_4$, and $Na_2SO_4/PbCl_2$

treatments was significantly higher than in the unamended controls. This would not be expected in $FeCl_2$ or $FeSO_4$ plus Na_2MoO_4 amended treatments, and was not observed. These results suggest that sulfate initially stimulates growth of the dechlorinating population but inhibits dechlorination, which commences once sulfate is depleted.

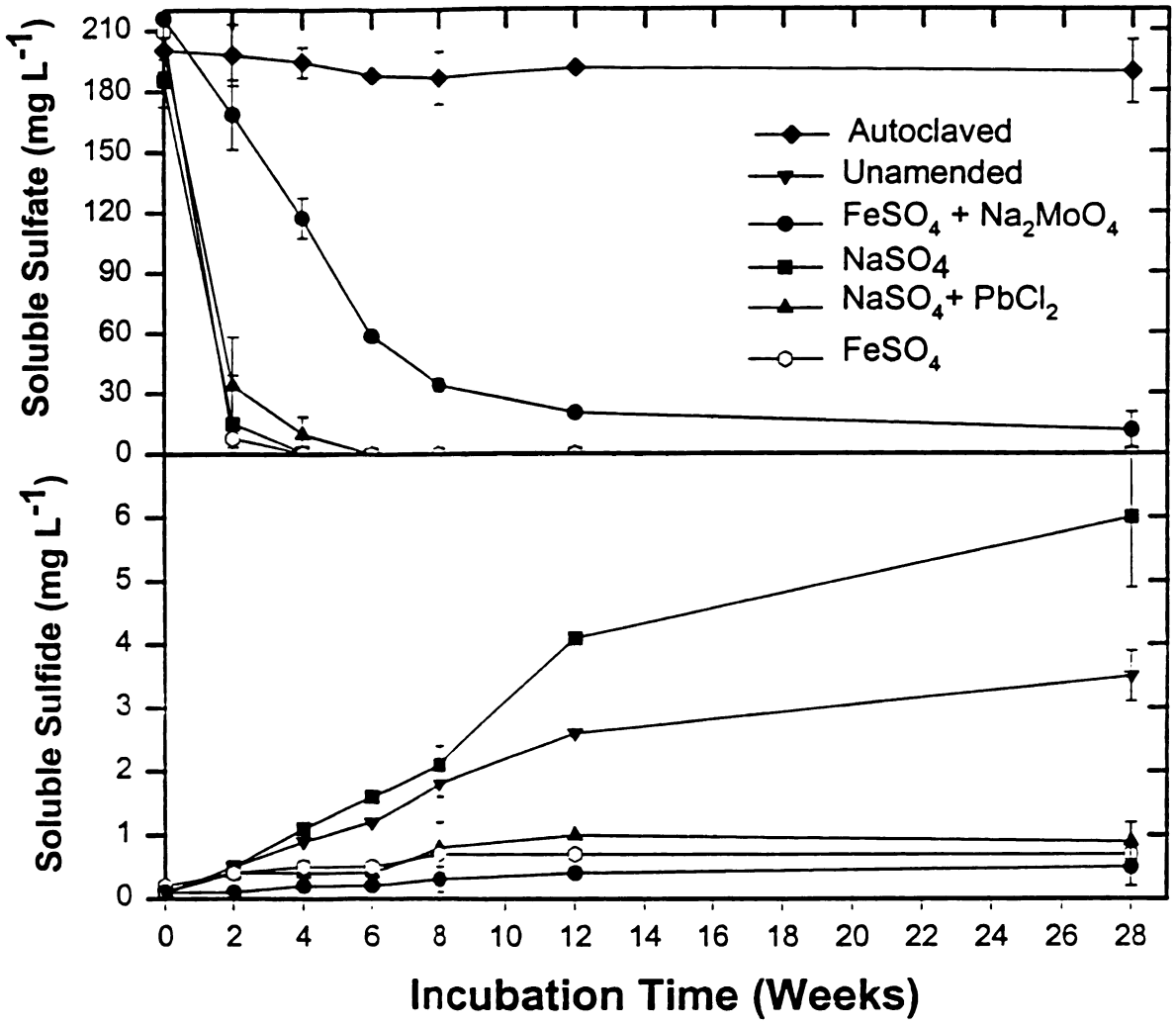

Figure 3. Soluble sulfate and sulfide concentrations in assay vessels over time. Data plotted are averages of triplicate samples except those of 6 and 12 weeks, which consisted of duplicates (error bars omitted). Sulfate and/or sulfide data is not shown for treatments in which their respective concentrations remained below 2 ppm and 1ppm over the course of the experiment. (Data omitted: sulfate and sulfide for $FeCl_2$ treated assays, sulfide in autoclaved controls and sulfate in untreated Hudson River assays).

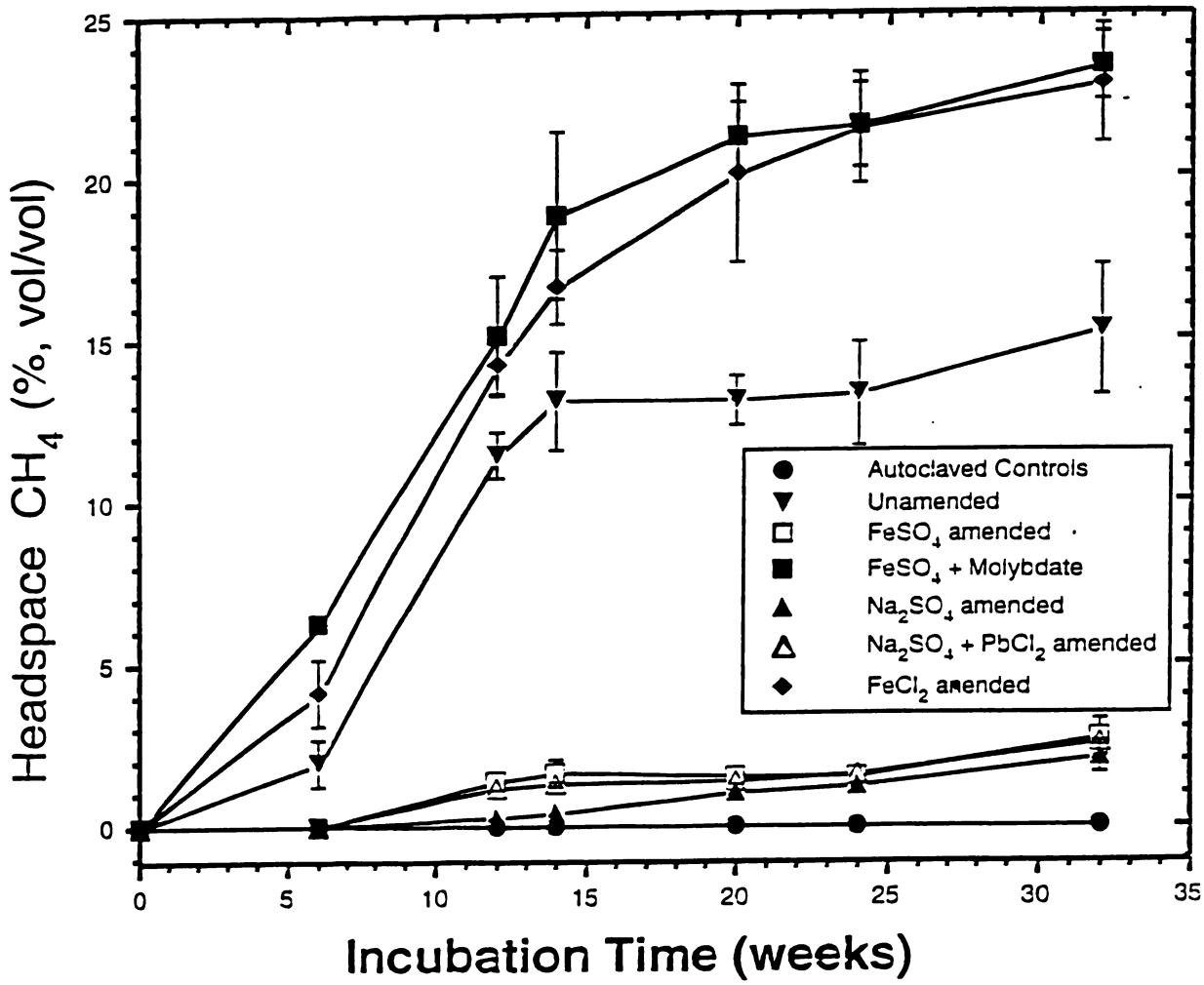

Figure 4. Methane content of assay vessel head space. No methane was detected in the headspace of autoclaved controls. Error bars indicate the standard error of triplicate samples.

Lastly, microbial community analysis via denaturing gradient gel electrophoresis (DGGE) of 16S rDNA revealed similar microbial community genotypic make-up in these two treatments which differed from each of the other treatments (unpublished data). This indicates that these two distinct treatments similarly effect the microbial community. This result further supports our interpretation of the underlying mechanisms involved in the stimulation of the *para* dechlorination of PCBs reported here.

Our interpretation of the underlying mechanisms are also consistent with other observations regarding the microbial physiology of microogranisms able to dechlorinate PCBs. In the anaerobic environment two of the main metabolic pathways are sulfate reduction and methanogensis. Both *meta* nor *para* dechlorination activities obtained from HR sediments occured in the absence of measurable methanogenic activity, suggesting that the responsible organisms are not methanogens.[11,31] In addition May *et al.*[31] have had some success subculturing HR organisms on solid media with the ability to *para* dechlorinate PCBs. Consistent with our observations, these subcultures were based on a primary enrichment for sulfate reducers. In addition these culture did not express dechlorination activity until sulfate was depleted.

The data presented herein demonstrates that $FeSO_4$ addition to sediment slurries containing PCB dechlorinating bacteria stimulates the *para*

dechlorination of PCBs. This in conjunction with the more stable and widely distributed *meta* dechlorination activity of the unamended controls, resulted in nearly complete removal of *meta* and *para* chlorines from Aroclor 1242, and the accumulation of 2-CB and 2',2-CB/2,6-CB as terminal products. $FeSO_4$ appears to be an effective, inexpensive and innocuous amendment for stimulating extensive PCB dechlorination. This greatly improves the potential to utilize PCB dehalogenation as a practical and effective remediation method both *in-situ* and *ex-situ*.

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