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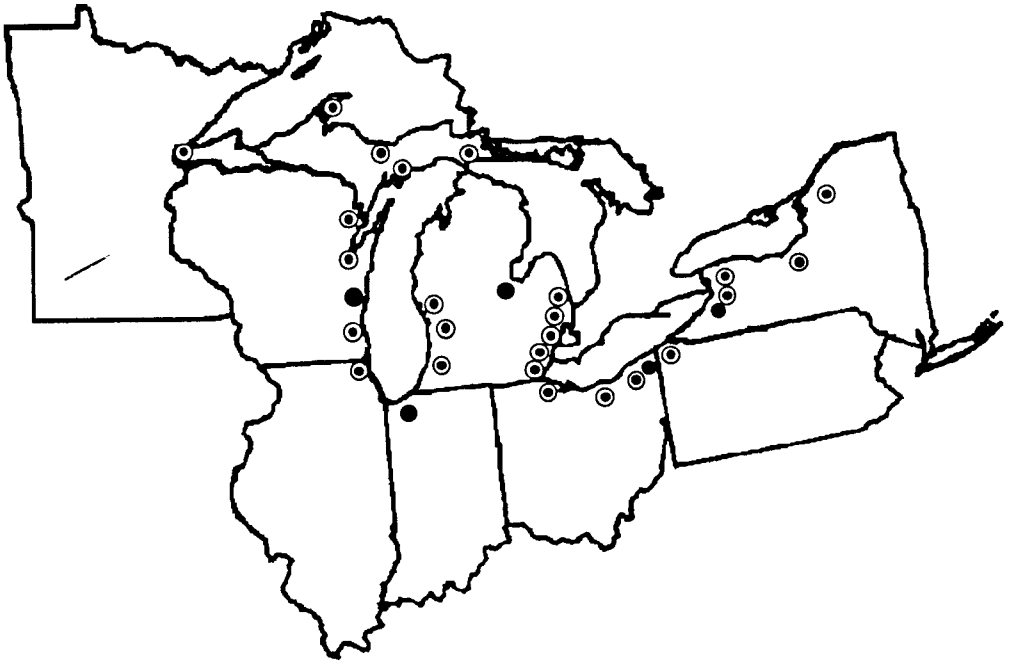
BENCH-SCALE EVALUATION OF BIOREMEDIATION FOR THE TREATMENT OF SEDIMENTS FROM THE ASHTABULA, BUFFALO, SAGINAW, AND SHEBOYGAN RIVERS



◉ United States Areas of Concern

● ARCS Priority Areas of Concern

Bench-Scale Evaluation of Bioremediation for the Treatment of Sediments from the Ashtabula, Buffalo, Saginaw and Sheboygan Rivers

Final Report

Prepared by

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For the

Assessment and Remediation of Contaminated Sediments (ARCS) Program
Great Lakes National Program Office
U.S. Environmental Protection Agency
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Bench-Scale Evaluation of Bioremediation for the Treatment of Sediments from the Ashtabula, Buffalo, Saginaw and Sheboygan Rivers

ABSTRACT

The potential of microbial degradation for the reduction of organic contaminants was examined in sediments from four Areas of Concern in the Great Lakes Region: the Ashtabula, Buffalo, Saginaw and Sheboygan Rivers. Only indigenous organisms were tested in the bioremediation study; organisms from other sites or genetically engineered bacteria were not employed. The ability of anaerobic bacteria to remove chloride ions (reductive dechlorination) from polychlorinated biphenyls (PCBs) was tested at three of the four sites. Separate tests were undertaken to assess the ability of aerobic microorganisms to degrade polynuclear aromatic hydrocarbons (PAHs) in sediments from the Buffalo River.

Bench-scale studies were performed to determine the dechlorinating potential of natural bacteria for biotransformation of historically contaminated (PCB) sediments and to assess enhancement of dechlorinating activity via nutrient addition. PCB-contaminated sediments from the Ashtabula River, the Saginaw River, and the Sheboygan River were tested for their ability to dechlorinate "historical" PCBs and highly chlorinated PCB congeners (added to samples) under anaerobic conditions. PCB-contaminated Ashtabula River sediments were spiked with 2,3,3',4,4'-pentachlorobiphenyl (Penta-CB), 2,3,3',4,4',5-hexachlorobiphenyl (Hexa-CB), and 2,2',3,4,5,6,6'-heptachlorobiphenyl (Hepta-CB). Dechlorination of the added Penta-CB, Hexa-CB, and Hepta-CB congeners was observed after respective lag periods of 5, 3, and 4 months. Significant dechlorination occurred after 6 months when the sediment was spiked with combinations of either two or all three congeners. The average number of chlorines per biphenyl decreased from 5.9 to 3.1 in Ashtabula River sediments amended with a combination of all three congeners. PCB-contaminated sediments from the Saginaw River were amended with Aroclor 1260 in the absence and presence of inorganic nutrients [revised anaerobic mineral medium (RAMM)]. Sediments amended with Aroclor 1260 plus modified RAMM exhibited an increase in the mole percent of mono-, di-, and trichlorobiphenyl (CB) homologs and a decrease in the mole percent of homologs with higher numbers of chlorine atoms per biphenyl when compared to unamended controls. In experiments amended with Aroclor 1260 only, an increase was observed in the mole percent of only the mono- and di-CB homologs; the mole percent of the remaining homolog groups decreased. PCB contaminated Sheboygan River sediments were amended with 5, 10 and 20 mg/L of 2,2',3,3',4,5,6,6'-octachlorobiphenyl (Octa-CB). The percentages of Octa-CB remaining in the samples after anaerobic incubation for 8 months ranged from 10 to 35 percent. A decrease in the higher chlorinated (5 to 8 chlorines per biphenyl) homologs,

including the added Octa-CB, and a corresponding increase in the lower chlorinated (1 to 3 chlorines per biphenyl) homologs, was observed. The major products of reductive dechlorination of Octa-CB amended sediments were di-CB congeners.

Sediments from Buffalo River were found to be contaminated with PAHs at concentrations ranging from 0.37 mg/kg for fluorene and benz(b)fluoranthene to greater than 10 mg/kg for fluoranthene and pyrene. The indigenous microorganisms from Buffalo River were capable of quickly degrading two- and three-ring PAHs and slowly degrading four-ring compounds, but showed little activity on compounds with greater than four rings. Each of the two- and three-ring compounds supported growth of sediment bacteria in mineral media; the compounds with four rings and higher did not support growth.

An enrichment culture was developed by exposing Buffalo River sediments to each compound in a mixture of 16 PAHs and combining the cultures adapted to each of the compounds to form an enrichment mixture with a wide range of degradative activity. Oxygen did not limit degradation of PAHs by the enrichment culture in sediment slurry experiments. Additions of phosphate, either alone or with nitrogen, enhanced the rate of degradation as measured by the mineralization of pyrene; additions of nitrogen as ammonium nitrate decreased the rate slightly. The addition of organic matter extracted from sediment increased the initial rate of pyrene mineralization by supporting faster growth of the degrader populations. Trace metals were not inhibitory to degradation of PAHs in aerated systems. The presence of additional PAHs decreased the rate of mineralization of pyrene; this inhibition could be reduced by treating the sediment slurries with hydrogen peroxide.

The enrichment culture, when added back to non-sterile sediments (bioaugmentation), did not enhance degradation at inoculation densities below 10^6 cells/ml. Above that density, mineralization of pyrene was first-order, indicating that growth of the bacteria was not needed to initiate degradative activity.

Sediment had both an inhibitory and a stimulatory influence on the rate of degradation of pyrene. The stimulatory effect could be explained by the utilization of sediment organic carbon as a growth substrate by the bacteria. However, at sediment slurry concentrations above 5%, the rate and extent of degradation decreased with increasing sediment slurry concentrations due to sorption of the compound.

Triton X-100, at concentrations both above and below the critical micelle concentration (CMC), inhibited the mineralization of pyrene when added during the growth phase of the organisms. When the surfactant was added after mineralization of the available phase of the PAH, "solubilization" of the remaining PAH by the surfactant resulted in slight increases in mineralization.

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List of Abbreviations/Acronyms

Ace	Acenaphthene
Acy	Acenaphthylene
Ant	Anthracene
ARCS	Assessment and Remediation of Contaminated Sediments
BaA	Benz(a)anthracene
BaP	Benzo(a)pyrene
BgP	Benzo(ghi)perylene
Bip	Biphenyl
BRS	Buffalo River sediment
CB	Chlorinated biphenyl
Chy	Chrysene
CWA	Clean Water Act
DbA	Dibenz(a,h)anthracene
ET	Engineering/Technology Work Group
Flr	Fluoranthene
Flu	Fluorene
GLNPO	Great Lakes National Program Office
Hepta-CB	2,2',3,4,5,6,6'-heptachlorobiphenyl
Hexa-CB	2,3,3',4,4',5-hexachlorobiphenyl
HMW	Higher molecular weight
LMW	Lower molecular weight
MSB	Minimal salts broth
Octa-CB	2,2'3,3',4,5,6,6'-octachlorobiphenyl
PAH	Polycyclic aromatic hydrocarbons
PCB	Polychlorinated biphenyl
Penta-CB	2,3,3',4,4'-pentachlorobiphenyl
Phe	Phenanthrene
Pyr	Pyrene
RAMM	Revised anaerobic mineral medium

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1.0 INTRODUCTION

The Great Lakes National Program Office (GLNPO) leads efforts to carry out the provisions of Section 118 of the Clean Water Act (CWA) and to fulfill U.S. obligations under the Great Lakes Water Quality Agreement with Canada. Under Section 118(c)(3) of the CWA, GLNPO was responsible for undertaking a 5-year study and demonstration program for the remediation of contaminated sediments. Five areas were specified for priority consideration in locating and conducting demonstration projects: Saginaw River and Bay, Michigan; Sheboygan River, Wisconsin; Grand Calumet River/Indiana Harbor Canal, Indiana; Ashtabula River, Ohio; and Buffalo River, New York. GLNPO created the Assessment and Remediation of Contaminated Sediments (ARCS) Program to implement these demonstration projects. The ARCS Program is managed by an Advisory Committee comprised of federal, state, and public interest representatives, and an Activities Integration Committee made up of the chairpersons of various technical work groups.

To make informed decisions concerning the design of demonstration projects, the Engineering/Technology (ET) Work Group within the ARCS Program was charged to evaluate and test available removal and remedial technologies for contaminated sediments, to select promising new technologies for further testing, to demonstrate alternatives at priority consideration areas, and to estimate contaminant losses during remediation. The ET Work Group was to select technologies that were available, implementable, and economically feasible. As part of this effort, the effectiveness of selected developmental sediment remediation technologies was to be tested at the bench scale.

The Environmental Research Laboratory, Athens, Georgia (AERL), as a member of the ET Workgroup, provided technical assistance in the areas of bioremediation. AERL conducted bench-scale studies to examine the degradation of polychlorinated biphenyls (PCBs) in Saginaw River and Bay, Sheboygan River, and Ashtabula River sediments and the degradation of polycyclic aromatic hydrocarbons (PAHs) in Buffalo River sediments. As part of this effort a workshop entitled "Biological Remediation of Contaminated Sediments, with Special Emphasis on the Great Lakes" was held in Manitowoc, Wisconsin in June of 1990 (Jafvert and Rogers, 1991). The research results presented in this report were designed to address key areas of research needs identified at this workshop.

1.1 BACKGROUND

In June 1990, a workshop, "Biological Remediation of Contaminated Sediments, with Special Emphasis on the Great Lakes," was held to identify research that would advance the use

of bioremediation to cleanup contaminated sediments. Two general research areas were identified as information gaps: (1) The specific processes and mechanisms controlling observed degradation rates and patterns, and (2) issues associated with extrapolation of bench-scale studies to pilot- or full-scale field studies.

Clearly, a significant amount of information about the biological transformations of pollutants already is known from process research. Much of this research is at the phenomenological level. The results of these studies have helped identify empirically, or allude to mechanistically, the interactions among microorganisms, pollutants, and sedimentary and aqueous media in which they exist. These interactions can be complex, even for simple systems, such as the transformation of a single compound by a pure microbial culture in homogeneous solution. In this simple system, characterization of the degradation process requires an understanding of nutrient and growth requirements, the kinetics of transformation reactions, degradation pathways, pollutant concentration dependencies, effects of alternative substrates and electron acceptors, temperature dependencies, the effects of metabolic inhibitors, and in some cases, the effects of varying carbon sources. The additional complexity associated with investigating the same microbial degradation process in natural or manipulated sediments is obvious. Additional consideration must be given to organic and inorganic inhibitor availability, combined inhibitory effects, pollutant bioavailability and the kinetics of this availability, and amended microorganism competition or cooperation with the indigenous bacteria.

Although a complete understanding of how these processes interact at specific sites would result in the most direct approaches to treatability, a comprehensive understanding may not always be necessary. In many cases, biological treatment efficiency may be significantly enhanced above background levels by regulating a few limiting factors. These factors can be identified at the bench-scale through simple process studies. In many cases, differences in these controlling factors are reasons for the site- or sediment-specific nature of biological treatability successes or failures. Clearly, while much is known, a better definition of the chemical, physical and biological processes or factors controlling observed transformation rates and pathways in natural and manipulated sediments will enhance the frequency and degree of bioremediation successes.

AERL has concentrated its efforts on determining the processes that limit the degradation of PCBs and PAHs in sediments. These bench-scale studies were conducted using actual sediment from the Ashtabula River, Sheboygan River and Saginaw River for PCBs, and the Buffalo River for PAHs.

1.2 SEDIMENT DESCRIPTIONS

The sediments used in the AERL bench-scale studies were collected from four of the five ARCS priority consideration Areas of Concern (AOCs). The sediments were located in areas in which serious impairment of beneficial uses of water or biota are known to exist. The sediments were collected from locations where future pilot and/or field demonstration projects may be conducted. The primary contaminants in the sediments were PAHs and PCBs. The map provided in Figure 1 shows the locations of the ARCS Priority AOCs.

1.2.1 Site Names and Locations

Sediments were collected by GLNPO from the Saginaw River, MI, the Sheboygan River, WI, the Grand Calumet River/Indiana Harbor, IN, the Ashtabula River, OH, and the Buffalo River, NY. AERL conducted bench-scale studies with sediment samples from the Ashtabula River, Sheboygan River and Saginaw River in the PCB studies and from the Buffalo River in the PAH studies. Specifics of the sample locations for the Ashtabula River, Buffalo River, Saginaw River, and Sheboygan River are shown in Figures 2, 3, 4 and 5, respectively.

The Ashtabula River is located in northeast Ohio and is primarily fed by a rural and agricultural drainage basin covering an area of approximately 350 square kilometers (Figure 2). The Fields Brook is a major tributary of the Ashtabula River; the large and diverse concentration of chemical plants located on the Fields Brook was a primary source of chemical pollutants to the Ashtabula River. Numerous chlorinated organic compounds, including PCBs, hexachlorobenzene, and chlorinated aliphatics, as well as heavy metals, are known pollutants in this area (Sanders, 1991). The Fields Brook site was placed on the National Priorities List in 1983.

The Buffalo River is located in western New York state (Figure 3). Heavy industrial activity, including chemical synthesis and power generation, has resulted in the contamination of Buffalo River sediment with PAHs, PCBs, chlordane, heavy metals and cyanide (Lee *et al.*, 1991).

The Saginaw Bay watershed (Figure 4) consists of 22,550 square kilometers and serves as the source of drinking water for over 300,000 people (Goudy, 1991). Industry located along the River is quite diversified; the major industries include automobile manufacturing, metal fabrication, chemical production, and food processing. A 25-30 cm depth section of a sediment core collected in the Bay had a total PCB concentration of 574 µg/ml, and a recent study showed that the major PCB contaminants are a mixture of Aroclor 1242, 1254 and 1260 (Brandon *et al.*, 1991).

The Sheboygan River and Harbor site (Figure 5) is located at the western shore of Lake Michigan. The site includes approximately 22 kilometers of the Sheboygan River and the 38 hectare harbor (Eleder, 1991). Contaminants of concern at this site include PCBs, dioxins, PAHs and heavy metals. Analysis of the sediments from this site indicates that the original PCB mixtures used in this area were Aroclors 1242, 1248 and 1254 (Skogerbee *et al.*, 1991).

1.2.2 Sediment Acquisition and Homogenization

Sediment samples were collected with a mechanical dredge by the Army Corps of Engineers from a site in the upper Buffalo River that had previously been identified as a "hot spot" of PAH contamination. The samples were identified as station 47, corresponding to site number 747 on the map insert. Samples collected by the Corps were shipped to the U.S. EPA's Environmental Research Laboratory at Duluth, Minnesota, where they were screened, homogenized, and then shipped to AERL.

Samples were stored and shipped on ice. Upon receipt of samples at AERL, the sediments (in 5 gal. plastic buckets) were mixed thoroughly, screened for debris (2 mm sieve) and divided into 0.5- and 1-liter subsamples. All subsamples were individually labelled and identified in a logbook. Samples were stored at 4°C until use.

Ashtabula, Saginaw, and Sheboygan River sediments were collected as grab samples that were immediately placed in glass containers (filled to capacity) and sealed with Teflon-lined caps. The sediments were characterized primarily as mixtures of silt and sand contaminated with PCBs and low concentrations of oils and greases. Samples were shipped on ice and were stored at 4°C until use.

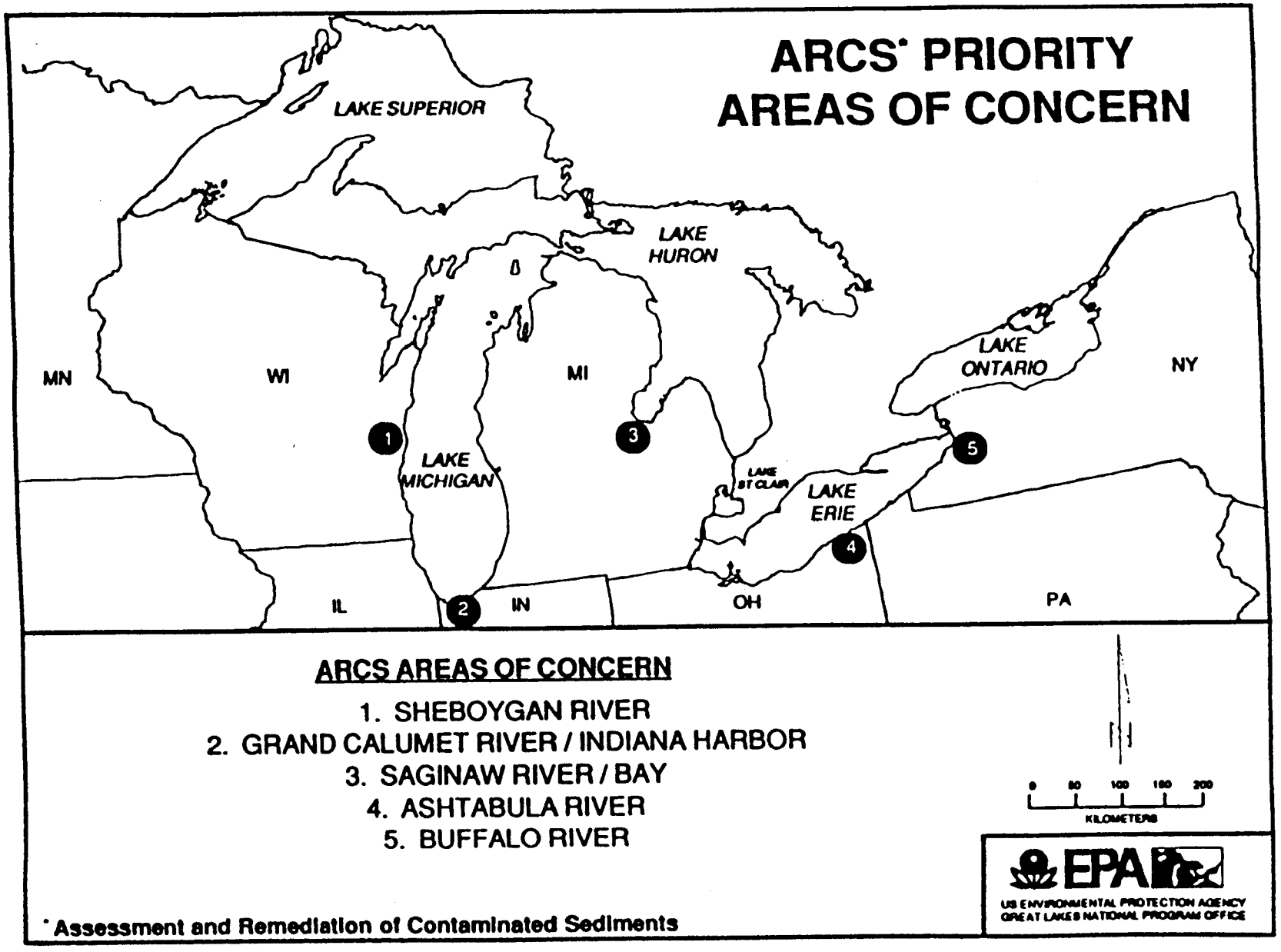


Figure 1. ARCS priority areas of concern.

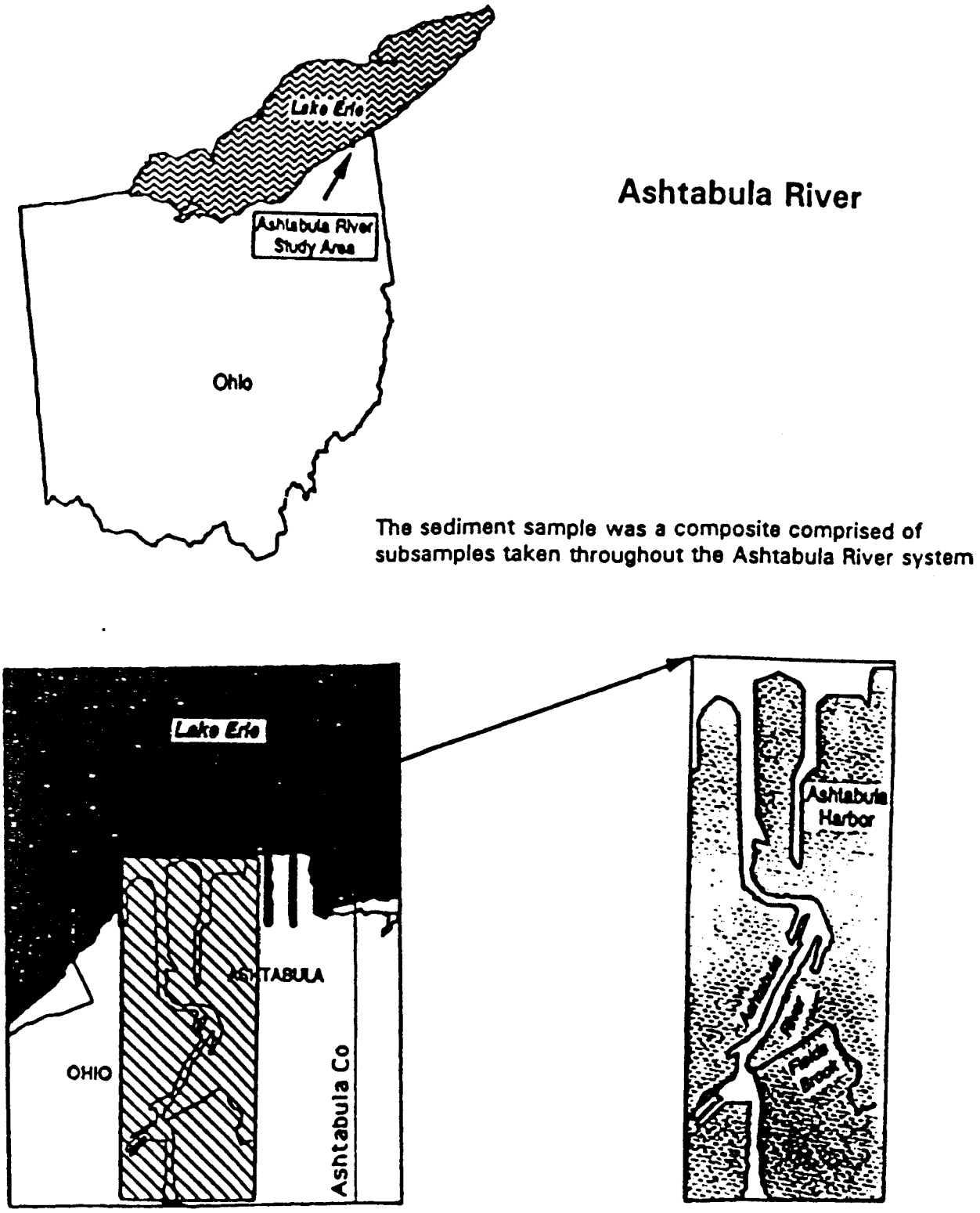


Figure 2. Map of Ashtabula River area of concern.

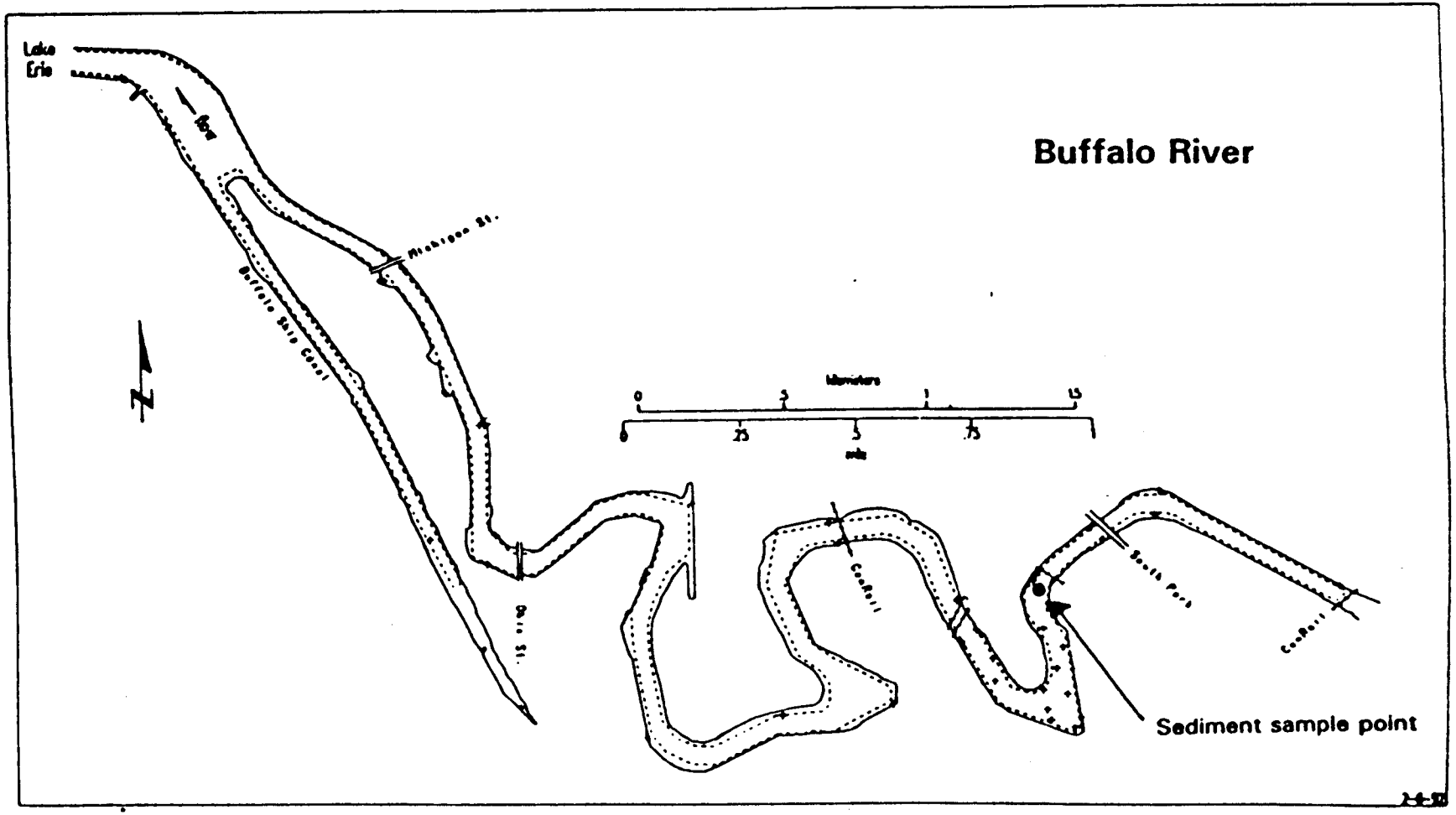


Figure 3. Map of Buffalo River area of concern.

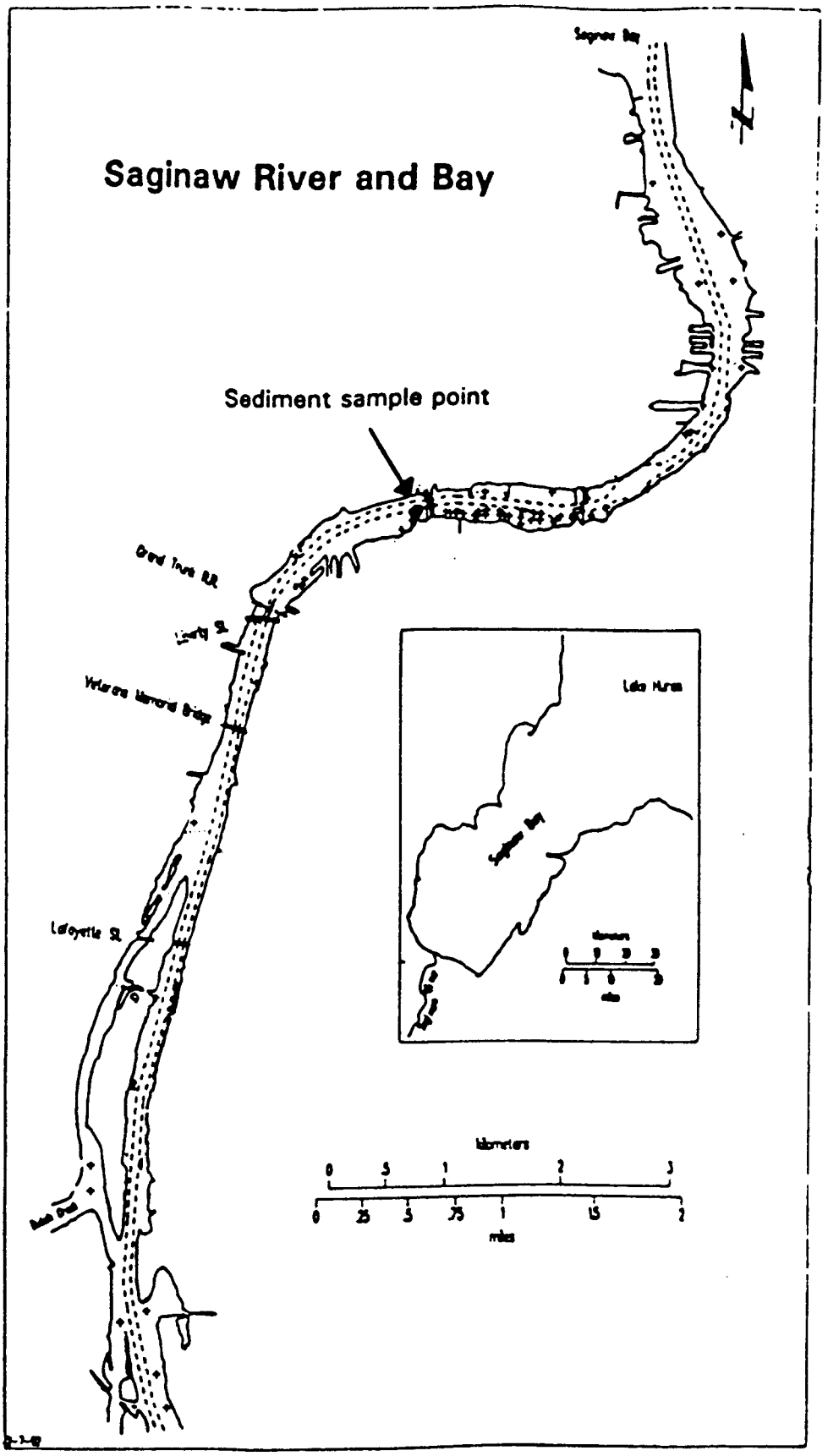


Figure 4. Map of Saginaw River area of concern.

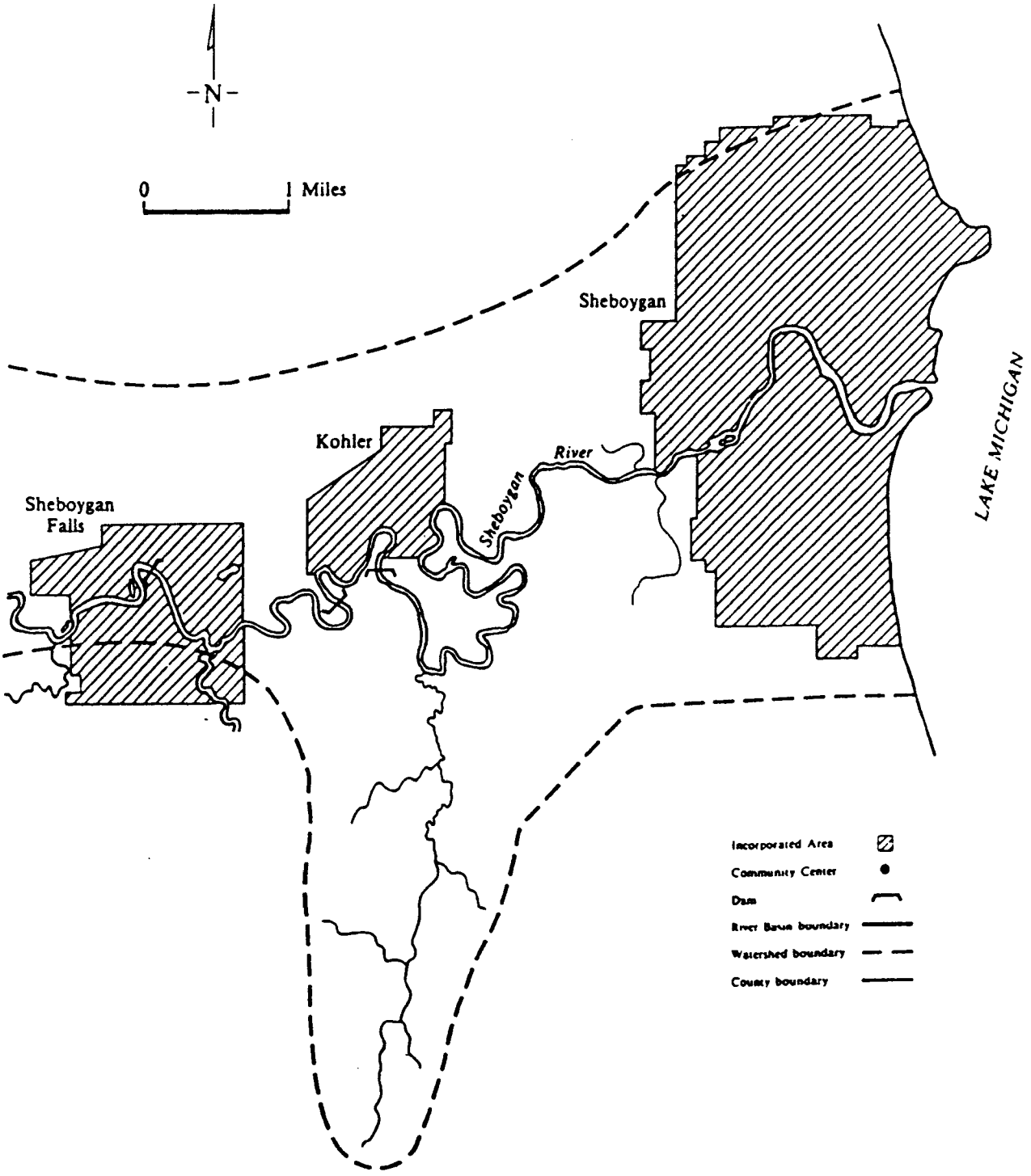


Figure 5. Map of Sheboygan River area of concern.

1.3 BACKGROUND ON BIOREMEDIATION TECHNOLOGY

A major challenge in today's world is to maximize the benefits that accrue from the industrial, agricultural and domestic use of xenobiotic chemicals while minimizing any adverse effects on natural ecosystems. Some of the means to achieve this goal are waste minimization, proper management of waste disposal, and clean up of sites already contaminated. Contaminated sites can include surface and subsurface soil, groundwater, and freshwater and marine sediments with their associated water. Often the cleanup or remediation of such sites may employ techniques from a number of disciplines, including biology, chemistry, geology, hydrology, and engineering.

For decades, biological treatment has been utilized in composting and in treatment of municipal and industrial wastewater. Within the past decade, interest has grown in the use of microorganisms for treatment of hazardous wastes that contaminate a variety of environments. Biological treatment, either alone or combined with other technologies, compares favorably with physical and chemical treatments on the bases of cost and completeness of chemical transformation. The cost of bioremediation is usually less than the cost of other methods of waste disposal.

The use of indigenous microorganisms to clean waste sites has the potential to make bioremediation a very cost-effective technology. This is especially true for cases in which only minor adjustments to the environment, such as addition of nutrients, are required to achieve cleanup. Biological treatment also offers the possibility of complete transformation of organic waste. Physical treatment of wastes, including extraction, adsorption, solidification, encapsulation, and filtration, only separates or immobilizes but does not destroy waste. Thermal processes, such as incineration, either completely destroy waste or reduce its volume, but may produce toxic ash and volatile compounds. Both chemical and biological treatment of wastes may result in hazardous and stable components. However, biological treatment offers the most flexible technology option to destroy hazardous waste components to acceptable environmental levels. In contrast to biological treatment, alternate technologies may require further treatment of wastes or extended storage of treated waste.

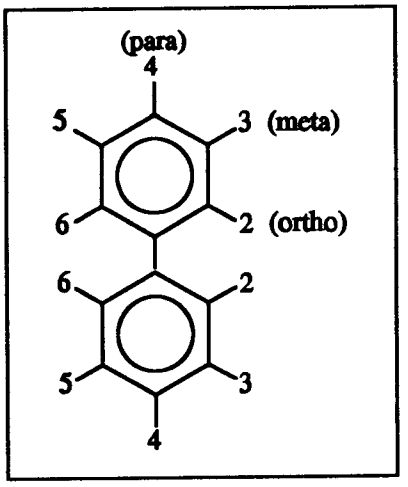
Bioremediation in aquatic environments often requires treatment of sediments, pore water, and surface waters. Sediments can serve as long-term sources of pollutants. Contaminated water or sediment may be biologically remediated *in situ* or in bioreactors. Although *in situ* treatment may be less expensive than bioreactor treatment, it often requires longer time periods (months to years) to achieve clean-up standards and may be extremely limited by physical constraints at the site. Treatment

in bioreactors may be complete within a shorter time (on the order of weeks) because optimal conditions (i.e., pH, temperature, nutrient concentrations, etc.) for biodegradation can be more easily maintained. However, physical removal of the contaminated material to the bioreactor is required and much higher operating expenses and maintenance costs are an additional consideration as are the risks associated with the removal and transportation of the contaminant.

1.3.1 PCB Biodegradation

For PCB-contaminated sediments, reductive dechlorination reactions (as mediated by anaerobic bacteria) preferentially transform the more highly chlorinated PCB congeners to less-chlorinated derivatives that are more amenable to aerobic degradation. In this instance, the anaerobic and subsequent aerobic processes are complementary and result in a reduction of toxic (higher chlorinated, coplanar) PCB congeners, and possibly even the complete biological destruction of PCBs through subsequent aerobic oxidation (Harkness et al., 1993). An important prerequisite for using this method at a remediation site is to assess the ability of indigenous microorganisms to transform the pollutants at the site and to develop techniques to enhance their activities.

PCB transformation in anaerobic environments such as the sediments of lakes and rivers was inferred in the mid 1980's from the studies of Brown and coworkers (1987a,b). These investigators noted an alteration in the expected profile of PCB congeners in contaminated sediments from the Hudson River compared to the congener profile of the original contaminating Aroclor. The alterations were characterized by a reduction in the concentration of the more highly chlorinated PCB congeners and an increase in the concentration of the more lightly chlorinated congeners. Further, the observed shifts in the congener profiles were congener specific, with selective or preferential removal of *meta*- and *para*-chlorines from the more highly chlorinated congeners.



PCB congener showing the numbering system and chlorine positions

The transformed sediments were thus enriched with *ortho*-substituted, lightly chlorinated PCB congeners. It was therefore proposed that dechlorination of the more highly chlorinated PCB congeners was catalyzed by anaerobic microorganisms residing in the contaminated sediment. The biologically mediated reductive dechlorination of PCBs from contaminated sediments was subsequently demonstrated in several laboratory investigations (Quensen *et al.*, 1988,1990; Bedard and Van Dort, 1992). In some studies, the microbial inoculum was obtained by "washing" PCB-contaminated sediments with anaerobic medium and collecting the supernatant (Quensen *et al.*, 1990; Nies and Vogel, 1990). Other investigators demonstrated reductive dechlorination of PCBs in actively methanogenic sediment slurries spiked with PCB mixtures (Aroclors) or specific PCB congeners (Van Dort and Bedard, 1991; Wubah and Rogers, 1992). In each study, evidence for reductive dechlorination was demonstrated by observing changes in the PCB congener profile with time.

To date, there have been only a limited number of studies that attempt to understand the factors that affect the reductive dechlorination of PCBs in historically contaminated sediments. Abramowicz *et al.* (1990) reported that addition of inorganic nutrients enhanced reductive dechlorination of endogenous PCBs in laboratory incubations of Hudson River sediments. In a recent study using methanogenic sediment slurries contaminated with Aroclor 1260, Bedard and Van Dort (1992) reported that dehalogenation of amended brominated biphenyl congeners significantly stimulated the reductive dechlorination of endogenous (historical) PCBs. In an earlier study, Bedard and coworkers (1990) reported that amendment of sediment from Woods Pond (Housatonic River, Massachusetts) with a high concentration (350 µM) of either 2,3',4',5-chlorobiphenyl (CB) or 2,3,4,5,6-CB stimulated reductive dechlorination of "historical" PCBs; transformation of congeners with *para* chlorines was especially evident.

One of the many objectives of this study was to determine the reductive dechlorination potential of PCB-contaminated sediments from the Saginaw, Sheboygan, and Ashtabula Rivers and to examine the effects of addition of PCB congeners and mineral nutrients on the reductive dechlorination of endogenous (historical) PCBs by the resident microbial populations.

1.3.2 PAH Biodegradation

PAHs are of environmental significance because of their resistance to biodegradation, their chronic toxic effects on humans and other species, and their widespread occurrence at contaminated waste sites. Low solubilities and high partition coefficients dominate the behavior of PAHs in biological and environmental systems. The high partition coefficients of PAHs determine their high relative concentrations in aquatic animals

and sediments as compared to the water column. With log K_{ow}s (octanol/water partition coefficients) in the range of 2.5 - 6.5, PAHs may be present in sediments at a hundred to nearly a million times their aqueous concentrations. Sediments, then, serve as a long-term source of PAHs to the water column and to biota.

The persistence of PAHs in sediments is a function of their chemical structures (Figure 6). Low molecular weight (LMW) PAHs, such as naphthalene, are both more soluble and more volatile than are the higher molecular weight (HMW) PAHs, and are lost from sediments more rapidly through volatilization, dissolution and biotransformation. High molecular weight PAHs are relatively insoluble and resistant to microbial degradation, and may persist indefinitely (Cerniglia and Heitkamp, 1989).

Given the tendency of PAHs to adsorb to sediments, there has been a great deal of interest in the potential for PAH degradation under anoxic conditions. Anaerobic degradation of monoaromatic compounds by anaerobic respiration, fermentation and photometabolism has been demonstrated (Young, 1984). Naphthalene and acenaphthene have been degraded to nondetectable levels under denitrifying conditions where water is the oxygen source for oxidation of the ring (Mihelcic and Luthy, 1988). Such activity is of limited utility in bioremediation; no anaerobic degradation of higher molecular weight PAHs has been reported to date.

Under aerobic conditions, molecular oxygen is the source of oxygen for the degradation of PAHs. Degradation may follow either a dioxygenase or a monooxygenase pathway (Figure 7). In bacteria, dioxygenases incorporate both atoms of oxygen, leading to the formation of dihydrodiols, with subsequent ring fission and assimilation, whereas fungi hydroxylate PAHs as a prelude to detoxification (Dagley, 1981).

Aerobic bioremediation also has been tested as a cleanup strategy for materials, primarily soils, from contaminated environmental sites where soils have been highly contaminated with PAHs from creosote and other wood-preserving compound mixtures. At such sites, high levels of PAHs are found in conjunction with other organics such as pentachlorophenol and organic solvents, with the total organic pollutants often comprising 5 to 10% by weight.

Both slurry reactors and *in situ* remediation have been attempted with PAH-contaminated soils. Mueller *et al.* (1991) reported that indigenous organisms successfully degraded phenols and LMW-PAHs in creosote-contaminated soil slurries within 30 days. The addition of nutrient amendments did little to enhance the effectiveness of degradation of HMW-PAHs. In soils that contained 4-, 5- and 6- ring PAHs in concentrations ranging from 10.4 mg/kg (benzo(*b*)fluorene) to 61.7 mg/kg (benzo(*b*/*k*)fluoranthene), the addition of nutrients to slurry

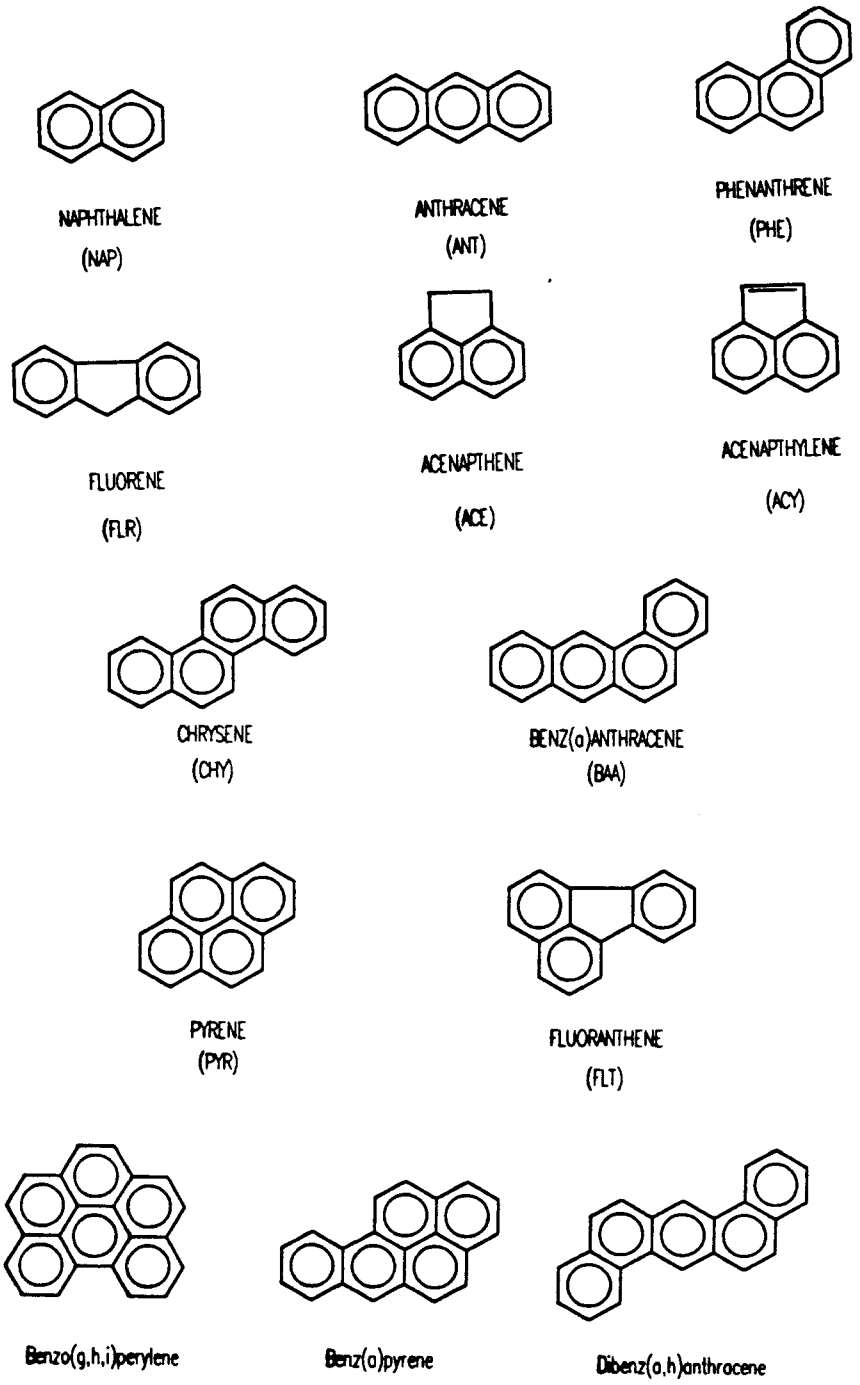


Figure 6. The chemical structures of PAHs.

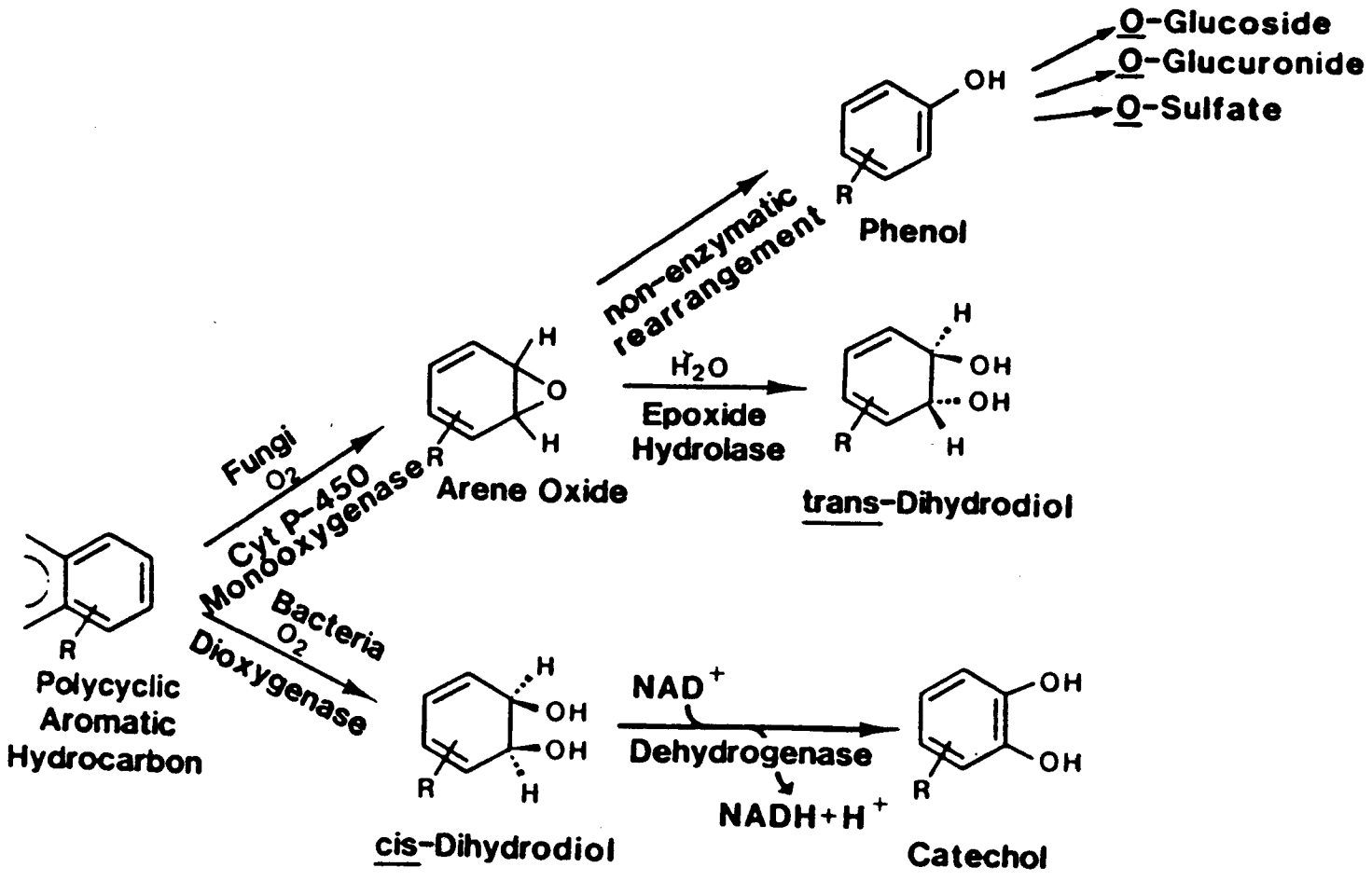


Figure 7. Metabolism of PAHs (from Cerniglia and Heitkamp 1989).

reactors resulted in the reduction of the HMW-PAHS by 48 percent after 12 weeks of incubation. The same group of compounds was reduced by only 35 percent in reactors operated without nutrient supplement. The *in situ* degradation rate for PAHs and phenols in soils was much slower; 8 to 12 weeks were required for 50 percent removal of organic contaminants, with little reactivity of HMW-PAHs.

Few studies have specifically addressed the issue of bioremediation in sediments. Most field applications of bioremediation have attempted to decontaminate soil, and usually soil with relatively high concentrations of PAHs. Soil sites have usually been contaminated directly by disposal of wastes, by spills, or by application of sewage sludges (Wild *et al.*, 1991), whereas sediments are typically contaminated as a result of sorption of the compounds via an aqueous exposure. Although the latter may result in concentrations that pose environmental threats, they are seldom as high as those resulting from direct dumping.

Sediments present several challenges to bioremediation: relatively low concentrations of PAHs, predominantly anoxic environments, an overlying water column that both limits physical access and disperses any soluble additions that may be added (e.g. nutrients or oxidants), and high concentrations of organic matter that both sorb the PAHs and support competitive consumption of added nutrients and oxidants.

2.0 TREATABILITY STUDY APPROACH

2.1 STUDY OBJECTIVES AND RATIONALE

2.1.1 PCB Biodegradation

The objectives of this study were to determine the extent of past (*in situ*) dechlorination activity, to assess the potential for further biotransformation of PCBs, and to examine the effects of addition of specific PCB congeners and mineral nutrients on the reductive dechlorination of amended and historical PCBs by the resident sediment microbial populations in sediment samples from the Ashtabula, Saginaw and Sheboygan Rivers. The primary goal was to identify factors that might influence the rate and extent of biotransformation of the historical PCBs in the contaminated sediments.

2.1.2 PAH Biodegradation

The objectives of this study were to identify the environmental parameters that constrained the biodegradation of PAHs in Buffalo River sediment and to assess the potential for their manipulation, either *in situ* or *ex situ*, to enhance the rate of remediation. The study considered the mechanisms

documented in the literature, as derived from both laboratory and field experiments, in light of the specific characteristics of the contaminated sediments themselves. Rates of degradation in sediment were compared and contrasted with those reported for other media and processes that enhanced the rate and extent of degradation were identified and investigated.

2.2 EXPERIMENTAL DESIGN

2.2.1 PCB Biodegradation

Enhancement of PCB biotransformation in sediments has been reported to occur in laboratory investigations via addition of a limiting nutrient, such as an inorganic mineral or other specific organic compounds, or via addition of specific PCB and/or polybrominated biphenyl (PBB) congeners. Experiments were therefore conducted to examine the effects of inorganic nutrient and PCB congener addition on PCB biotransformation in the various contaminated river sediments.

Slurries prepared with Sheboygan River sediments were amended with 2,2'3,3',4,5,6,6'-octachlorobiphenyl (Octa-CB) to yield a final Octa-CB concentration of 5 to 20 mg/L wet sediment slurry (approximately 0.2 g dry weight sediment/ml wet slurry). Sterile controls were prepared both with and without addition of Octa-CB. Live controls (non-sterilized) without addition of Octa-CB were also prepared.

Slurries prepared with Ashtabula River sediments were amended with 2,3,3',4,4'-pentachlorobiphenyl (Penta-CB), 2,3,3',4,4',5-hexachlorobiphenyl (Hexa-CB), and 2,2',3,4,5,6,6'-heptachlorobiphenyl (Hepta-CB) either alone (individual congeners, 10 mg/L) or in all congener combinations (total of 10 mg/L amended congeners). Sterile controls were also prepared as described previously.

Slurries prepared with Saginaw River sediments were amended with Aroclor 1260 (final concentration of 50 mg/L) both in the absence and presence of revised anaerobic mineral medium (RAMM). RAMM (see Appendix) was prepared according to the procedures of Shelton and Tiedje (1984) and was added to the sediment slurries as a concentrated, anoxic solution. Sterile controls were prepared as previously described.

2.2.2 PAH Biodegradation

Enhancement of bioremediation of PAHs has been reported in field and reactor studies. Although enhancement can be associated with manipulations such as nutrient additions, many variables affect the rate of PAH transformation. Thus, an understanding of the processes that impact the rate of degradation is essential

for predicting the feasibility and effectiveness of bioremediation of these compounds on other than a case-by-case basis.

In light of the objectives of this project, experiments were designed to: 1) develop an enrichment culture of PAH-degrading bacteria derived from Buffalo River sediment, 2) characterize the activity of the enrichment culture and contrast it with the activity of indigenous organisms, 3) determine whether the enrichment culture maintained and expressed its activity when reintroduced into sediments, and 4) investigate the processes that affected the rate of transformation of PAHs by the enrichment culture in sediments.

The factors that determine the rate of degradation of PAH compounds in the environment can be considered to be intrinsic, that is, controlled by properties of the chemical itself; extrinsic, controlled by the environment; or combinations of the two. Thus, oxygen or nutrient concentrations and bacterial densities are extrinsic limitations, toxicity to bacteria is an intrinsic limitation, and sorption of low-solubility PAHs to sediment organic matter is a combination of the two. Extrinsic factors are more readily modified, and were investigated first as possible limitations on the rates of biodegradation. Environmental processes considered in these investigations focus on the extrinsic factors that may limit the rate and extent of biodegradation of PAHs and the interactions between bacteria and the sediment environment that may determine that activity of degrader organisms on the substrates of interest.

The processes that influence the rate and extent of PAH degradation in sediments were investigated by comparing the kinetics of degradation under controlled conditions. The potential for degradation of PAHs and the range of compounds susceptible to transformation were assessed by amending sediments with mixtures of PAHs. The concentrations of PAHs in Buffalo River sediments as sampled were low. Thus, PAHs were added to these sediments in order to elevate concentrations to levels that supported the analysis of differences in rates of degradation in response to experimental treatments. Moreover, given the relative unavailability of hydrophobic materials that have been associated with organic sediments for long periods, the amendment of sediments with PAHs permits the optimization of the environmental conditions, other than sorption, that control the rate of degradation. The added mixture consisted of up to 16 compounds at individual concentrations of approximately 20 mg/kg sediment, with representative compounds from each size class of PAHs, based on number of rings. The structure of the compounds added is shown in Figure 6. The transformation of the PAHs was assessed by extracting the entire sediment slurry from among sets of replicate treatments at a specified time, and analyzing the extract by gas chromatography. The sampling time periods

depended on the concentrations of the PAHs and the relative activity of the sediments or inoculum.

The processes that influence the rates of degradation were investigated by measuring the kinetics of degradation of a model compound. Pyrene was selected as the model on the basis of its relative resistance to degradation in unmodified sediments. Pyrene degrades slowly in sediments, and few organisms that utilize pyrene as a growth substrate have been identified. Pyrene is also available in radiolabelled form, which facilitates frequent data collection over short time periods needed for kinetic studies. Thus the trapping of radiolabelled CO_2 yielded data amenable to kinetics interpretation; specifically, the time series data provided by radiorespirometry permit accurate rate comparisons as well as indications of bacterial acclimation times and growth. The enrichment culture developed from Buffalo River sediment was able to mineralize pyrene and was used as the inoculum in the mineralization studies.

2.3 EXPERIMENTAL METHODS

2.3.1 PCB Biodegradation

Anoxic slurries of the PCB-contaminated sediments were prepared in an anaerobic glove box containing an atmosphere of 5 percent hydrogen and 95 percent nitrogen. For all sediment samples, one volume of wet sediment was combined with two volumes of anoxic (N_2 sparged) distilled water and the mixture stirred for approximately 5 minutes. Approximately 50 milliliters of the sediment slurry were added to 120-ml amber serum bottles and amendments were added within 1 hour. Serum bottles were crimp-sealed with a Teflon-lined septum and aluminum cap and vigorously mixed to distribute the amended PCB congener evenly. Live control (no sterilization) sediment slurries were prepared without PCB congener amendment. Sterile controls were prepared for all experiments by autoclaving sediment slurries at 121°C for 30 minutes on 3 consecutive days, followed by PCB congener amendment. Experiments were performed in triplicate. All vessels were incubated in the dark at room temperature (25°C) inside the anaerobic glove box.

At specified time intervals (usually 1-2 months), the sediment slurries were thoroughly mixed. Slurry samples (approximately 5 ml) were withdrawn, transferred to screw-cap tubes, and sealed with a Teflon-lined cap. Five milliliters of acetone and 100 µl of surfactant [10% (v/v) Triton X-100] were added to each sample and the sediment slurry was shaken thoroughly. Surfactant was added to enhance the recovery of PCBs from the contaminated sediments. Samples were then placed in an ice bath, sonicated for 10 minutes at 30-35W using a titanium microtip probe (Ultrasonic Power Corp., Freeport, Illinois), and

centrifuged at 3500 x g for 10 minutes. The supernatant was transferred to a 250-ml separatory funnel and 10 ml of hexane-acetone (9:1, v/v) were added. The separatory funnel was vigorously shaken and the aqueous layer was discarded. The remaining organic phase was sequentially washed with 5 ml each of 2% (w/v) NaCl, concentrated H_2SO_4, and 2% (w/v) NaCl. A detailed description of the PCB extraction procedure is presented in the Appendix.

Congener-specific PCB analysis was performed using an HP-5890 gas chromatograph (Hewlett-Packard Co., Palo Alto, California) equipped with a DB-1 (J & W Scientific, Folsom, California) capillary column [30m by 0.25mm (inside diameter); 0.25µm film thickness] and an electron capture detector. Identification and quantitation of PCB congeners were performed by comparing the areas and retention times of individual peaks with those in a standard mixture of Aroclors 1232, 1248 and 1262 in the ratio 2.5:1.8:1.8 (Mullin, 1985). Congener identities were confirmed by comparing the order of elution with that published by Mullin *et al.* (1984). A three point calibration curve was used to quantitate added PCB congeners as well as historical PCBs. Data were acquired and analyzed using Maxima 820 software (Dynamics Solutions, Ventura, California) on an NEC PowerMate 386SX computer connected to the gas chromatograph through a system interface module (Waters Corp., Milford, Massachusetts). A detailed QA/QC plan for the congener-specific PCB analysis is presented in the appendix.

2.3.2 PAH Biodegradation

An enrichment culture that could degrade the widest range of PAHs was developed by enriching the sediment bacterial population for individual PAH degraders, and then combining the individual cultures to form a mixed enrichment culture. The individual enrichments were developed by incubating 10% slurries (w/v) of Buffalo River sediment in minimal medium with each of the PAHs shown in Figure 6, except for the 6- ring compounds. Each of the 2- and 3- ring PAHs were added to the sediment slurry at 50 mg/L; the 4- and 5- ring PAHs were added at 20 mg/L. The compounds were added at concentrations substantially higher than environmental concentrations in order to maximize the population of degrader organisms. The 6-ring PAHs were not added to the enrichment media because these compounds do not support microbial growth and would likely be toxic to bacteria. The sediment slurries were incubated on a shaker (150 rpm) at 30 C for 3 weeks. After 3 weeks, a subsample of the sediment slurry was transferred to minimal medium (Appendix) containing the same PAH compound on which the organism had been enriched. Each of the PAH-degrading strains was evaluated for activity by plating the enrichment on agar plates containing the PAH to be tested.

The activities of the indigenous organisms and the enrichment culture were assessed by introducing each source of bacteria to 10% sediment slurries to which had been added a mixture of all of PAHs shown in Figure 6, each at a nominal concentration of 20 mg/kg sediment (dry weight of sediment). The amount of PAH remaining was determined throughout the period of 2 to 6 weeks following dosage.

PAHs were added to sediments as a primary mixture made by combining 2 mg each of acenaphthene, acenaphthylene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo(*a*)pyrene, dibenz(*a,h*)anthracene, and benzo(*g,h,i*)perylene in 10 ml CH_2Cl_2. The spiking mixture was stored at -4°C. Sediment slurries were spiked by adding 50 µl of the PAH mixture to wet sediment (as received, equivalent to 20 g dry weight) in a 250-ml flask. The solvent was allowed to evaporate overnight at 4°C prior to addition of the experimental medium (e.g., minimal salts broth) to form the slurry.

Sampling intervals depended on the concentration of PAHs added to the sediments and the activity of the bacterial inoculum. Estimates of the appropriate intervals were based on outcomes of prior experiments. At the time intervals indicated in the figures for each experiment (2-6 weeks), the entire contents of replicate flasks were extracted. The sediment slurries, which had been amended with PAHs, were centrifuged to remove water, treated with methanol or sodium sulfate to remove residual water, and extracted twice with methylene chloride on a wrist-action shaker overnight. Detailed procedures for extraction and GC analysis are presented in the appendix.

The recovery of PAHs extracted from sediments was calculated from the additions of a mixture of six deuterated PAHs, (Table 1). Deuterated compounds are those that differ from the non-deuterated compound by the replacement of a number of hydrogen atoms with the heavier form of hydrogen, or deuterium. By virtue of the greater molecular weight, the deuterated form elutes from the gas chromatograph slightly later than the standard form. The identification of separate peaks for the extracted compound and the recovery spike permits the calculation of recoveries from a single analysis, rather than the two analyses required by the method of standard additions. Recoveries were calculated to assess the effectiveness and the consistency of the analytical procedures used; the PAH data presented herein have not been corrected for recovery. The procedure for analysis of PAHs by gas chromatography also is presented in the appendix.

Table 1. Recovery of deuterated PAHs extracted from Buffalo River sediment slurries.

Compound	% Recovery[1]	S.D.
d8- naphthalene	32.85	4.3
d10-acenaphthene	46.15	6.5
d10-phenanthrene	60.38	4.9
d12-chrysene	70.27	5.3
d12-perylene	76.77	6.3

[1]Recoveries calculated as mean and standard deviation (S.D.) of 13 samples.

In experiments in which the PAH concentration was determined by extraction and GC, each treatment was run in duplicate or triplicate. At each sampling, at least one treatment replicate was sampled in triplicate, in order to compare the variance due to sampling to that due to differential performance of replicates within treatments. All data are represented as averages of replicate or triplicate determinations.

Radiolabelled PAHs can be readily quantified with a high degree of precision and were used for several types of experiments in this project in a technique known as radiorespirometry. No extraction is required to measure the radiolabelled compounds, either as the parent compound in the sediment slurry or as carbon dioxide produced by respiration of the parent. This permits frequent, precise sampling, which is necessary for comparing the rates of transformation of PAHs in response to treatments. Moreover, although disappearance of a parent PAH can indicate the occurrence of a transformation, the detection of radiolabelled carbon dioxide from labelled PAHs can indicate the full mineralization of the parent and hence the potential for supporting the growth of bacteria capable of their degradation.

Mineralization experiments were performed in biometer flasks or Erlenmeyer flasks modified to function as biometer flasks (Figure 8). The sediment slurry or liquid sample was incubated in the main chamber of the flask and CO_2 was trapped in 0.1 N NaOH in a 25-ml vial suspended in the flask by wire from a rubber stopper. A 3-ml syringe with a needle pierced through the stopper was connected to tubing and was used to replace the NaOH. An aliquot of NaOH (1 ml) was combined with 5 ml scintillation cocktail (ICN Ecolite) and radioactivity was determined on a Beckman L56000LL scintillation counter (Fullerton, California) after chemiluminescence had subsided. Samples were counted for 5 minutes or to 2% counting precision. The counts were corrected

by the external standards method. Data are reported as the cumulative number of counts trapped as radiolabelled carbon dioxide ($^{14}CO_2$), expressed as the percentage of counts added to the experimental flask as labelled PAH. Thus, the data represent the fraction of the PAH transformed and respired by the bacteria. The percent $^{14}CO_2$ seldom approaches 100% because a fraction of the original PAH is incorporated into biomass or metabolic products. Thus, a characteristic yield of $^{14}CO_2$ results from the utilization of any given substrate by a given bacterium under similar growth conditions. All data are the mean of triplicate measurements unless otherwise specified.

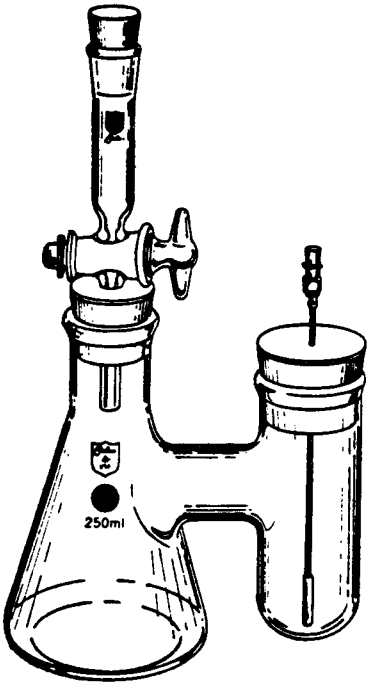


Figure 8. The design of biometer flasks used for radio-respirometry.

To determine whether specific PAHs were mineralized by the enriched culture, approximately 0.025 µCi of the labeled compound and 2 ppm unlabeled substrate were added to sediment slurries and $^{14}CO_2$ evolution was monitored over time. The compounds tested were: phenanthrene, fluoranthene, pyrene and benzo(a)pyrene, which were obtained in radiolabeled form ([9-^{14}C]phenanthrene (Sigma Chemicals), [3-^{14}C]fluoranthene (Chemsyn Labs, Lexana, Kansas), [4,5,9,10-^{14}C]pyrene (Chemsyn Labs) or [7,10-^{14}C]benzo(a)pyrene (Sigma Chemicals)). Sediments were inoculated with the enrichment culture.

3.0 RESULTS AND DISCUSSION

3.1 SUMMARY OF PCB BIODEGRADATION RESULTS

3.1.1 Bench-scale experiments with Sheboygan River sediments.

The most prominent PCB homologs detected in the contaminated Sheboygan River sediments were trichlorobiphenyls and tetrachlorobiphenyls (Figures 9 a-d, week 1); the total PCB concentration was 65.6 ± 5.3 mg/L. In unamended control and autoclaved control sediments (Figure 9a), no appreciable change in the profiles of historical PCBs was evident after 30 weeks of anaerobic incubation at 25°C. The average number of chlorines per biphenyl (3.9) in the live, unamended control sample remained essentially constant over the course of the 30-week incubation period (Figure 10). However, in all experiments amended with Octa-CB, there was a decrease in the concentration of hepta-, hexa-, penta-, tetra- and tri-CB congeners and an increase in the concentration of di- and mono-CBs. The mole percentage of mono-CBs was less than 1% at the onset of the experiment (week 1); after anaerobic incubation for 30 weeks, this homolog group accounted for approximately 8% of the total PCB congeners in sediments amended with 20 mg/L Octa-CB (Figure 9b). The major products of reductive dechlorination were di-CB congeners; this homolog group increased from 2.5 to 40 mole percent after 30 weeks of incubation. The most prominent di-CB peak detected in Octa-CB amended sediments consisted of two *ortho*-substituted congeners (2,2'- and 2,6-CB).

Two additional homolog groups, tri- and tetra-CBs, initially accounted for approximately 80% of the total PCB homologs in the contaminated sediments (week 1, Figures 9 a-d). After anaerobic incubation for 30 weeks, these two CB homolog groups were reduced to less than 50% of the total mole percent of homologs in the Octa-CB amended sediments. The mole percentage of penta-, hexa- and hepta-CB homologs were reduced from 8, 5 and 2% to 4, 3 and 1%, respectively.

In addition to the changes observed in the PCB homolog distribution after anaerobic incubation for 30 weeks, there also were changes in the average number of chlorine atoms per biphenyl in the various treatments (Figure 10). In the unamended and sterile Sheboygan River sediments, the average number of chlorine atoms per biphenyl remained constant throughout the experiment, indicating little or no detectable dechlorination activity. However, in the Octa-CB amended sediments, the average number of chlorines per biphenyl (total of historical plus amended PCBs) decreased from 4.2 to 2.8 (± 0.1), 2.5 (± 0.3) and 2.2 (± 0.3) in experiments amended with 5, 10 and 20 mg/L Octa-CB, respectively.

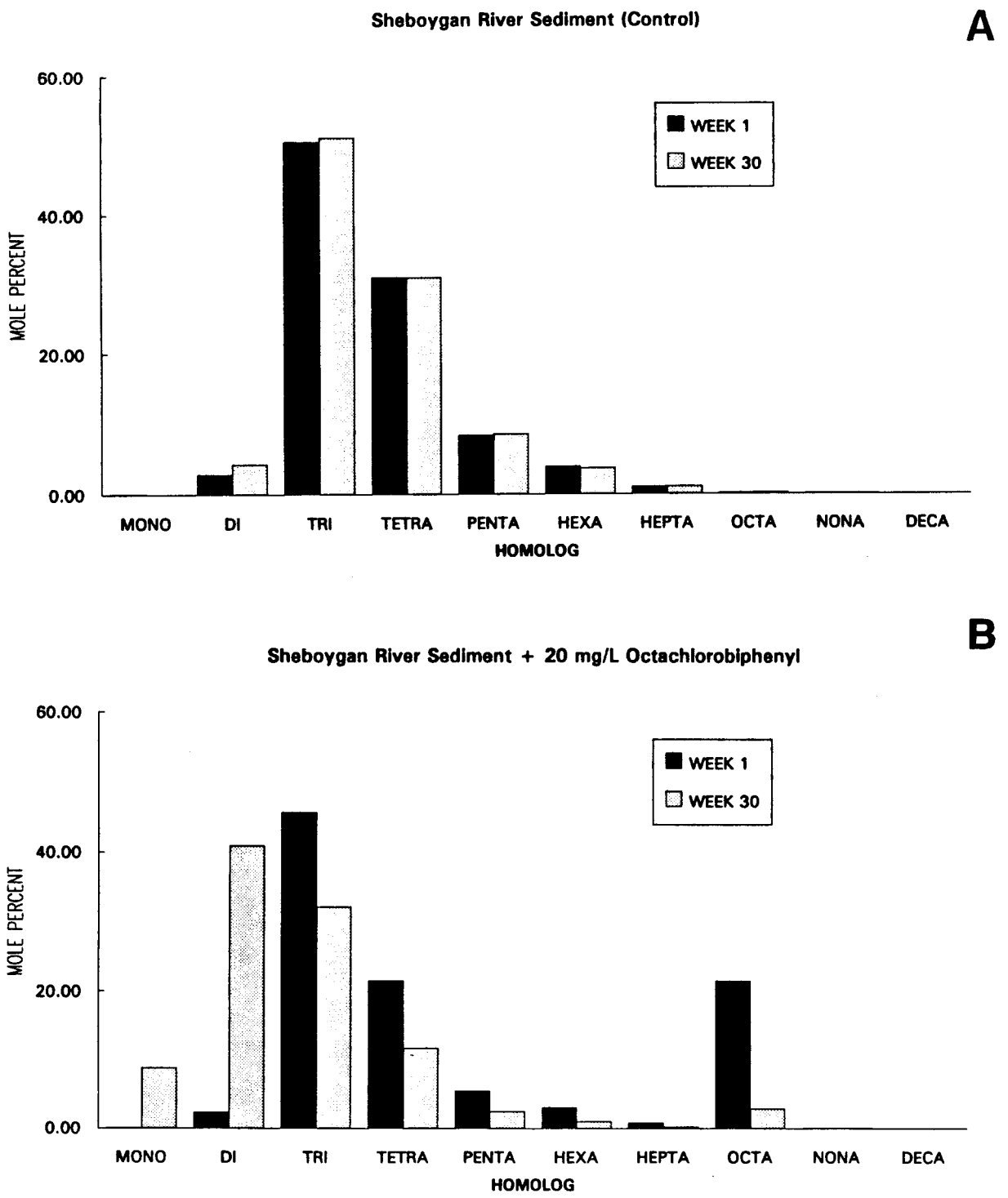
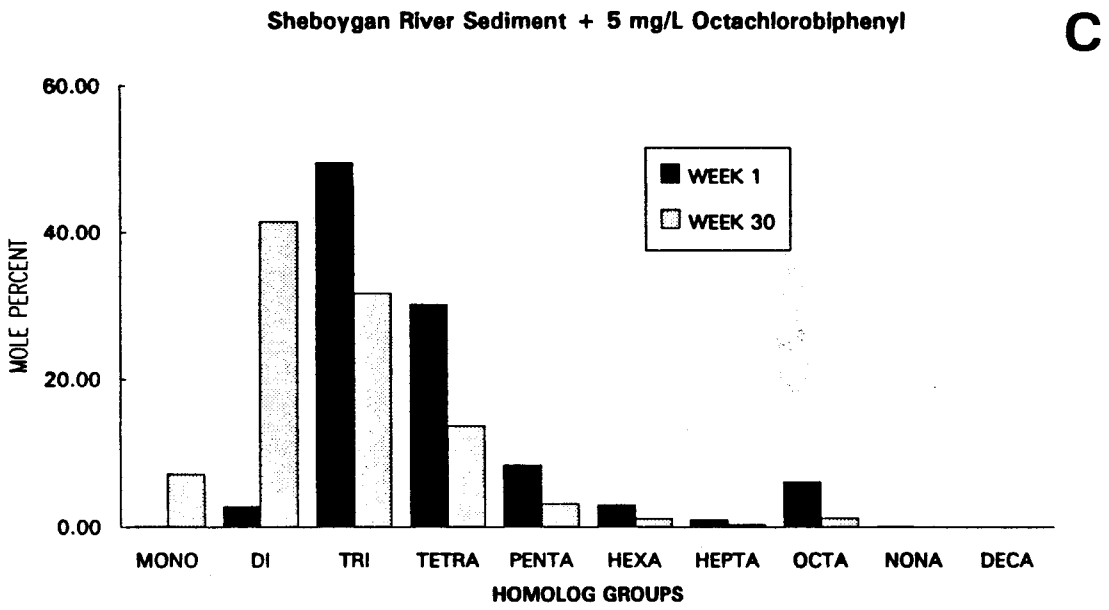


Figure 9. Profile of amended and historical PCB biotransformation in (a) unamended control (sterile and nonsterile) sediments and (b) 20 mg/L 2,2',3,3',4,5,6,6'-octachlorobiphenyl (Octa-CB) amended sediments. Experiments were conducted under anaerobic conditions using PCB-contaminated sediment from the Sheboygan River.



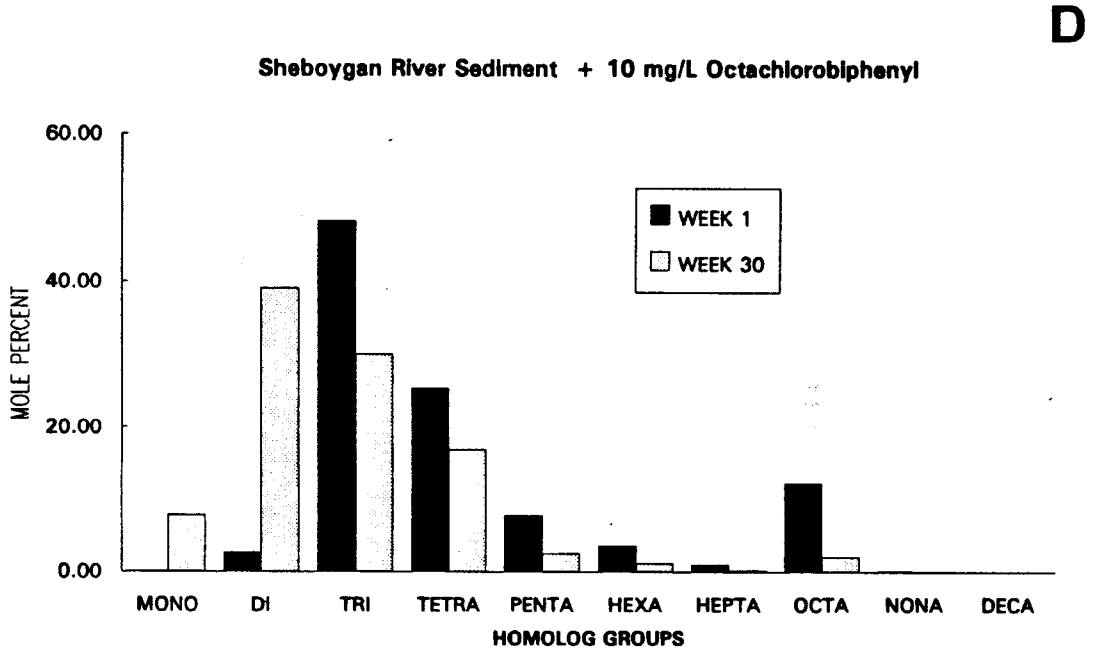


Figure 9. Profile of amended and historical PCB biotransformation in (c) 5 and (d) 10 mg/L Octa-CB amended sediments. Experiments were conducted under anaerobic conditions using PCB-contaminated sediment from the Sheboygan River.

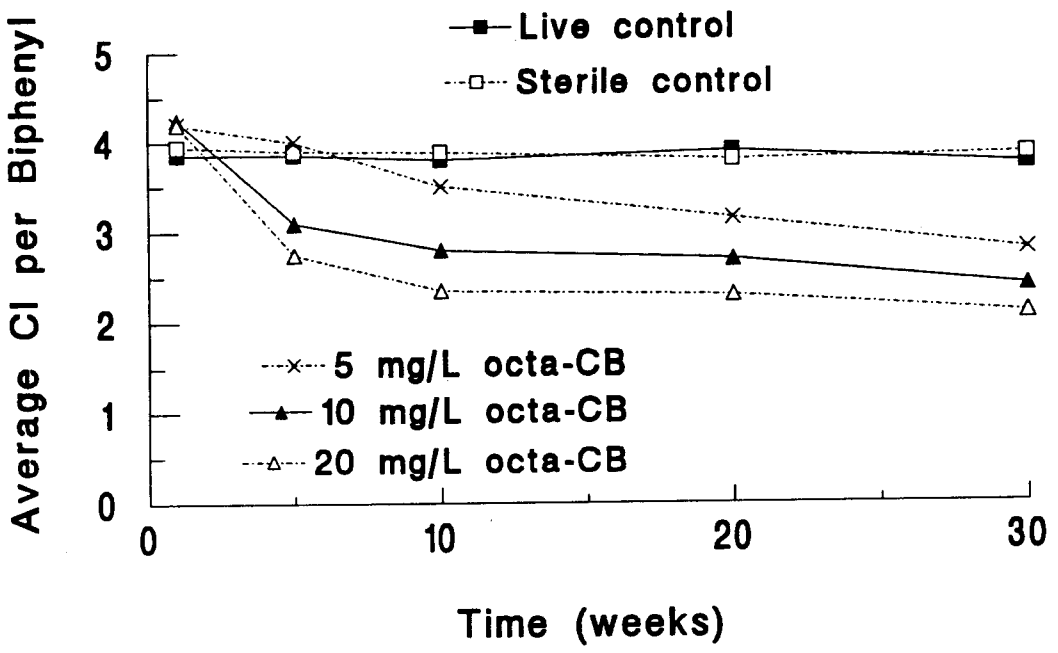


Figure 10. Evidence for the reductive dechlorination of PCBs as indicated by the reduction in the average number of chlorines per biphenyl in unamended (live and sterile controls) and in Octa-CB amended (5-20 mg/L) sediments (Sheboygan River).

In all 'live'(non-sterile) experiments, the concentration of the added PCB congener (Octa-CB) was appreciably reduced over the course of the experiment. The onset of transformation (dechlorination) of the added Octa-CB preceded the onset of dechlorination of the historical PCB congeners by 1-2 months (data not shown). Initial products of *meta*- or *para*- directed reductive dechlorination of the Octa-CB congener did not accumulate to an appreciable level; thus, the disappearance of Octa-CB over time was used to determine the rate of Octa-CB dechlorination (Table 2). The estimated rates of dechlorination of the added Octa-CB were directly proportional to the concentration of the amended Octa-CB.

Table 2. Rate of dechlorination of amended octachlorobiphenyl (Octa-CB) in Sheboygan River sediment.

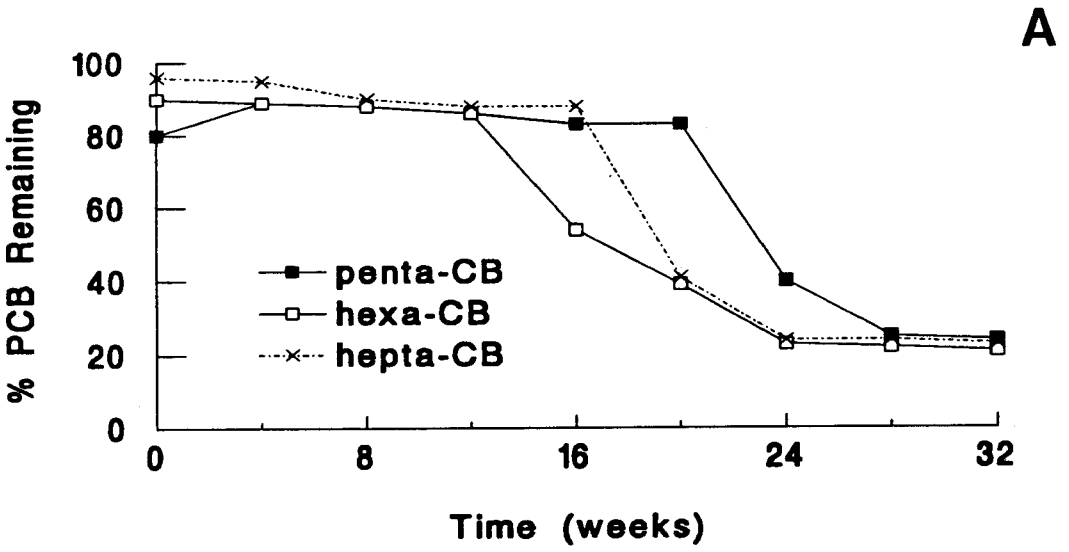
Experiment	Rate (mean ± S.D)*
Autoclaved control	ND
Unamended control	ND
Sediment + 5 mg/L Octa-CB	0.22 ± 0.01
Sediment + 10 mg/L Octa-CB	0.35 ± 0.01
Sediment + 20 mg/L Octa-CB	0.43 ± 0.01

* Rate measured as µg atoms of chlorine removed per gram sediment per month

ND = no dechlorination

3.1.2 Bench-scale experiments with Ashtabula River sediments.

Unamended Ashtabula River sediments and sediments to which a single chlorobiphenyl congener had been added exhibited no appreciable dechlorination activity during the first 3 months of anaerobic incubation (Figure 11a). Lag periods of 3, 4 and 5 months were observed before the onset of dechlorination of the amended Hexa-CB, Hepta-CB and Penta-CB congeners, respectively. After 6 months incubation, approximately 75% of the amended Hexa-CB and Hepta-CB were transformed while the same amount of the Penta-CB was transformed after 7 months incubation. No further dechlorination was observed after incubation for an additional 2 months. Approximately 20 to 30% of the original concentration of all amended PCB congeners remained at the end of the incubation period.



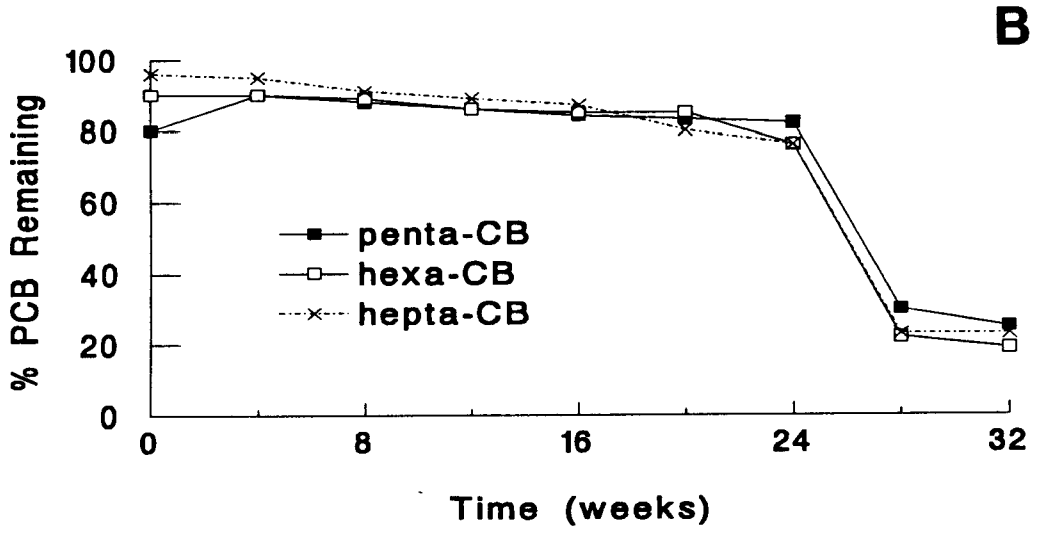


Figure 11. Reductive dehalogenation of amended PCB congeners [either 2,3,3',4,4'-pentachlorobiphenyl (Penta-CB), 2,3,3',4,4',5-hexachlorobiphenyl (Hexa-CB), or 2,2',3,4,5,6,6'-heptachlorobiphenyl (Hepta-CB)] added (a) individually or (b) as a mixture in Ashtabula River sediment slurries. Results are expressed as the percent of the amended PCB congener concentration.

In experiments amended with two congeners, a lag period of 6 months was observed before the onset of dechlorination of all amended congeners except for sediments amended with a combination of Penta-CB and Hexa-CB. In this case, significant dechlorination was detected after incubation for 5 months. The Hexa-CB congener was transformed more rapidly in sediments amended with either Penta-CB or Hepta-CB compared to sediments amended with Hexa-CB alone. In sediment slurries amended with a mixture of all three congeners, a lag period of 6 months was observed before appreciable dechlorination occurred (Figure 11b). In this case, all three congeners were transformed at a similar rate. Approximately 20 to 30% of the original concentration of all amended congeners remained after 8 months incubation.

Addition of the Penta-CB, Hexa-CB, and Hepta-CB congeners, singly or as mixtures, to Ashtabula River sediment slurries resulted in enhanced reductive dechlorination of historical PCB congeners in a manner similar to that observed for Sheboygan River sediment amended with Octa-CB. The results presented in Figure 12 are representative of the PCB homolog distributions for Ashtabula River sediment slurries after 32 weeks incubation amended with either the Penta-, Hexa-, or Hepta-CB congener or combinations thereof. Figure 12a depicts the results for the live control sediment. Figure 12b depicts results for sediment amended with Penta-CB plus Hexa-CB.

The average number of chlorine atoms per biphenyl (total of historical plus amended PCBs) decreased in all experiments amended with PCB congeners, but not in sterile and live (unamended) control experiments (Figure 13a). A representative plot of the average number of chlorines per biphenyl for experiments amended with all three congeners is presented in Figure 13a; the average number of chlorines/biphenyl lost for experiments amended with individually added congeners is presented in Figure 13b. No appreciable changes were observed in the congener profile or the total PCB concentration of the historical PCBs for both sterile and live controls. However, the molar increase in lower chlorinated congeners was consistent with the molar decrease in higher chlorinated congeners (historical PCBs) for all experiments amended with specific PCB congeners.

3.1.3 Bench-scale experiments with Saginaw River sediments.

Unamended Saginaw River sediment slurries and sterile controls (Figures 14a-b) exhibited little or no appreciable changes in the profile of PCB congeners after incubation at 25°C for 25 weeks. In experiments amended with Aroclor 1260 plus RAMM, a significant increase in the mole percentage of mono-, di- and tri-CB homologs and a decrease in the mole percentage of homologs with higher numbers of chlorine atoms per biphenyl was evident (Figure 15b). Significant reductions in the mole percentages of tetra-, hexa-, hepta-, octa-, and nona-CB were also evident.

Concomitant with the reduction in the mole percentage of the higher chlorinated homolog groups, the mole percentage of the mono-, di-, and tri-CB homolog groups increased from 1, 7.5, and 18% to 4.5, 15, and 20%, respectively. These data clearly indicate that active, PCB-dechlorinating populations were present. In experiments amended with Aroclor 1260 alone, an increase was evident in the mole percentage of mono- and di-CB homologs only (Figure 15a). The mole percentage of the tri-CB through the nona-CB homolog groups decreased significantly. The mono- and di-CB congeners, which accounted for 1% and 7.5% of the total PCBs at week number 1, increased to 8% and 28% after anaerobic incubation for 25 weeks.

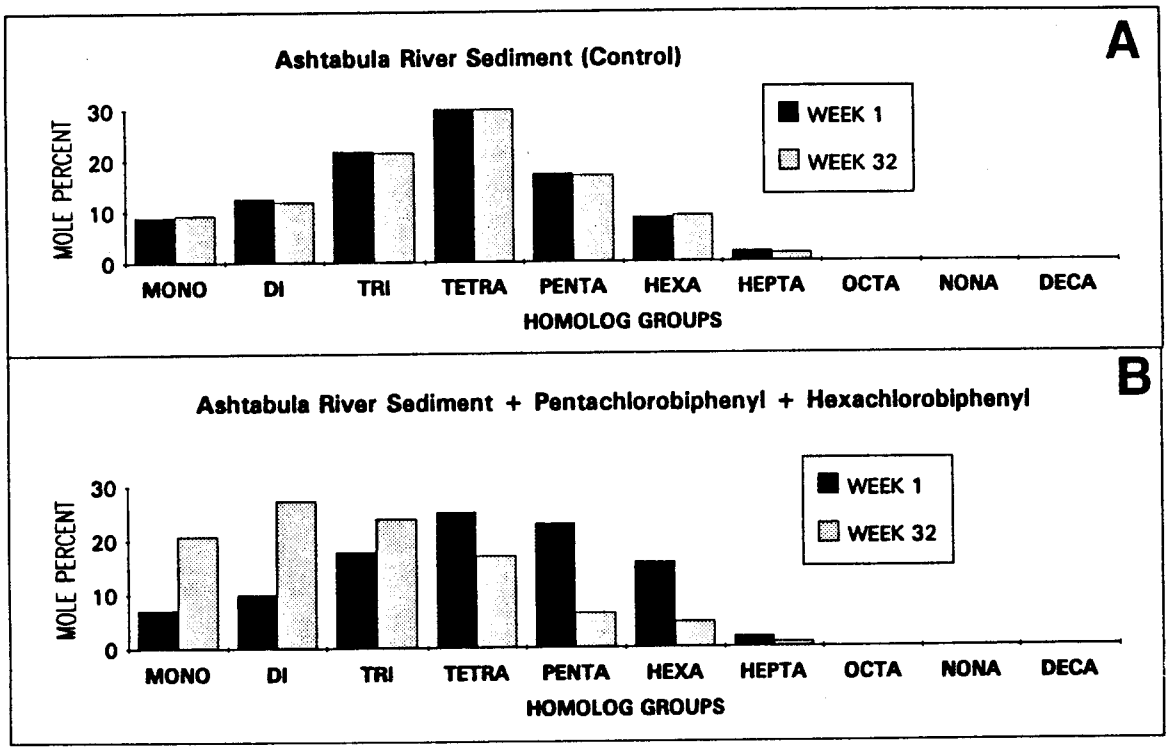


Figure 12. Profile of biotransformation of historical and amended PCBs in (a) live control experiments and in (b) 2,3,3',4,4'-pentachlorobiphenyl (Penta-CB) plus 2,3,3',4,4',5-hexachlorobiphenyl (Hexa-CB)-amended sediment slurries. Experiments were conducted under anaerobic conditions using PCB-contaminated sediment from the Ashtabula River. Sterile control samples behaved identically to the live control sample.

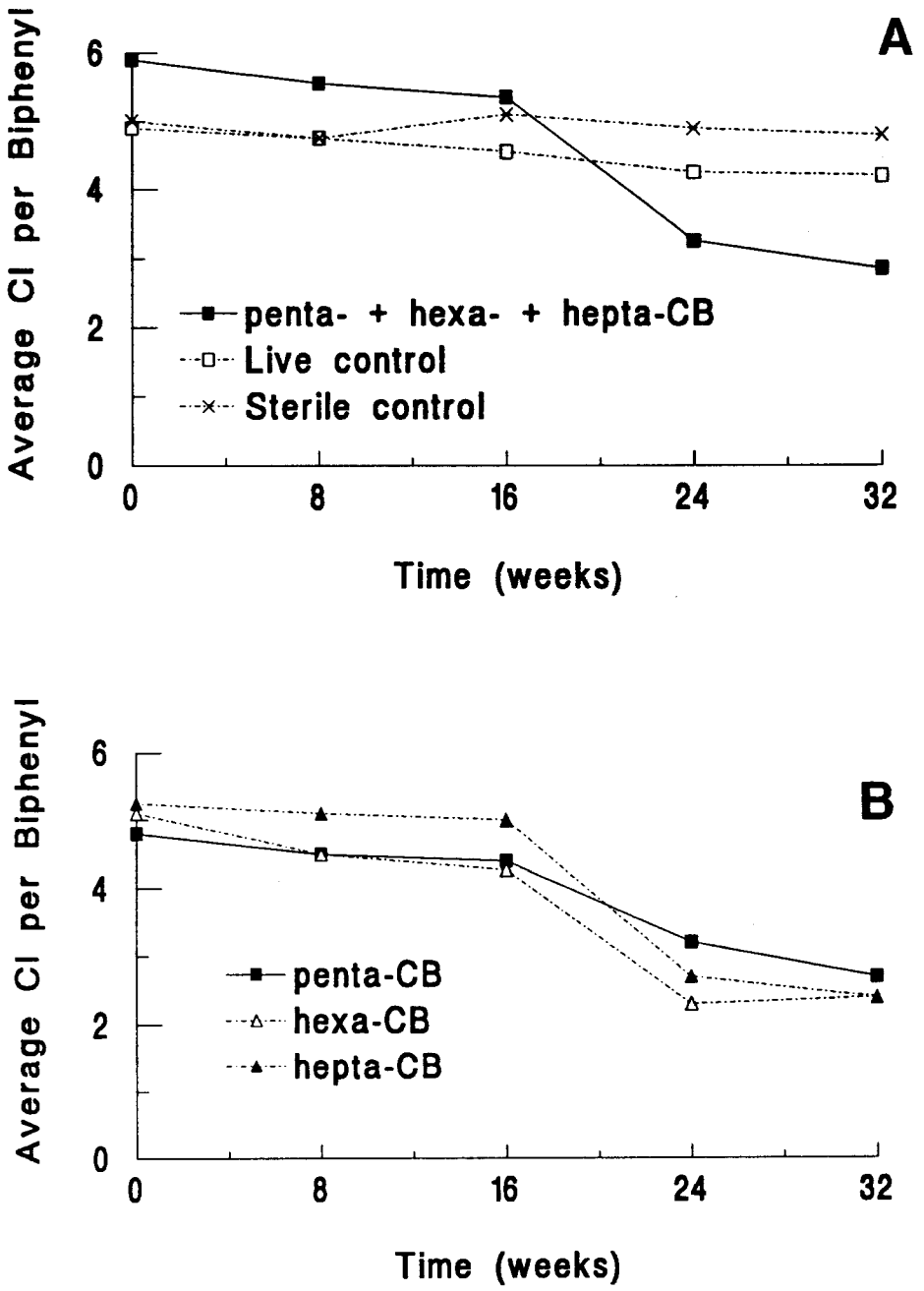


Figure 13. Profile of reductive dehalogenation of historical and amended PCBs in Ashtabula River sediment slurries as measured by the reduction in the average number of chlorines per biphenyl. (a) Sediments were incubated without amendments (live and sterile controls) or with a mixture of Penta-CB plus Hexa-CB plus Hepta-CB. (b) Sediments were amended with individual PCB congeners.

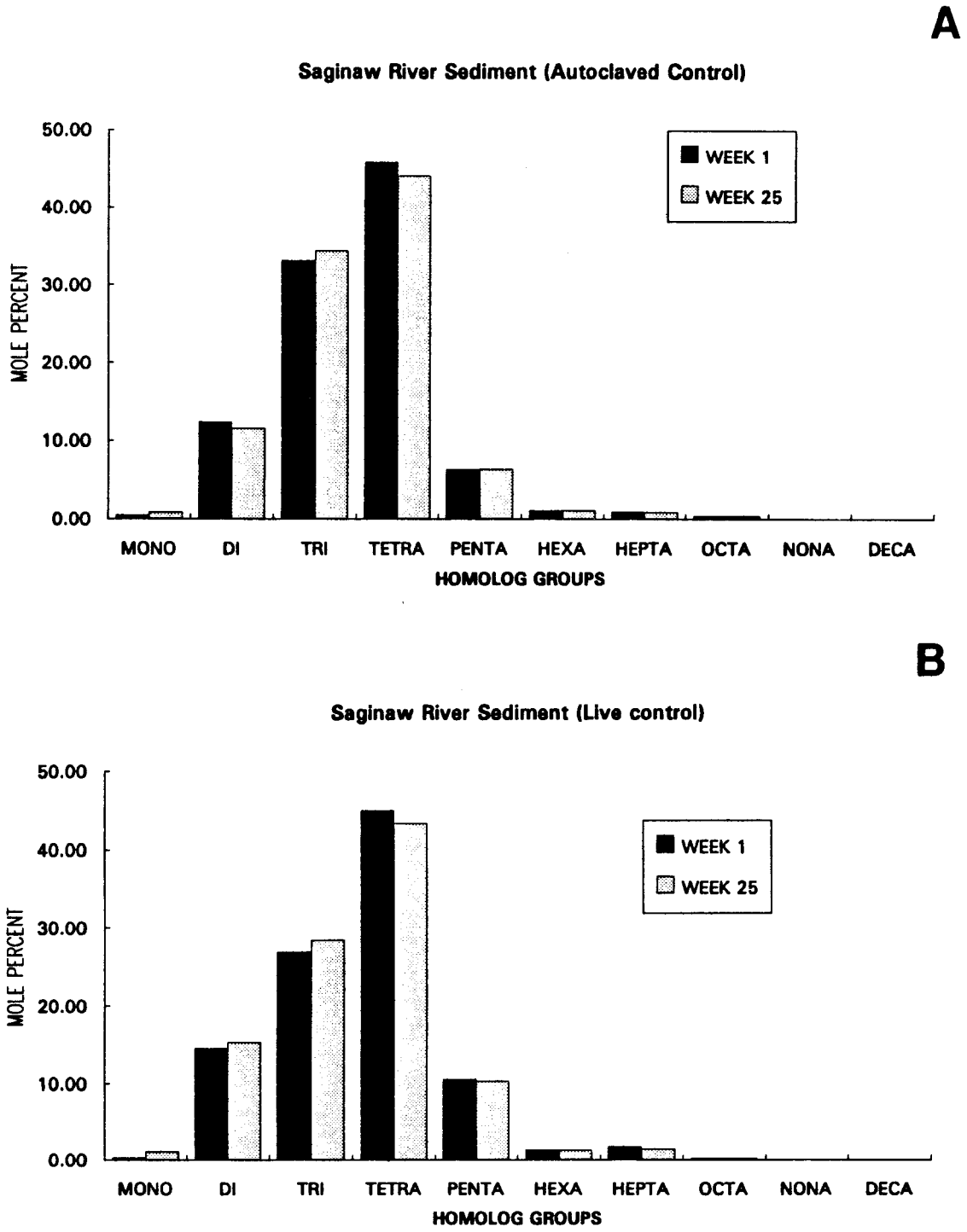
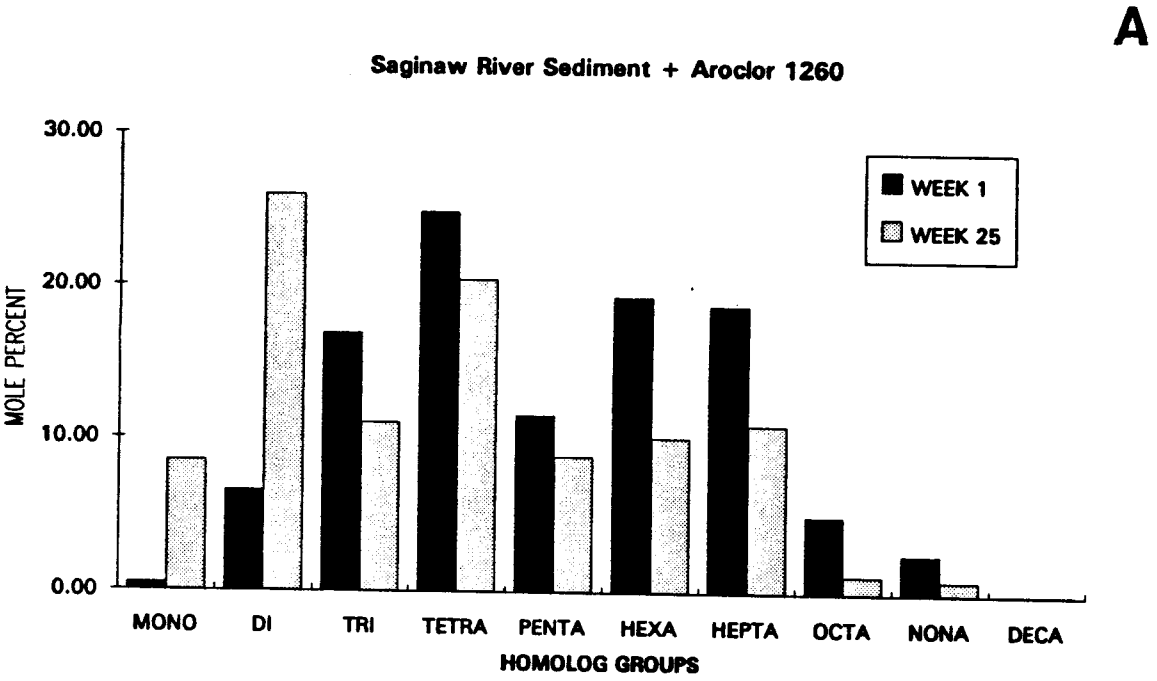


Figure 14. Reductive dehalogenation of PCBs in (a) sterile control and (b) unamended (live) Saginaw River sediments after anaerobic incubation for 25 weeks.



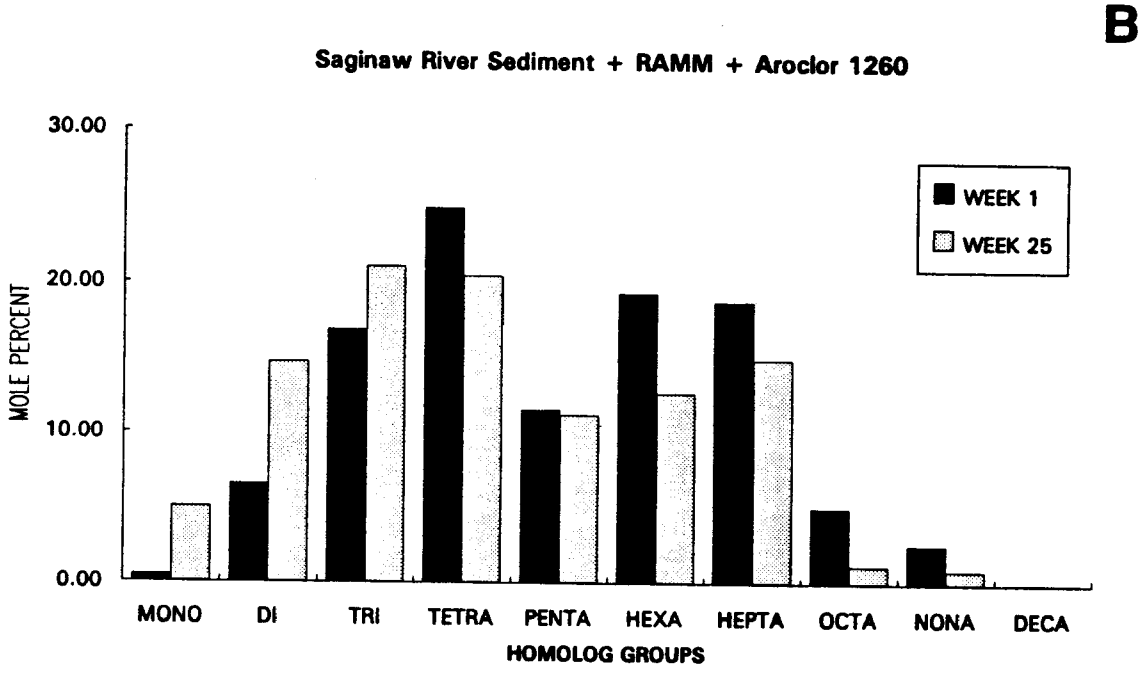


Figure 15. Reductive dehalogenation of PCBs in Saginaw River sediments after anaerobic incubation for 25 weeks and amended with (a) Aroclor 1260 and (b) Aroclor 1260 plus RAMM.

3.2 SUMMARY OF PAH BIODEGRADATION RESULTS

3.2.1 Assessment of potential for biodegradation of PAHS

3.2.1.1 PAH concentrations in homogenized sediment material

The concentrations of PAHs in Buffalo River sediment are given in Table 3. The compounds present in highest concentrations are pyrene and fluoranthene, both four-ring PAHs. Other studies that have analyzed samples from several locations in the Buffalo River have noted similar patterns of PAH concentrations (USACE Buffalo District, 1993). Thus, because it is present at high concentrations, and for the reasons described earlier, pyrene was selected as a model PAH for subsequent rate experiments.

Table 3. Concentrations of PAHs in homogenized bulk Buffalo River sediment.

Compound	mg/kg (dry weight)[1]	S.D.[2]
Fluorene	0.37	0.08
Phenanthrene	5.83	1.33
Fluoranthene	12.10	2.44
Pyrene	11.66	2.18
Benz(*a*)anthracene	2.55	0.65
Chrysene	5.26	0.64
Benz(*b*)fluoranthene	0.37	0.08
Benz(*k*)fluoranthene	0.08	0.11
Benz(*a*)pyrene	1.28	0.13
Indeno(*1,2,3-d*)pyrene	0.53	0.37
Dibenz(*a*)anthracene	ND	
Benz(*g,h,i*)perylene	0.72	0.35

[1] Mean of triplicate determinations.
[2] Standard deviation of triplicate determinations.

3.2.1.2 Aerobic degradation of PAHs by both indigenous bacteria from Buffalo River sediments and enrichment cultures derived from Buffalo River sediments

Organisms from the Buffalo River are capable of aerobic growth on biphenyl, naphthalene, acenaphthene, acenaphthylene, fluorene, phenanthrene, and anthracene, as demonstrated by colony formation and turbid growth in media derived from sediment slurries (10% w/v) amended with 10 mg/L of individual PAHs (Table 4). The fluorene and anthracene degraders produced very little turbidity and no colonies on agar, which suggests very low

activity on those compounds. Aerobic growth on phenanthrene produced an orange transformation product in the medium, which subsequently turned yellow, suggesting that an oxidized product may be a metabolic intermediate, which is further oxidized. One microbial species may carry out the sequential metabolic transformations, or the intermediate compound may be used as a substrate by other organisms. No bacterial growth was detected with fluoranthene, pyrene, chrysene, or benz(a)anthracene as the sole substrate, or with sediment organic extract as a co-substrate.

Table 4. Indicators of aerobic growth of indigenous microorganisms from Buffalo River sediments on PAHs as sole substrate and with sediment organic matter as co-substrate.

Compound	MSB/PAH[1]	MSB/PAH/ Sediment Extract[2]	Isolated Colonies on PAH + MS Agar
Biphenyl	++	++++	+
Naphthalene	++	++++	+
Acenaphthene	++	+++	+
Acenaphthylene	++	+++	+
Fluorene	w	+	-
Phenanthrene	++/orange	++++/yellow	+
Anthracene	w	++	-
Fluoranthene	-	-	-
Pyrene	-	-	-
Chrysene	-	-	-
Benz(a)anthracene	-	-	-

[1]Growth in broth was determined visually (+++) >(++) > (+) > (w) > (-) = turbid growth to no growth. Growth on agar was determined by observing colony formation.
[2]Sediment extract was prepared by adding 50g Buffalo River sediment and 150ml water. The mix was autoclaved, then shaken for 24 hours on a wrist-action shaker. Sediment was centrifuged and the supernatant retained as sediment extract.

When Buffalo River sediment slurries (20% w/v) were amended with a mixture of 16 PAHs (20 mg/kg each), all of the compounds for which degraders were detected (Table 4) were measurably degraded after 6 weeks (Table 5). Likewise, those PAHs for which no degraders were detected were not degraded.

Table 5. Aerobic biodegradation by indigenous Buffalo River microorganisms of PAHs (20 mg/kg) added to sediment slurries.

Compound	Concentration (mg/kg dry weight) Start	6 weeks
Acenaphthene	13.2	1.1
Acenaphthylene	8.9	ND[2]
Fluorene	16.3	3.2
Phenanthrene	19.4	4.1
Anthracene	21.2	4.6

[1]No degradation of fluoranthene, pyrene, benzo(*a*)anthracene, chrysene, benzo(*a*)pyrene, dibenz(*a,h*)anthracene, and benzo(*g,h,i*)perylene occurred within the 6-week period.
[2]ND = not detected

Table 6 shows that the enrichment culture degraded a wider range of PAHs at a faster rate than did the unenriched sediment. After 2 weeks of incubation, the degradation of 4- ring PAHs by the enrichment culture was more than double that of the sediment, although neither inoculum was capable of transforming the 5- and 6- ring compounds during that period.

Table 6. Comparison of rate of aerobic PAH degradation by an enrichment culture added to Buffalo River sediment slurries to that by indigenous organisms.

PAH Class	Concentration (mg/kg dry weight)		
	Start	14 days	
		Enrichment	Indigenous
2-, 3- ring	80.9	8.1 (86%)[1]	13.6 (77%)
4- ring	58.9	21.7 (58%)	44.7 (23%)
5-, 6- ring	46.3	43.4 (0%)	42.9 (0%)

[1]Values in parentheses are percentages of parent PAH compounds lost.

3.2.2 Processes affecting the rate of aerobic biodegradation of PAHs.

To determine whether oxygen was limiting in flask experiments incubated on a bench-top shaker, the rates of degradation of PAHs using three methods of aeration were compared: diffusion (stationary flasks), agitation, and air infusion (sparging). Sediment slurries, either shaken or left stationary, were monitored for phenanthrene mineralization and for PAH degradation. Mineralization was rapid and leveled off quickly. Thus measurements of mineralization were terminated after 14 days; the rate was determined by the slope of the initial increase. The extent of PAH degradation was measured by extracting the slurries after 28 days of incubation. The shaken sediment slurry mineralized phenanthrene more rapidly (7.68%/day) and to a greater extent than did the stationary sediment slurry (3.17%/day), as shown in Figure 16 and Table 7. However, the difference in PAH degradation after 4 weeks between aerated (shaken, sparged) and non-aerated (stationary) was small. Air sparging, using airstones to mix and aerate sediments, did not increase the extent of degradation after 28 days. The 2- and 3-ring PAHs were readily degraded in all three treatments (55%, 42% and 44% respectively; Table 8). The 4-ring PAHs were also degraded (47%, 37% and 22%, respectively). Although slightly more loss of the 5- and 6-ring PAHs occurred after 28-day incubations than after the 14-day incubation, they were relatively resistant to degradation.

Table 7. The effect of aeration method on the rate of degradation of PAHs in Buffalo River sediment slurries.

PAH Class	Concentration (mg/kg dry weight)			
	Start	28 days		
		Shaking	Stationary	Sparged
2-, 3- ring	29.9	14.1 (55%)[1]	18.0 (42%)	17.3 (44%)
4- ring	42.0	22.5 (47%)	26.5 (37%)	33.1 (22%)
5-, 6- ring	37.0	27.4 (16%)	30.1 (8%)	27.6 (15%)

[1]Values in parentheses indicate percentage loss of parent PAH compound.

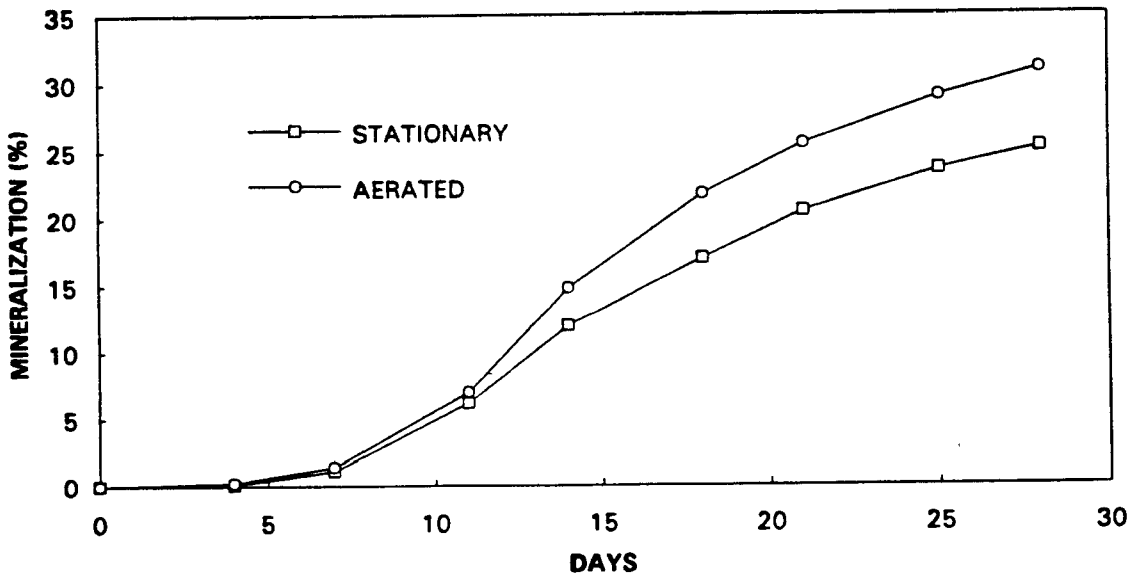


Figure 16. Effect of aeration on the mineralization of phenanthrene by the indigenous bacteria in 10% sediment slurries prepared with Buffalo River sediments. Values represent the mean of triplicates.

The addition of nitrogen (1000 mg/L $(NH_4)_2NO_3$)and phosphorus (1000 mg/L K_2HPO_4 and 200 mg/L KH_2PO_4), or phosphorus alone, increased mineralization of phenanthrene. However, addition of nitrogen alone decreased the mineralization of phenanthrene (Figure 17). Sediment slurries without any inorganic nutrient additions mineralized phenanthrene at nearly the same rate (7.7%/day) as with nitrogen and phosphorus added (9.9%/day), following an increased lag period. Pyrene, likewise, was mineralized at comparable rates in media with or without inorganic nutrient addition (Figure 18).

Although pyrene can be mineralized by the enrichment culture, the addition of sediment extract increased the initial rate of degradation at all additions tested (Figure 19). The addition of sediment extract also transformed the shape of the mineralization curve from logarithmic to first order, suggesting that the presence of the sediment organic matter either shortened an induction period or greatly increased the rate of growth of the degrader bacteria.

To test whether the increased rate of degradation of pyrene in the presence of sediment organic extract was due to the growth of a larger active degrader population, different numbers of bacteria were inoculated into sediment containing pyrene (Figure 20). An inoculum of 10^6 produced rapid mineralization of pyrene, and the first-order shape of the curve resembled that of the previous experiment. Smaller inocula (1 - 10^4) resulted in much slower mineralization. The shape of the slower curves was nearly linear, suggesting that growth either did not occur, or occurred very slowly. This observed threshold may result from failure of the enrichment culture to successfully compete with indigenous organisms when introduced at low inoculation densities.

Both the rate and extent of mineralization of pyrene by the Buffalo River enrichment culture decreased as the concentration of sediment in the slurry increased (Figure 21) due to sorption of pyrene on the sediment. Triton X-100, a neutral surfactant, was added to pyrene sorbed to sterile sediment. In all cases, that is, above and below the critical micelle concentration (CMC), the presence of the surfactant reduced the rate of mineralization of the compound by the enrichment culture (Figure 22). The extent of inhibition was inversely related to the amount of surfactant added.

When the pyrene was sorbed to the sediment surfaces for a period of time prior to the addition of the surfactant, and the bacteria had utilized all of the available form of the compound (as indicated by a leveling off of the mineralization curve), the addition of surfactants did not consistently increase the amount of PAH degraded (Figure 23).

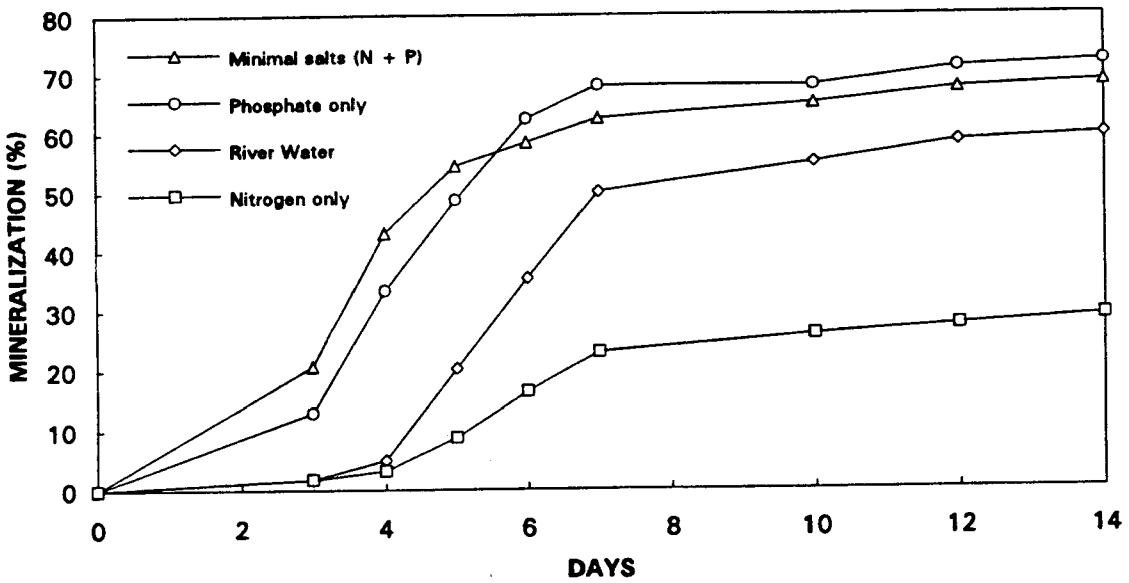


Figure 17. Effect of nutrient additions on the aerobic mineralization of phenanthrene by the indigenous bacteria in 10% sediment slurries prepared with Buffalo River sediments. Values represent the mean of triplicates.

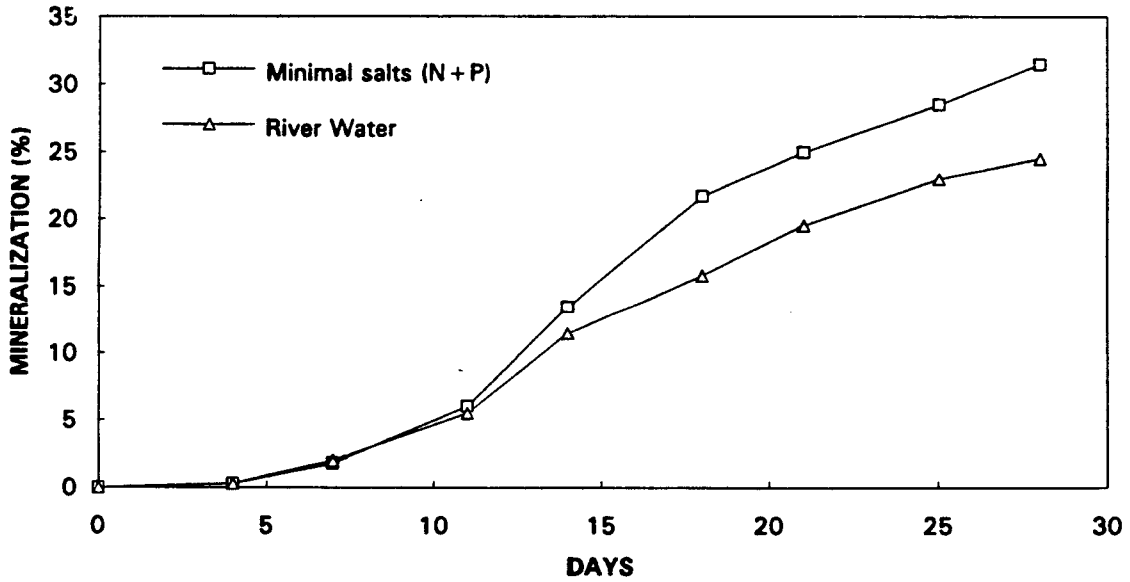


Figure 18. Effect of nutrient additions on the mineralization of pyrene when Buffalo River enrichment culture was added to 10% sediment slurry. Values represent the mean of triplicates.

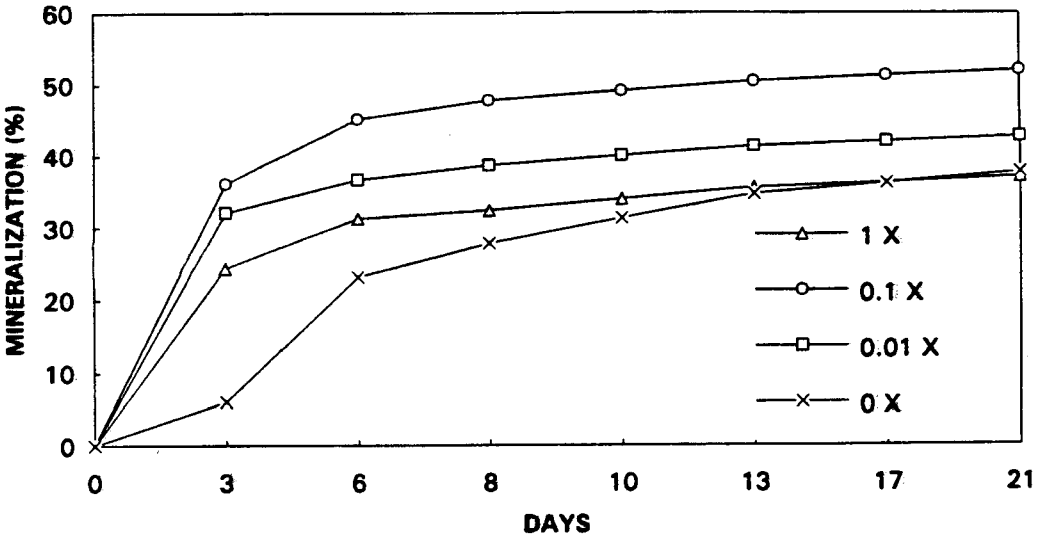


Figure 19. Effect of addition of sediment organic matter on the aerobic mineralization of pyrene by a Buffalo River enrichment culture in MSB containing added organic carbon. The cultures were prepared with MSB and 100%, 10%, and 1% by volume of the supernatant solution of a 10% sediment slurry prepared with MSB. Values represent the mean of triplicates.

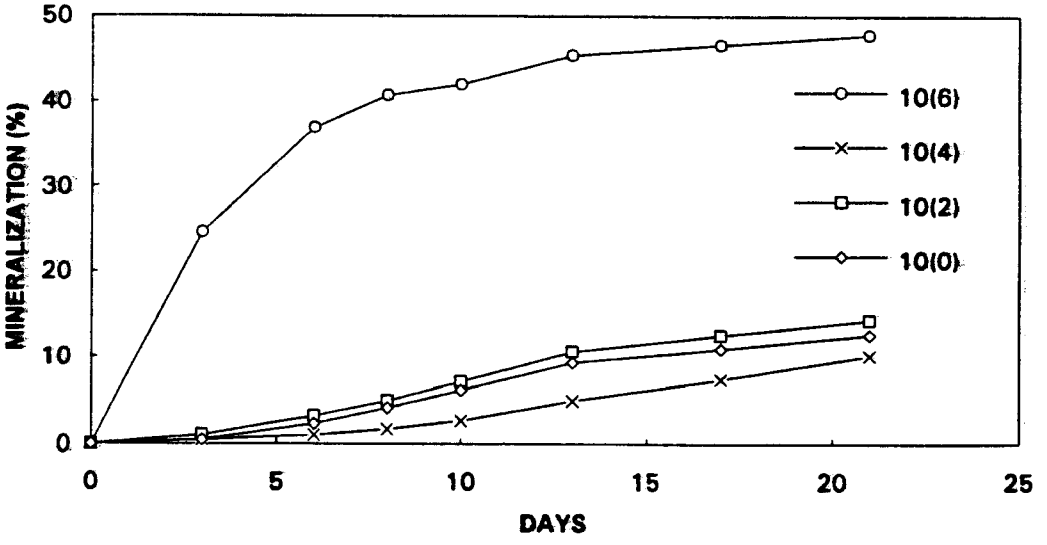


Figure 20. Effect of inoculum size on the aerobic mineralization of pyrene when Buffalo River enrichment culture was added to 10% sediment slurry. Values represent the mean of triplicates.

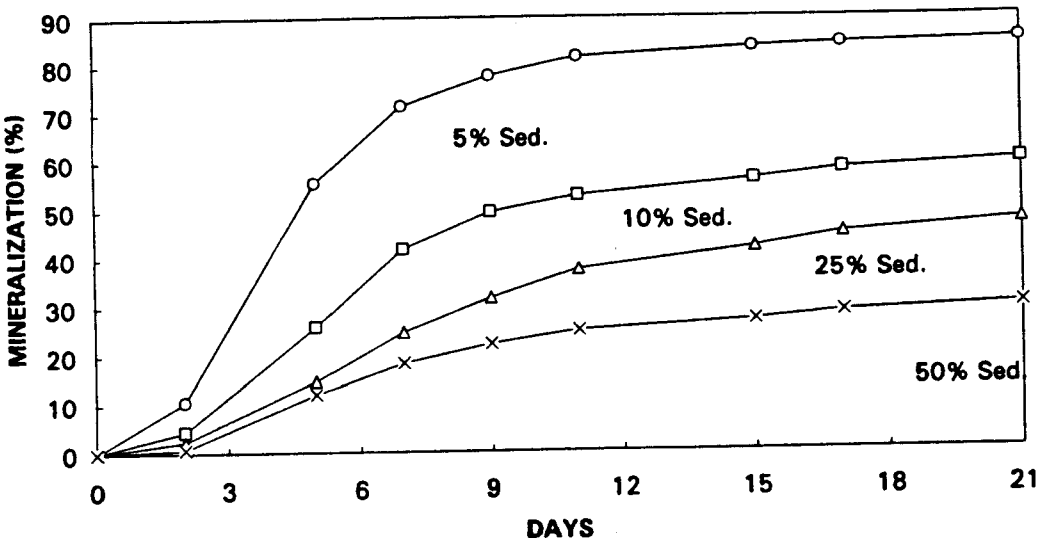


Figure 21. Effect of sediment concentration on the aerobic mineralization of pyrene in sediment slurries when Buffalo River enrichment culture was added to 0%, 5%, 10%, 25%, and 50% sediment slurries. Values represent the mean of triplicates.

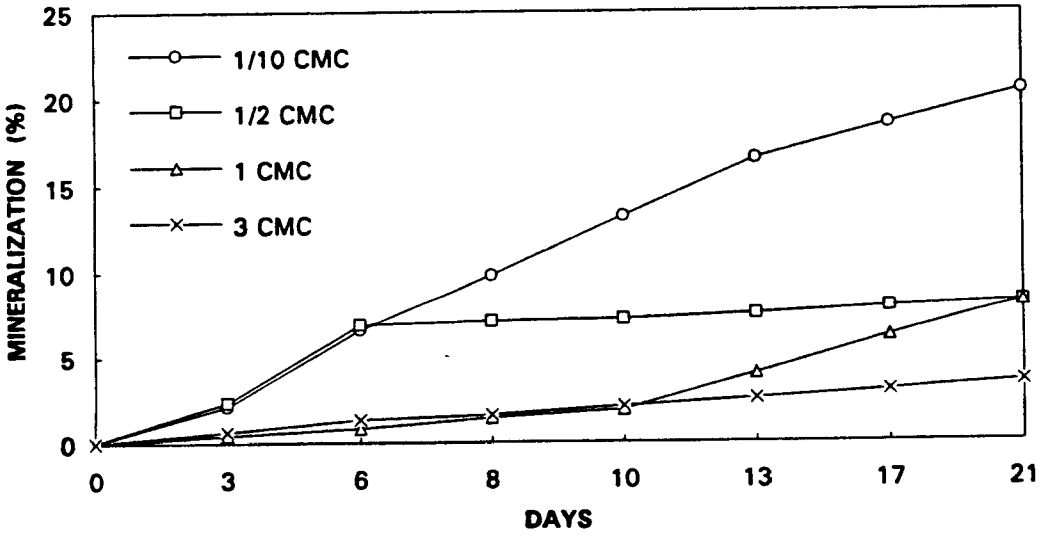


Figure 22. Effect of initial addition of Triton X-100 (CMC = 130 mg/L) on the aerobic mineralization of pyrene when Buffalo River enrichment culture was added to 10% sediment slurry. Triton X-100 concentrations were 10%, 50%, 100%, and 300% of CMC. Values represent the mean of triplicates.

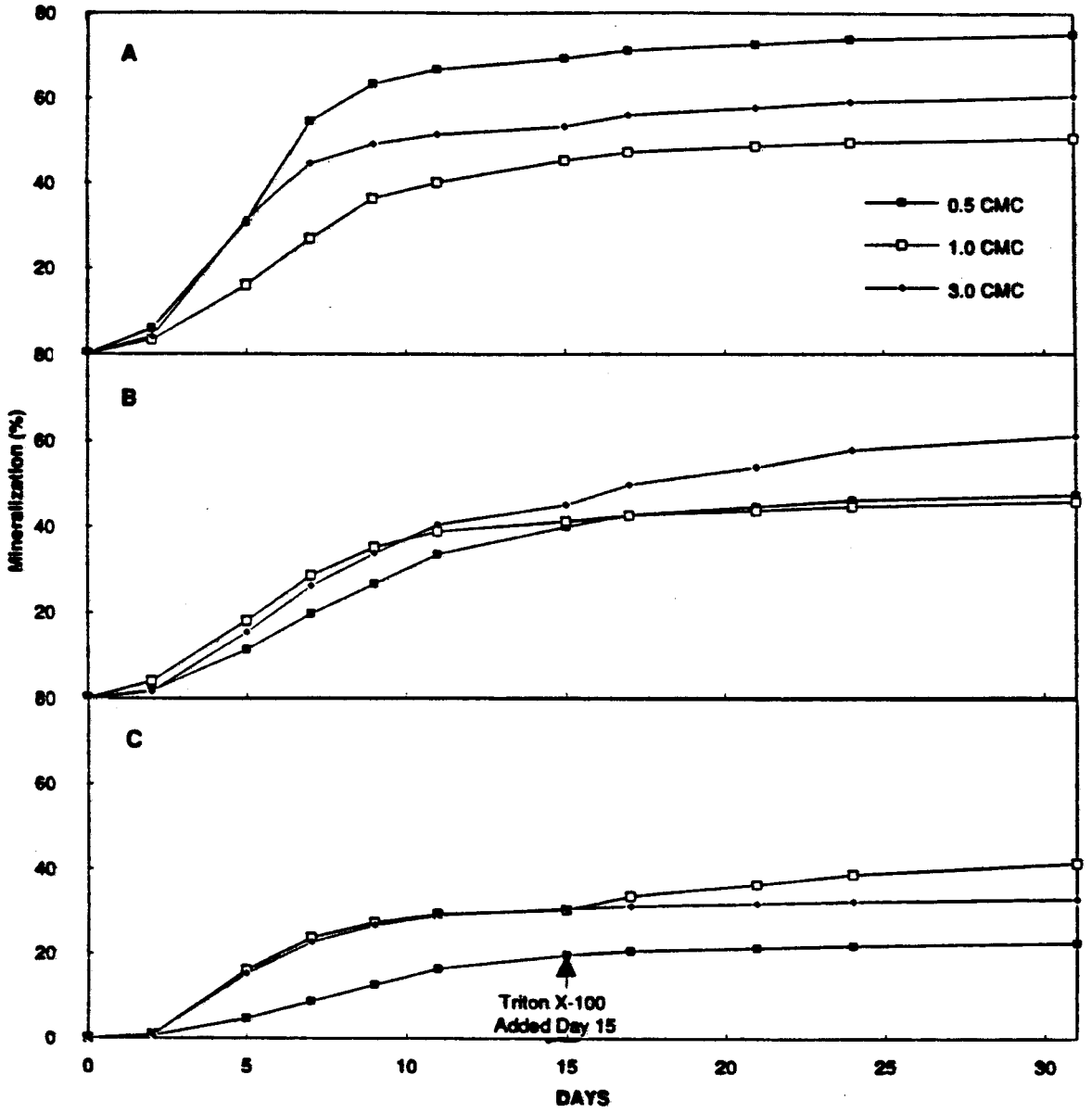


Figure 23. Effect of addition of Triton X-100 (0.5 CMC = 62.5 mg/L) after the aerobic mineralization of available pyrene in 10% (A), 25% (B) and 50% (C) sediment slurries to which Buffalo River enrichment culture was added. Surfactant was added at day 15, after mineralization has leveled off. Values represent the mean of triplicates.

The addition of Triton X-100 at day 15 produced an increase in the subsequent rate of mineralization in sediment slurries of either 25 or 50%, although no clear relationship between the amount of surfactant added and the extent of degradation increase was observed. The linearity of the mineralization curve following the addition of the surfactant suggests that a first-order process, in this case, solubilization of the compound, is controlling the rate of mineralization.

Figure 24 shows that even very high concentrations of heavy metals in the oxidized state do not affect the rate of mineralization of pyrene by sediment bacteria. The metals were added as the most soluble complex under aerobic conditions, at concentrations well above the concentrations measured in Buffalo River sediments (Table 8).

Table 8. Trace metal concentrations in Buffalo River sediments. (from Dial, 1994)

Trace Metal	mg/kg (dry weight)
Cu	33
Cd	1.9
Ni	57
Fe	3.9(%)
Cr	110
Zn	200
Pb	94

Figure 25 shows that the degradation of pyrene by the enrichment culture is slower in the presence of several combinations of 2-, 3-, and 4- ring PAHs. The enrichment culture is able to mineralize pyrene; therefore, it can derive energy from that compound. The addition of 2- and 3- ring PAHs did not increase the rate of growth, and hence the rate of degradation, for the culture, but rather slowed the rate, suggesting that the smaller compounds may be preferred as substrates by the bacteria. Additions of 4-ring PAHs repressed pyrene mineralization to a greater extent than did the additions of the smaller compounds. The inhibition caused by additions of both classes of PAHs was partially ameliorated by the pretreatment of the sediment slurry with hydrogen peroxide (7.5 ml per flask).

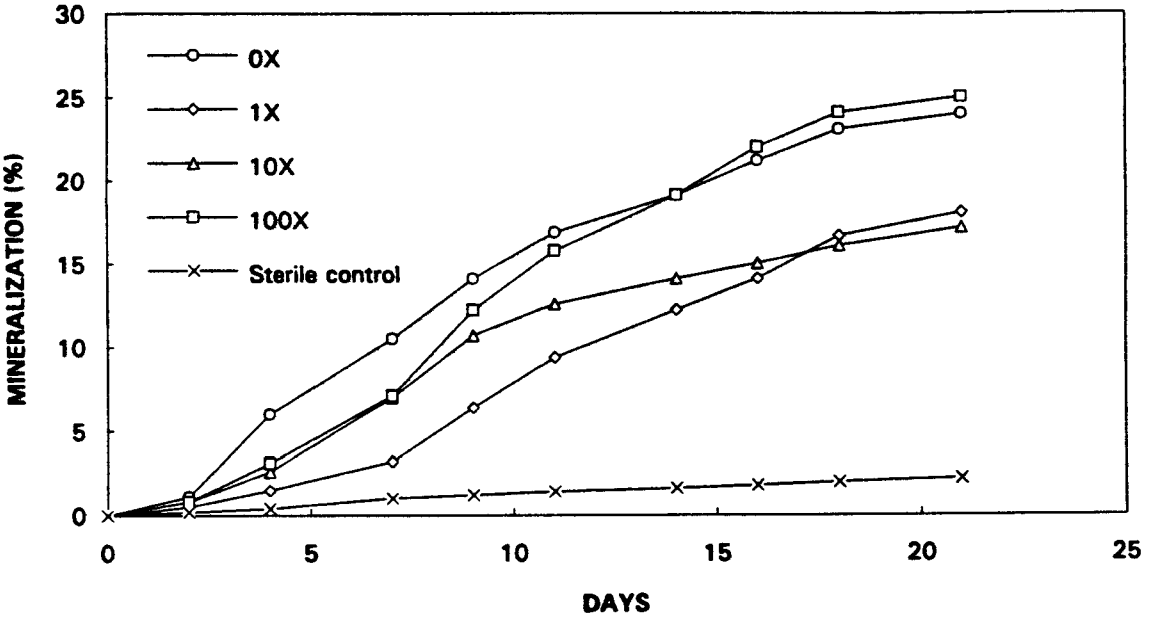


Figure 24. Effect of additions of heavy metals (X = 25 ug/ml Pb, 25 ug/ml Cu and 2.5 ug/ml basal concentration) on the aerobic mineralization of pyrene when Buffalo River enrichment culture was added to 10% sediment slurry. Metal concentrations were 0, 1X, 10X, and 100X. Values represent the mean of triplicates.

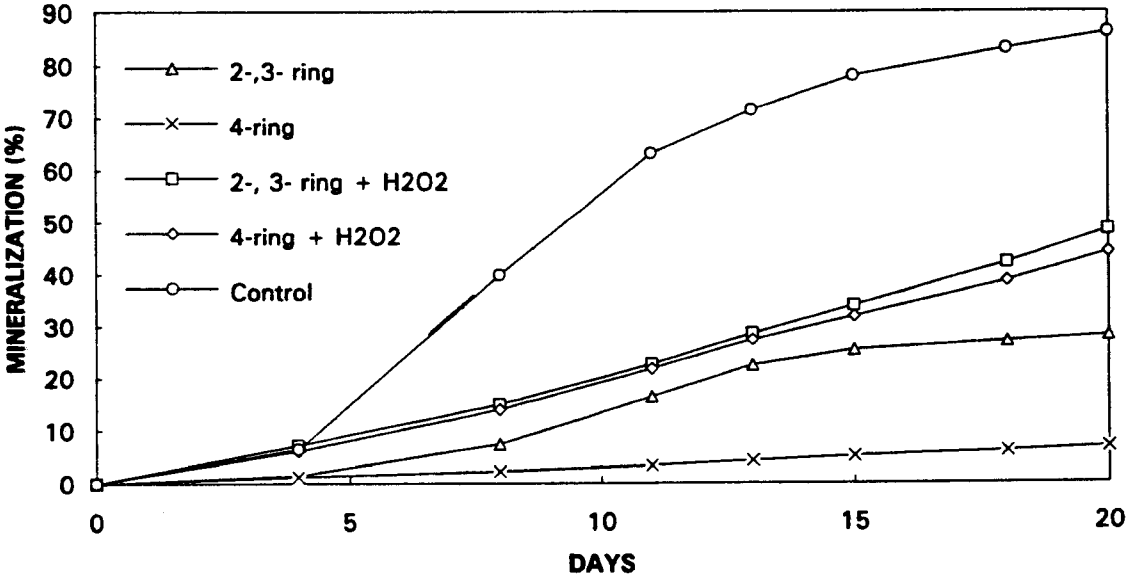


Figure 25. Effect of additions of secondary PAHs on the aerobic mineralization of pyrene when Buffalo River enrichment culture was added to 10% sediment slurry, with and without pretreatment with hydrogen peroxide. Values represent the mean of triplicates.

3.3 DISCUSSION AND INTERPRETATION

3.3.1 PCB Biodegradation

The Sheboygan River sediments used in this study were originally contaminated with hydraulic fluids containing Aroclors 1248 and 1254 (Sonzogni and David, 1991); the most prevalent PCB congeners in a mixture of Aroclors 1248 and 1254 are tetra-, penta- and hexachlorobiphenyls. Our initial analyses of the PCB congener profile of Sheboygan River sediment used in this study revealed a higher concentration of lower chlorinated congeners and a lower concentration of highly chlorinated congeners than would be found in the original contaminating material. This indicated that the PCB contaminants had been transformed since their discharge. Further, based on the distribution and weight percentages of indigenous PCB congeners in contaminated Sheboygan River sediments, Sonzogni and David (1991) suggested that microbial transformation of PCBs occurred *in situ*.

Unamended sediment slurries prepared from Sheboygan River sediments exhibited no appreciable changes in PCB homolog distribution after 30 weeks of anaerobic incubation. These results suggest that rates of reductive dechlorination of the historical PCB congeners are extremely slow and it is likely that some factor (nutritional, physiological, physicochemical) limits PCB degradation in natural sediments. In contrast to the unamended Sheboygan River sediments, addition of Octa-CB to these sediments resulted in the accumulation of *ortho*-substituted congeners, indicating that dechlorination had occurred at the *meta* and *para* positions. This dechlorination pattern has also been described in laboratory incubations of actively dechlorinating, PCB-contaminated Hudson River sediments (Brown *et al.*, 1987a,b; Ye *et al.*, 1992). In our experiments, the pattern of dechlorination of historical PCBs in sediments with added Octa-CB was similar; the concentration of PCB homolog groups with three or more chlorine atoms decreased with time and the major products were mono- and di-CBs. These results illustrate the preferential dechlorination of the more highly chlorinated PCB congeners by the indigenous anaerobic microorganisms. The rate of dechlorination of Octa-CB was estimated in experiments with 5 to 20 mg/L of added Octa-CB, and the results indicated that dechlorination was directly proportional to the concentration of Octa-CB added.

In a previous investigation using Sheboygan River sediments, reductive dechlorination of PCBs was not observed in experiments in which bacteria eluted from contaminated sediments were used as the inoculum (Sonzogni and David, 1991). Results from our study indicate that naturally occurring microorganisms mediate the reductive dechlorination of both amended and historical PCBs in Sheboygan River sediments incubated anaerobically under laboratory conditions. It has been suggested that different

physiological groups of dechlorinating bacteria are present in PCB-contaminated sediments (Quensen *et al.*, 1990; Ye *et al.*, 1992). The use of 'eluted' microorganisms as inocula in dechlorination experiments may exclude some microbial populations that are attached to sediment matrices and that may be important for transformation of complex organic chemicals. This hypothesis can only be tested through additional studies with PCB-contaminated sediments in which reductive dechlorination has been demonstrated.

Addition of PCB congeners to Ashtabula River sediments also resulted in enhanced dechlorination of historical PCBs as was observed for Sheboygan River sediments. The congeners added in this study, Penta-CB, Hexa-CB and Hepta-CB, contained one, one, and four *ortho*-chlorines per biphenyl, respectively; the onset of dechlorination of these congeners, either alone or in combinations, occurred prior to dechlorination of the historical PCBs. Although all amended congeners were dechlorinated in Ashtabula River sediment slurries, it was expected that Hepta-CB would be more resistant to anaerobic transformation than the other amended congeners since reductive dechlorination of *meta* and *para* chlorines are preferred. This was not the observed result. The onset of dechlorination of the amended congeners occurred in the order: Hexa-CB, followed by Hepta-CB and Penta-CB. A possible explanation for the longer lag period before onset of dechlorination of Penta-CB is the lack of a significant population of Penta-CB dechlorinating microorganisms in the sediment. In experiments with mixtures of the amended congeners, the results are less clear. Because *meta*-dechlorination of the Hexa-CB congener on the less chlorinated ring produces the identical Penta-CB congener used in our studies, the calculated rates of dechlorination may be biased (an underestimate for Penta-CB dechlorination) and account for the apparent slower rate of dechlorination of Penta-CB in the presence of Hexa-CB. The slower degradation rate of Hepta-CB may be attributed to the presence of four *ortho*-chlorine atoms, which likely makes this congener more resistant to anaerobic biotransformation.

The results from the present study demonstrate the dechlorination capacity of PCB-contaminated Sheboygan River and Ashtabula River sediments and support the theory of Sonzogni and David (1991) that a threshold PCB concentration exists for reductive dechlorination of chlorobiphenyls. No appreciable dechlorination of historical PCBs was observed in unamended sediment slurries. Three explanations are proposed for the stimulation of reductive dechlorination of historical PCBs in sediments by addition of specific PCB congeners: (a) the bioavailability of PCBs was enhanced, thus providing an available electron acceptor for oxidation reactions; (b) the growth of indigenous PCB dechlorinating microorganisms was stimulated; (c) amended PCB congeners induced dechlorinating activity of indigenous microbial populations. It has recently been

demonstrated that the reductive dechlorination of 3-chlorobenzoate by the anaerobic isolate *Desulfomonile tiedjei* provides energy for growth (Dolfing, 1990; Mohn and Tiedje, 1990). If microorganisms that reductively dechlorinate PCBs derive energy from the process, then this would provide a selective advantage for their growth in PCB-contaminated environments. Further experiments are necessary to examine the questions of PCB bioavailability, growth of dechlorinating populations in historically contaminated sediments, and to determine the threshold concentration for PCB biotransformation.

3.3.2 Aerobic PAH Biodegradation

The present-day concentrations of PAHs in Buffalo River sediments indicate that some transformation of PAHs has probably occurred. Ideally, residual concentrations should be compared to a previously measured concentration, or absent that, a weight-averaged mass loading, to determine whether losses of compound have occurred. Historical data for Buffalo River are sparse and variable spatially. Nonetheless, patterns of compound relative abundance indicate biological activity. The lower molecular weight and more soluble compounds, such as naphthalene, may be lost from sediment by either biological or physical processes, but the loss of the 3- and 4-ring compounds relative to the 5- and 6- ring compounds is an indication of biological activity.

PAHs may persist in sediments due to limitations of bacterial metabolic activities or because the compounds are present in a form that is not available to the bacteria. Bacterial activity may be limited by inherent metabolic capability, by inadequate supplies of nutrients and/or electron acceptors, or by inhibitory interactions with other organisms (e.g. competition, predation) or other compounds (e.g. toxic metals).

The native microorganisms of the Buffalo River demonstrate the capability to degrade PAHs in sediment slurries amended with PAHs, yet those compounds persist in river sediments. One possible explanation for the restricted activity of indigenous bacteria in sediments is the limitation of growth of the degrader population by other factors, such as competition with other organisms for substrates or nutrients. Alternatively, indigenous bacteria may not express the metabolic capability to degrade some of the PAH compounds in the presence of preferred substrates.

PAHs of less than five rings were degraded by an enrichment culture derived from Buffalo River sediments. These bacteria utilized a greater range of compounds than did the indigenous organisms, and were able to grow using some PAHs as energy-yielding substrates. Rates of transformation of PAHs by the enrichment culture were faster than the rates for indigenous

bacteria. Since the enrichment culture was derived from the Buffalo River sediment, this suggests that the genotypes for PAH degradation are present in the sediment, yet some environmental conditions prevent their natural growth and/or expression.

It has been well established that PAH biodegradation is far more efficient under aerobic conditions. However, except for the surface layer, sediments are primarily anaerobic environments. Moreover, efficient aeration is difficult to attain under *in situ* field conditions, where sediment oxygen demand often exceeds mixing capabilities. Without appropriate physical constraints, the high rates of mixing needed to achieve aeration under field conditions result in unacceptable levels of sediment resuspension. Under such conditions, alternate oxygen delivery systems, such as liquid oxidants, may be more efficacious. Mechanical aeration is more feasible for engineered systems.

Experiments indicated that degradation was slightly faster in aerated slurries than in non-agitated slurries. Dilute slurries were tested in these experiments; the differences between aerated and non-aerated systems is expected to be more pronounced in the more concentrated slurries characteristic of field conditions. On the other hand, aeration of slurries by sparging did not increase the rates of degradation; rather, a slight decrease was observed. This suggests that not only is there a maximal aeration rate corresponding to other rate limitations on the compound transformations, but that higher rates of agitation of the sediment slurries may be detrimental to microbial activity by shearing bacteria from sediment surfaces.

Mineralization of PAHs indicates complete conversion of the parent compound to CO_2 and H_2O, yielding energy for growth of the bacteria. There have been few reports of the mineralization of PAHs with four or more aromatic rings; most transformations of larger PAHs reported in the literature have been supported by co-metabolism of other substrates from such sources as sediment organic matter. The second substrate may be required by the organisms as a source of energy, either because the metabolism of the PAH does not yield energy or because the compound is so insoluble that too little is available to the bacteria to support growth and/or activity.

In the former case, cometabolism may result in incomplete transformation of the parent compound. Cometabolism of PAHs in the environment may result in the accumulation of toxic by-products. Thus, the enhancement of mineralization of these compounds by the introduction of enriched degrader bacteria may reduce the residual toxicity associated with their partial transformations. The higher rates of mineralization of pyrene resulting from the additions of sediment-derived organic matter suggest that additional substrates may be utilized by the enrichment culture to enhance growth. However, the concurrent

mineralization of the pyrene indicates that such utilization of additional substrates does not preclude PAH degradation, but rather enhances it to a slight extent.

PAH compounds may affect the metabolism of other PAH compounds in a number of ways, including inhibition (or toxicity) or cometabolism. Most of the large-ring PAHs are mutagenic and/or carcinogenic; they are toxic to bacteria as a result of their intercalation in cell membranes. Such toxicity may contribute to their persistence in the environment. Conversely, given the importance of cometabolism in the degradation of larger PAHs, it is possible that the smaller PAHs may support cometabolism of the larger PAHs by providing energy to the degrader bacteria. In these investigations, the additions of additional PAHs, either 2-, 3- or 4- rings, inhibited the mineralization of pyrene by the enrichment culture.

Additions of nitrogen did not significantly enhance the rate of mineralization of PAHs, which suggests that nitrogen is not growth-limiting in Buffalo River sediments. Phosphate additions, either alone or with nitrogen (as MSB) increased the rate and extent of degradation. In the absence of additional phosphorus, 20% mineralization is not achieved in 5 days; with phosphorus, that level of mineralization is achieved in 3 days, suggesting that phosphorus additions either decrease the acclimation period for degradative activity or increase the rate of growth of the bacteria.

When degradation results from the activity of the enrichment culture alone, that is, in sterile sediment, degradation is proportional to bacterial numbers, but when the enrichment culture is added to sediment with a viable indigenous bacterial population, a threshold population of the enriched bacteria must be achieved to effect degradation. At population densities below 10^6, the mineralization of pyrene was slow and nearly linear, even if incubation times were long enough to allow growth of the smaller populations. The inability of the enrichment to grow may have resulted from competition with indigenous sediment organisms or predation by sediment protozoa. Ramadan *et al.* (1990) have reported a similar occurrence; the introduction of a *p*-nitrophenol degrading pseudomonad into nonsterile lake water was unsuccessful at population densities below 4×10^4. Given the much higher densities of indigenous microflora in sediment, it is reasonable that the threshold density for successful introduction is higher in sediment than in lake water. Thus, bioaugmentation of degrader bacteria into sediments may only be successful if large numbers of active bacteria can be introduced and maintained.

Several other processes could be manipulated to increase the rate of degradation, but the system response was not straightforward. The concentration of sediment in the slurry was

critical in determining the rate of the reaction. An optimal concentration resulted from the competing influences of sediment as a source of nutrients and organic co-substrates to the degrader bacteria and as a sorbent that decreased the availability of the PAHs for degradation. In the Buffalo River sediments used in this study, the optimal slurry concentration was approximately 5%; this number will likely be different for different sediments.

Sediments are frequently contaminated with high concentrations of heavy metals. In river bottom sediments, due to the prevailing anoxic conditions, metals are predominantly in their reduced state. Most heavy metals are non-toxic in the reduced form, but may be toxic in the oxidized state. Biodegradation of PAHs requires oxygen and their transformation has been shown to proceed most rapidly under well-aerated conditions. Thus, those conditions that best favor biodegradation of PAHs will also convert sediment metals to their most potentially toxic states. When soluble forms of metals were added at concentrations that exceeded the ambient concentrations in Buffalo River sediments, no consistent inhibition of degradation occurred. This suggests that the bacteria may have a mechanism for resistance to trace metals in sediments, or that the metals may be made unavailable, and hence nontoxic, to the bacteria by another mechanism.

The rate of degradation of the larger PAHs may be limited by their availability to the bacteria. Their low solubilities dictate that very low concentrations will exist in the aqueous phase; unless the bacteria can enhance the rate of solubilization, the available concentrations of compound may be too low to support microbial growth.

The addition of a nontoxic surfactant slightly increased the availability of sediment-bound PAHs if the surfactant was added after the bulk of the available compound had been degraded. When the surfactant was added prior to initiation of mineralization of pyrene, degradation was inhibited; it is likely that the surfactant itself was utilized by the bacteria as a growth substrate, precluding adaptation of the bacteria to metabolism of the PAH. The observed increase in turbidity supports this explanation. Tsomides et al. (1995) tested the effects of several nonionic surfactants on the degradation of PAHs sorbed to sediments; all of the surfactants tested, except for Triton X-100, were toxic to the bacteria and inhibited degradation of phenanthrene.

Although surfactants can increase the apparent solubility and degradability of PAHs from solid crystalline form (Volkering et al., 1995), there is little evidence for enhancement of degradation of PAHs sorbed to sediments. Additions of Triton X-100, at concentrations well above the CMC, increased the apparent

solubility of phenanthrene but did not significantly increase its mineralization. The compound within the surfactant micelle structure is apparently not available for biodegradation (Volkering et al., 1995; Roch and Alexander, 1995). On the other hand, an increase in biodegradation by the addition of surfactants at sub-CMC concentrations has been reported (Aronstein *et al.*, 1991); the mechanism for this result has not been elucidated.

PAHs appear to be less available for degradation after long contact with soils or sediments. Hatzinger and Alexander (1995) report that, when phenanthrene was incubated with sterile soils for varying lengths of time prior to inoculation with a bacteria capable of degrading the compound, longer incubation times resulted in less degradation. The extent of degradation corresponded to the amount of phenanthrene that could be extracted with organic solvents or by exposure to earthworms, and this "available" fraction decreased with soil resident time. This phenomenon is known as aging, and is more pronounced in soils with higher organic content. The addition of nutrients slightly increased the rate and extent of degradation in both aged and unaged soils, but did not reverse the effects of aging. The authors concluded that aging may be due to slow diffusion of nonionic organic chemicals within the solid-phase organic matter in soils, or to entrapment and retarded diffusion within the small pores in soils aggregates. Sonication increased the initial rates of degradation in aged and unaged soils, but did not appreciably increase the extent of degradation. This strong association between an organic contaminant and soil is likely to limit the effectiveness of *in situ* bioremediation. Given the high organic content of Buffalo River sediments, it is very likely that aging of PAHs has rendered them resistant to degradation via a similar mechanism.

Most of the processes found to increase the rate of degradation of PAHs could be utilized either *in situ* or in an *ex situ* engineered facility. Nutrients, oxidants, and competent bacteria could be added to sediments *in situ* with injection pumps or with the construction of semi-enclosed weirs. Increasing the availability of the more recalcitrant PAHs, on the other hand, requires the formation of less concentrated sediment slurries and enhancement of PAH availability, either by disruption of sediment particulate matter (e.g. sonication) or the addition of surfactants. Such treatments can best be carried out in *ex situ* contained facilities.

4.0 CONCLUSIONS

Several conclusions can be made from these studies. They are:

- For the three PCB contaminated sediments examined, intrinsic rates of PCB degradation were not observed during the course of the investigation under the experimental approaches used herein. No evidence for further PCB dechlorination was detected in these historically contaminated sediments despite evidence that PCB dechlorinating microorganisms were present.

- Reductive dechlorination of the historical PCBs were stimulated by the addition to the sediments of single or multiple PCB congeners containing five or more chlorines. Both the added PCB congener(s) as well as congeners already present in the sediments were dechlorinated primarily to mono- and di-chlorinated PCBs.

- Under aerobic conditions, microorganisms native to Buffalo River sediments mineralized PAHs with less than five rings and partially degraded some PAHs with five and six rings. The activity of native bacteria can be increased by enrichment on specific substrates and reintroduction to contaminated sediment (bioaugmentation).

- Degradation of PAHs was not stimulated by the addition of surfactants at levels above or below the CMC. Surfactants generally inhibited PAH degradation in this study.

- Microbial degradation of PAHs was not limited by nitrogen and was not inhibited by potentially toxic metals also found in these sediments. Inhibition of degradation by the large ring PAHs may be partially alleviated by treatment with peroxide.

- Bioaugmentation of PAH-degrading bacteria into PAH-contaminated sediments may only be successful if large numbers of active microorganisms can be introduced and maintained in an active state.

An evaluation of these results with respect to effective bioremediation of the contaminated sediments suggests to the authors that *in situ* treatment of these contaminated sediments is not practical. Removal of the sediments to a treatment facility or alternative treatment location would be required. PCBs could be treated by a combination of anaerobic pretreatment, to convert the highly chlorinated PCB congeners to congeners containing one or two chlorines, followed by aerobic degradation of the less chlorinated PCB congeners. Treatment of PAH contaminated

sediments could best be accomplished and controlled by aerobic, above-ground treatment systems. Bioaugmentation with competent PAH degrading bacteria is an alternative treatment option for PAH remediation but may be costly due to the large amount of microbial inocula required for effective and timely treatment. Complete degradation of high MW PAHs will require treatment to enhance their availability to bacteria.

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6.0 APPENDIX

QUALITY ASSURANCE PROJECT PLAN

BIOLOGICAL REMEDIATION OF PCB AND PAH
CONTAMINATED SEDIMENTS

U.S. ENVIRONMENTAL PROTECTION AGENCY

ECOSYSTEMS RESEARCH DIVISION

NATIONAL EXPOSURE RESEARCH LABORATORY

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INTRODUCTION

The Biological Remediation of PAH and PCB Contaminated Sediments project is a research activity of the Biology Branch of the EPA Environmental Research Laboratory in Athens, Ga. The Biology Branch has a long history in research on the degradation and remediation of hazardous organic wastes under anaerobic and aerobic conditions. The Athens laboratory researchers are working with the Engineering/Technology Workgroup of the Assessment and Remediation of Contaminated Sediments (ARCS) program and the Bureau of Mines to investigate bioremediation technologies as remediation alternatives for contaminated sediments.

The ARCS program is authorized as part of the Water Quality Act of 1987 and is administered by the Great Lakes National Program Office of the EPA. The two major objectives of the ARCS program are to assess the extent of sediment pollution in designated areas of concern and to identify and demonstrate options for the removal and/or treatment of the contaminated sediments. The ARCS program is to be completed in the years 1988-1992.

In 1991, Congress directed the Department of the Interior, Bureau of Mines (BOM) to investigate quality of bottom sediments in the North Branch of the Chicago River, and the potential for remediation of contaminated sediments. As part of this investigation, the BOM coordinated its research with other agencies and groups, including the U.S. Army Corps of Engineers, the Metropolitan Water Reclamation District of Greater Chicago, the Northeast Illinois Planning Commission, Friends of the River and the U.S. EPA. The current EPA research is supported by an interagency agreement with the BOM.

The Athens ERL effort will be predominantly laboratory research, with pilot and field studies to be designed based on outcomes of laboratory studies.

PROJECT DESCRIPTION

During a workshop held July 1990 (sponsored by the ARCS program and Environment Canada), an initial review was made of bioremediation methods and options for their use in reducing contamination of Great Lakes sediments. The workgroup determined that the biological remediation technologies for these classes of organic contaminants warranted examination by the ARCS Program because the technologies were in rapidly advancing stages of development. Major contaminants of interest included polychlorinated biphenyls (PCBs) and polyaromatic hydrocarbons (PAHs). PCB analytes will include total PCBs as well as individual PCB congeners. PAH analytes will include, but not be restricted to, benz[a]anthracene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene and chrysene.

PAHs and metals are the contaminants of concern in the North Branch of the Chicago River. In complementary research, the BOM will investigate bioremediation as an option for the treatment of metals in sediments, and the EPA will concentrate on the environmental factors that influence the rate of PAH degradation, with an emphasis on those factors that can be managed to enhance the rate and range of biotransformation in engineered systems.

PROJECT ORGANIZATION AND RESPONSIBILITY

The Athens ERL is one of twelve research laboratories operated by the USEPA Office of Research and Development. It is headed by a Director, with a

Program Operations Director and a Research Operations Director. The individual research groups report through Branch Chiefs to the Research Director.

The Biological Remediation of PAH Contaminated Sediments project is under the direction of Dr. John E. Rogers, Project Manager. Day-to-day technical direction is coordinated by Dr. Rochelle Araujo and Dr. W. Jack Jones, Co-Principal Investigators. Independent quality assurance oversight activities will be performed by the Biology Branch QA Officer, Mr. John Pope, and the AERL QA Officer, Dr. Robert R. Swank. Project staff includes a microbiologist and a post-doctoral researcher.

QUALITY ASSURANCE OBJECTIVES

The purpose of this quality assurance project plan is to describe the procedures to be taken to assure that the measurement data produced are of known quality based upon their intended use.

Completeness - The objective for completeness in this research effort is 90 percent.

Representativeness - After the samples have been mechanically mixed to visible homogeneity, representative subsamples from each sample lot will be extracted and analyzed for comparison upon sample characterization. Buffalo River and the North Branch of the Chicago River sediment are representative of PAH contaminated sediments. These two areas represent possible field demonstration sites. The Saginaw River/Bay sediment is representative of a PCB contaminated sediment.

Comparability - For this project, comparability for each test will be assured through use of the same analytical procedures for all similar samples. The analytical data should be reported in the same units for each test and for all samples collected from a site.

Precision and Accuracy - Known standards, QC samples, replicates, blanks, surrogate spike and treated matrix spike samples will be used to assess precision, accuracy and detection limit of all analyses.

Detection Limits - The detection limit for the analysis of TOC is 300 mg/kg, PAHs 0.2 mg/kg, and PCBs (Aroclor) 1.0 mg/kg. Detection limit determination for the ARCS program is done by the analysis of 15 low level standards or blanks with a concentration within a factor of 10 times the instrument detection limit. The limit is then set as three times the standard deviation of the multiple measurements. This determination will be done prior to the analysis of the experimental samples.

SAMPLING PROCEDURES

Initial procurement of the sediment samples is the responsibility of GLNPO for Buffalo River and PCB contaminated sediments and the Bureau of Mines for Chicago River. When the samples are received at Athens ERL, splitting will be accomplished by mechanically mixing the received material until visual homogeneity is observed, then the sample will be completely split into equal subsamples. These wet sample splits and original study site samples will be stored in the dark at 4° ± 2° C. Weekly temperature measurements will be recorded in a bound notebook. Wet-sample splits will be consumed through the course of the project, so holding times may be as long as one year. Periodic

storage checks to ensure the lack of breakdown of organics will be done on the subsamples when they are removed from the storage area and prior to testing. The initial baseline analysis of each subsample should be within ± 30% of the original analysis.

SAMPLE CUSTODY

Upon receipt of the samples at the Athens ERL, they will be logged into a sample logbook. The following information will be documented in the logbook:

* Date and time of sample receipt
* Project number
* Field sample number
* Laboratory sample number (assigned during log-in procedure)
* Sample matrix
* Sample parameters
* Storage location
* Log-in person's initials

As noted, the sample will be split into quantities suitable for characterization and testing, and each split will be numbered and labeled. When a researcher takes one or more of these splits for processing studies, its number, date, name of the researcher, and purpose will be entered in the log. As further subsamples are generated, they will be assigned numbers consistent with the original split. The principal investigators will serve as sample custodian for the purpose of receiving samples and verifying sample records.

INSTRUMENT CALIBRATION PROCEDURES AND FREQUENCY

Research equipment, capable of calibration, will be calibrated once daily during each period in which the device is used. The calibration standards will be from Ultra Scientific and will include the constituents of concern for the project. Required frequency and acceptance criteria for the ARCS program are listed in the Internal Quality Control section.

The following instruments will be utilized in this project in accordance with the working standards supplied by each operations manual:

1) Hewlett Packard 5890 Series II Gas Chromatograph
   Manual #5890-90110

2) Hewlett Packard 3396A Integrator
   Manual #03396-60920

3) Hewlett Packard 7673 Automatic Sampler
   Manual #07673-60995

4) Orion 611 Ph Meter
   Manual #502700-058

5) Mettler PM400 and PM4000 Balances
   Manual #ME-702395

6) Dohrman DC-85A Carbon Analyzer
   Manual #915-239

7) Hewlett Packard 3396 Series II Integrator
Manual #03396-90105

8) Hewlett Packard 5970 Mass Selective Detector
Manual #05970-90049

9) Hewlett Packard 59970C Chemstation
Manual #59970C-90034

10) Beckman LS6000LL Liquid Scintillation Counter with external standards source

Below is an overview of the calibration procedures for the analytical instruments that will be used for this project. The concentrations of the calibration standards for each method will be determined by the detection limit and linear response of the range.

Instrument	Procedure
GC (PAHs) Hewlett Packard 5890 with a 30-meter DB-5 and flame ionization detector (FID)	Meet criteria described above in a 3 point initial calibration with 20, 50, and 100 mg/L standards.
GC/MSD (PAHs) Hewlett Packard 5970	Meet MSD tuning criteria followed by chromatographic acceptance criteria (same as above) with the 3-point initial calibration with 20, 50, and 100 mg/L standards.
GC (PCBs) Hewlett Packard 5890 with a 30 meter DB-1 column (0.25 um i.d.) and electron capture detector (ECD)	Meet chromatographic acceptance criteria (such as degradation, peak shape, sensitivity, signal to noise ratio, and retention time stability) in a 3-point initial calibration with 6.1, 30.5, and 61 mg/L standards.
Analytical balance	Prior calibration check with class S weights in the gram and milligram range. Other checks as appropriate in expected weighing range.
HPLC (PAHs)	Meet chromatographic acceptance criteria (same as for the GC/MS) with a multipoint initial calibration at applicable concentrations.
pH meter	Three point calibration at Ph 4, 7, and 10. Calibration check every 10 samples.
TOC	Daily single-point calibration in triplicate. Check standard every 20 samples.
LSC	Daily checks for acceptable background and sample quench.

ANALYTICAL PROCEDURES

The research group will perform characterization and separation procedures on the samples. PAHs will be analyzed using methods adapted from EPA SW 846[1]. Quantitative analysis for PAHs will be preformed by GC/FID and GC/HPLC. PCBs will be analyzed using a modification of methods from EPA SW 846[1]. Quantitative analysis for PCBs will be performed by GC/ECD and qualitative analysis will be by GC/MSD. Measurements of pH will be in accordance with methods 9040 and 9045 from EPA SW 846[1]; measurements of TOC will adhere to method 9060 also from EPA SW 846[1].

Initial efforts will be directed toward ascertaining the appropriate methods for monitoring the losses of PAHs and PCBs from contaminated sediment incubated under aerobic and anaerobic conditions, respectively. Aerobic conditions will be maintained by mixing the reaction vessel on a temperature controlled reciprocal shaker (150 rpm). Anaerobic conditions will be maintained by conducting experiments in an anaerobic chamber containing a gas mixture of 95% N_2 and 5% H_2. Sterile controls will be prepared by autoclaving samples for 30 min on three consecutive days.

Extraction and Clean-up of Sediment Slurry Samples for Analysis of PAHs.

Extraction

1. Vortex sediment slurry for 1 minute. Immediately remove 5 ml samples to 50ml centrifuge tubes. Triplicate samples are collected for PAH analysis; another set of triplicates is collected for determination of sediment dry weights. Record amount of sediment in sample (grams dry weight/5 ml).
2. Centrifuge sample at 8000 rpm for 20 minutes in refrigerated (4° C) Sorvall Superspeed (RC-2) centrifuge. Pour off aqueous supernate and extract with 5 ml CH_2Cl_2. Combine organic phase with subsequent sediment extract.
3. Add 15µl of surrogate mixture containing 4mg/L each of d14-1,4-dichlorobenzene, d10-acenapthene, d8-naphthalene, d12-perylene, d10-phenanthrene, and d12-chrysene to sediment. Refrigerate sample at 4°C 2h-overnight to allow CH_2Cl_2 to evaporate and for PAHs to adsorb to the sediment. A surrogate spike of 15µl (60µg of each surrogate compound) yields a final analyte concentration of 10 mg/L after the sample has been split as described.
4. Dry the sediment by adding 10g sodium sulfite (15g of granular form). Mix immediately to a sandy texture. Water was removed from sediment samples of greater than 10 g by preliminary extraction with methanol (3X). The methanol was subsequently pooled with methylene chloride extracts and back-extracted with a 3% saline solution.
5. For sediments dried with sodium sulfite, extract first with 15ml of a 2:1 CH_2Cl_2/ acetone mixture on a wrist-action shaker (Burrell Model 75) at full-speed setting for 18h. Centrifuge sample (4°C, 8000 rpm, 20min.). Pour off supernate to a pennyhead centrifuge tube. Cap and store at 4°C.
6. Repeat extraction with 10ml CH_2Cl_2 to sediment and shake 18h.
7. Centrifuge sample (4°C, 8000 rpm, 20 min.). Combine supernatants in a graduated cylinder. Adjust volume of methylene chloride to 21 ml. Put sample into a storage tube.

Clean-up

1. Remove one third (7ml) of each sample to a separatory funnel. Add 10ml 1N NaOH. Shake well and vent.
2. Remove organic layer to pennyhead-stoppered centrifuge tube.
3. Exchange methylene chloride for hexane by adding 7 ml of hexane and evaporating methylene chloride under a stream of dry nitrogen. Repeat with a second aliquot (5ml) of hexane. Evaporate down to 5ml +/-.
4. Pass sample in hexane through silica columns. Preparation of silica columns will be as follows: Heat silica gel at 110°C for 24h to activate. Place silanized glass wool on bottom of 10 ml syringe fitted with a Teflon luerlock valve. Saturate silica gel with hexane and add to column to reach the 10 cc mark. Add ±1 g of sodium sulfate to top. Seal with aluminum foil and parafilm and refrigerate until use.) Wash with 20ml hexane.
5. Elute sample with CH_2Cl_2 (2 x 10 ml aliquots). Collect in test tubes and pool aliquots into a pennyhead-stoppered centrifuge tube.
6. Evaporate methylene chloride under a stream of dry nitrogen to 1.0-1.5ml.

GC Analysis

1. Prepare 1 hexane blank and 3 or 4 standards (1, 2.5, 5ppm, or 10ppm). Prepare standards with 1ml 2X concentration standard mixture. Add 10ppm surrogate mixture to each standard.
2. Add 200ul dibutyl chlorendate internal standard (10mg/ml) to each sample and to standards. Pipet internal standard to all samples in one group with the same pipet to increase accuracy.
3. Accurately top sample volumes to 2.0ml with CH_2Cl_2.
4. PAHs were quantitated by gas chromatography using an HP 5890 Series II (Hewlett Packard) equipped with a DB5 capillary column (30 m X 0.32 mm, J & W Scientific) and an FID detector. Helium (30 psi) was used as the carrier gas, while air (40 psi) and hydrogen (30 psi) supplied the FID detector. Injector and detector temperatures were 300°C. The temperature program was 40°C for 3 min., 10°C/ min to 125°C, 4°C/ min. to 310°C, and 310°C was held for 3 min. The injection volume was 1µl.
5. PAH concentrations were determined with a Chemstation software package from standard curves obtained from analysis of the standard mixture of PAHs.

Media

Bushnell-Haas Minimal salts medium (MSB)

K_2HPO_4	1000 mg	KH_2PO_4	1000 mg
$(NH_4)_2NO_3$	1000 mg	$MgSO_4$-$7H_2O$	200 mg
$CaCl_2$-$2H_2O$	20 mg	$FeCl_3$	5 mg

Stock solutions

K_2HPO_4	10 g	KH_2PO_4	10 g
$(NH_4)_2NO_3$	10 g	$MgSO_4$-$7H_2O$	2 g
$CaCl_2$-$2H_2O$	0.2 g	ddH_2O	1 L

Mix and aliquot into 100 ml volumes, then autoclave. Stocks are 10X.
$FeCl_3$ ..... 0.5 g in 100 ml ddh_2O, sterile filtered. Use 1 ml per 1L.

Sediment Extract MSB Medium

Sediment extract was prepared by combining 50g BRS and 150ml H_2O. The mix was autoclaved, then shaken for 24h on wrist-action shaker. Sediment was pelleted by centrifugation and the supernate was retained as sediment extract. MSB stock solutions (10X) were added to achieve a final MSB concentration of 1X.

Extraction of PAHs

Two methods were used to extract PAHs from sediments, depending on the concentration of PAHs and the amount of sediment to be extracted. Laboratory experiments in which PAHs were added to sediment slurries resulted in higher PAH concentrations than encountered in the field. Thus, smaller volumes of sediment needed to be extracted to yield measurable quantities of the compounds. Such sediment slurries were extracted by drying with sodium sulfate and extracting with methylene chloride, as described below.

Sediments that had not been spiked with additional PAHs contained lower concentrations of the chemicals; hence larger volumes of sediment were required. Such large quantities were not amenable to drying with sodium sulfate. In such cases, the methylene chloride extraction was preceded with methanol extractions until all water was removed.

Thus, water was initially removed from experimental sediment slurries by centrifugation, followed by drying with sodium sulfate (2:1 by weight). Unless otherwise specified, all centrifugation was carried out in a Sorvall RC-3 at 4000 x g at 10°C for 10 min. The sediment slurries were subsequently extracted with 15 ml of a mix of CH_2Cl_2:acetone (2:1) on a wrist action shaker (Burrell, Pittsburgh, PA) for 18-24 h. The sediment was pelleted by centrifugation and the supernate was retained. Extraction was repeated with 10 ml of CH_2Cl_2. All extraction supernatants were pooled, back-extracted with 1 N NaOH, and the total volume was adjusted to 21 ml with CH_2Cl_2 prior to clean-up.

When PAHs were to be determined in samples that had not been spiked with additional compound, sediment quantities of 10 g or greater were required to yield sufficient analyte. In such cases, the sediment was extracted three or four times with 10-ml quantities of methanol after the bulk water had been removed by centrifugation. The methanol was retained and combined with the subsequent methylene chloride extracts (two 10-ml extractions on the wrist-action shaker, as above). The pooled extract was back-extracted with 3% saline to remove the methanol, but retain any compound that may have been removed with that solvent.

A deuterated PAH mixture (J&W Scientific, Folsom, CA) was added (to yield 10 ppm in the final analyte) prior to extraction to evaluate extraction and clean-up efficiencies. Typical recoveries of deuterated PAHs were naphthalene 45%, acenaphthalene 60%, phenanthrene 80%, and perylene 95%.

Clean-up of PAH samples.

After either of the described extractions, the methylene chloride solvent was reduced in volume and a solvent exchange into hexane was performed. The sample, in hexane, was applied to an activated silica clean-up column (63-200 mesh) to remove sediment-derived organics, and the PAHs eluted in methylene chloride. Dibutyl chlorendate (J & W Scientific) was added as an internal standard at a final concentration of 20 ppm and the sample volume was adjusted to 2.0 ml.

PAH additions to sediment slurries.

PAHs were added to sediments as a primary mixture made by combining 2 mg each of acenaphthene, acenaphthylene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo(a)pyrene, dibenz(a,h)anthracene, and benzo(g,h,i)perylene (Sigma Chemicals, St. Louis, MO) in 10 ml CH_2Cl_2. The spiking mixture was stored at -4°C. Sediment slurries were spiked by adding 50 µl of the PAH mixture to wet sediment (equivalent to 20 g dry weight) in a 250 ml flask. The solvent was allowed to evaporate overnight at 4°C prior to addition of the experimental medium (e.g., Bushnell Haas minimal salts broth).

Mineralization of radiolabelled PAHs.

Radiolabelled PAHs can be easily quantified with a high degree of precision and were used for several types of experiments in this project. Although disappearance of a parent PAH can indicate the occurrence of a transformation, the detection of radiolabelled carbon dioxide from labelled PAHs can indicate the full mineralization of the parent and hence the potential for supporting the growth of bacteria capable of their degradation.

Moreover, the use of radiolabelled compounds, detected either as disappearance of the parent or production of carbon dioxide, permits frequent, precise sampling, which is necessary for comparing the rates of transformation of PAHs in response to treatments.

To determine whether specific PAHs were mineralized by the enriched culture, approximately 0.025 µCi of the labelled compound and 2 ppm unlabeled substrate were added to sediment slurries and $^{14}CO_2$ evolution was monitored over time. The compounds tested were: phenanthrene, fluoranthene, pyrene and benzo(a)pyrene, which were obtained in radiolabelled form ([9-^{14}C]phenanthrene (Sigma Chemicals), [3-^{14}C]fluoranthene (Chemsyn Labs, Lexana, KS), [4,5,9,10-^{14}C]pyrene (Chemsyn Labs) or [7,10-^{14}C]benzo(a)pyrene (Sigma Chemicals)). Sediments were inoculated with the enrichment culture.

Enrichment of PAH degraders from Buffalo River Sediments.

An enrichment culture of microorganisms from Buffalo River sediment (BRS) were developed by exposing the sediment to a mixture of PAHs for a period of three weeks. BRS was combined with mineral salts broth (MSB) and a mixture of PAHs (20 mg/kg each of the sixteen PAHs of concern) in 250-ml flasks, which were incubated shaking (200 rpm) at 28°C for 3 weeks. The culture was transferred (1%) every 3-4 weeks. After 3 months, in order to determine the activity of the enrichment on each of the PAHs as well as the mixture of PAHs, and to determine the dependence of the culture on nutrients derived from sediment, the enrichment was inoculated into: (1) BRS + MSB + each PAH individually, (2) BRS + MSB + PAHs, (3) MSB + PAHs, and (4) MSB + each PAH individually. In addition to testing the activity of each individual enrichment, a uniform inoculant with optimal activity was developed by combining all of the cultures. The resulting enriched culture was maintained by sequential transfer in BRS + MSB + PAHs every 3-4 weeks. It is this enriched culture that is the experimental inoculum unless otherwise specified.

Quality control and statistical analyses.

The procedures used to assure quality control and validity of statistical analyses are described. Deuterated PAHs were added to sediment samples to monitor extraction efficiency. The extraction efficiency varied with the molecular weight of the PAH.

In experiments in which PAH concentration was determined by extraction and GC, each treatment was run in duplicate or triplicate. At each sampling at least one treatment replicate was sampled in triplicate, in order to compare the variance due to sampling to that due to differential performance of replicates within treatments. All data are represented as averages of replicate or triplicate determinations.

Extraction and Cleanup of Sediment Samples for PCB Analysis.
Sediment preparation.

Transfer entire sample jar contents to a pre-weighed, labeled glass pie plate. Remove any visible large debris (stones), and reweigh the pan. Place pan in a hood to air dry for 3 days. Reweigh. Scrape contents of the pan into a mortar and grind to a consistent grain with a pestle. Sieve the sediment through three consecutive sieves and into a catch pan: first #4 - 1.40 mm, second #18 - 1.00 mm, and lastly #45 - 0.355 mm. Transfer the sieved sediment into a clean, labeled sample jar. Cap and store in the refrigerator at 4 ± 2°C.

CALCULATIONS

% dry weight:

$$\% \text{ dry weight} = \frac{\text{g of dry sample}}{\text{g of sample}} \times 100$$

PCB extraction of dried sediment.

Transfer 5.0 g of air-dried sediment into a 15.0 ml test tube and cap with a teflon-lined screw cap. Add 0.25 ml of 1% (v/v) Triton-X 100, 0.100 ml of the 100 ppm matrix spike solution (when applicable), and 10.0 ml acetone to the sample. Vortex, and place on the wrist-action shaker for 24 hours. Visually check to be certain that the sample is well mixed. Centrifuge the sediment/acetone mixture in the test tube at 2200-2400 rpm for 10 minutes at room temperature. Transfer all the supernatant solution into a 125 ml separatory funnel with a 5.0 ml graduated pipet. Record the volume recovered. Place test tube with sediment plug in a 105°C oven overnight; record weight and subtract test tube weight for dry sediment weight. Add 10.0 ml of the extraction solvent and 5.0 ml of 2% (w/v) sodium chloride solution and shake for 1 min. Allow the sample to settle before draining the lower layer. Add 5.0 ml of concentrated sulfuric acid and shake for 1 min, and allow the sample to settle. If there is still color in the non-aqueous layer, reshake and then drain. Add 5.0 ml of 2% (w/v) sodium chloride and shake for 1 min. Allow the lower layer to separate and drain. Transfer the non-aqueous layer with a 5.0 ml pipet to a labeled 15 ml test with a Teflon-lined screw cap. Record volume and dilute to exactly 10.0 ml with hexane.

Sample cleanup.

Prepare florisil spice tubes (Analtech #11-96) with the addition of approximately 2 g florisil. Transfer spice tubes to anaerobic glove box and add approximately 10 g cleaned (reduced) copper filings to each. Add approximately 2 g florisil to the top of the tube, and remove from the glove box. Pre-wet spice tubes with 2 rinses of 5 ml hexane. Transfer 5 ml of extract onto the spice tube column and vacuum drain slowly. Collect the extract. Rinse 3 more times with 5 ml hexane, collecting all rinses. Measure volume of collected extract, record, and dilute to 20 ml with hexane in a labeled glass scintillation vial. Transfer 4.5 ml of extract into a labeled shorty vial. Add 0.5 ml internal standard solution (10 ppm octachloro-naphthalene;OCN). Cap with Teflon-lined cap and shake to mix. Transfer 1-2 ml to an amber (labeled) autosampler vial. Cap with a Teflon-lined crimp seal. Seal and store remaining extract in the shorty vial in the freezer (-20C). Sample remaining in the scintillation vial may be disposed as hazardous waste. All solvents used should be pesticide grade or better.

Solutions for PCB extraction.

Extraction solvent (5 mg/L of congener #151). Make up 1 liter of 9:1 hexane/acetone by measuring 900 ml hexane and 100 ml acetone into a 1 liter amber volumetric flask. Completely rinse the contents of a 5 mg vial of BZ #151 (Ultra Scientific #RPC-051) into the volumetric flask using the 9:1 solvent. Mix thoroughly, cap, seal, and store in the refrigerator.

Matrix spike solution (100 mg/L of congener #158). Completely rinse the contents of a 5 mg vial of BZ #158 (Ultra Scientific #RPC-109) with hexane into a 50 ml amber volumetric flask. Fill the volumetric flask to volume with hexane. Mix, cap, seal, and store in the freezer.

2% (w/v) sodium chloride solution. 20 gm of high purity sodium chloride (Aldrich #22,351-4) is added to 1000 ml of distilled water. Mix, cap, and store in the refrigerator.

1% (w/v) Triton X-100 solution. 1 ml of Triton X-100 (Aldrich #28,210-3) is diluted to 100 ml with distilled water in a 100 ml volumetric flask. Mix, cap, and store in the refrigerator.

10% sulfuric acid solution. 10 ml of concentrated sulfuric acid (Baxter Diagnostics) is diluted to 100 ml with distilled water in a volumetric flask. Mix, cap, and store in the hood.

Internal standard solution (10 mg/L OCN). 20 mg of octachloronapthelene (OCN) (Ultra Scientific #RCN-012) is rinsed with hexane into a 20 ml amber volumetric flask. Fill the volumetric flask with hexane (this is now the 1000 ppm stock solution). Transfer 5 ml of the 1000 ppm solution to a 500 ml amber volumetric flask, and dilute to the mark with hexane. Mix, cap, seal, and store in the freezer.

Copper preparation. Transfer 500 g of copper metal - fine granular (Mallinckrodt/Baxter #4649-500) into a clean sample jar. Cover copper with 10% sulfuric acid, shake vigorously, and let sit a minimum of 24 hours. Pour off sulfuric acid, rinse 3 times with 200 ml distilled water, followed by 3 rinses with 200 ml acetone. Blow down remaining acetone with argon or nitrogen. Transfer copper to the anaerobic glove box immediately.

Florisil preparation. Transfer 100 gm Florisil 100-200 mesh (Mallinckrodt/Baxter #6876) into a glass container. Loosely cap and store in 120° oven.

Sediment preparation. Transfer entire sample jar contents into a labeled, pre-weighed glass pan. Remove any visible large stones. Reweigh. Allow sediment to air dry for 3 days. Scrape contents of the pan into a large mortar and grind to a consistent grain. Sieve the ground sediment through three consecutive sieves; first #14 - 1.40 mm, second #18 - 1.00 mm, and lastly #45 - 355 um. Pour final sieved sediment into a clean, labeled sample jar. Cap and store in the refrigerator. All solvents should be pesticide grade or better.

DATA REDUCTION, VALIDATION, AND REPORTING

Data Recording - All raw data will be put into laboratory notebooks, as mandated in AERL LOP 5400-3[2].

Data Reduction - Wherever possible, the initial data reduction will be computerized. Where data reduction is not computerized, calculations will be performed in permanently bound laboratory notebooks with carbon copy pages.

Data Validation - The individual analysts will verify the completion of the appropriate data records to verify the completeness and correctness of data acquisition and reduction. The principal investigator will periodically review computer and manual data reduction results and will inspect laboratory notebooks and/or data sheets to verify data reduction correctness and completeness and to ensure adherence to the specified analytical method protocols. Calibration and QC data will be examined by the individual analysts to verify that all instrument systems are in statistical control, and that QC objectives for precision, accuracy, completeness, and method detection limit are being met.

Reporting - Results will be given in standard units, as specified by the analytical methods. Reporting units will be given in mg/L for all liquid samples and in mg/kg for soils and other solid matrices. Before final reporting of results, the QC Monitor will review data for adherence to QC objectives. All results will be available in hard-copy and computer readable format to the ARCS or BOM Project Managers. All reports and documentation required, including chromatograms and mass spectra, calibration records, and QC results, will be clearly labeled with the laboratory sample number and associated field sample number.

INTERNAL QUALITY CONTROL CHECKS

At the discretion of the researcher, duplicate tests will be conducted to ensure the accuracy and reproducibility of the analyses. Typically, these replicate tests will be conducted on random samples. These repeat test analyses will be a double check on analytical work to help ensure that consistent results are obtained throughout the entire research program and should fall within 20%. The analytical internal quality control procedures listed in Table A will be taken to ensure the stated criteria for precision and accuracy (Table B).

QC control charts will be kept on file for blanks, QC samples, and mid-range calibration standards.

Triplicate sample analysis giving acceptable precision is ± 20% RSD.

Samples in a group of 20 or less is called a set. Data from a sample are acceptable when spike recoveries fall in the range of 70 to 130, except for highly volatile compounds. An analytical batch is a group of samples run with the same reagents on the same instrument under the same conditions. The analytical batch is acceptable when the QC chart shows the analysis to be in control and that the QC measures have been acceptable.

Conservation of mass will be used to validate mass measurement data.

PERFORMANCE AND SYSTEM AUDITS

Internal Performance Audits - An audit will initiated by the principal investigators using reference standards to assess adequacy of pollutant concentration measurements.

Internal Systems Audit - An audit will be performed by the project manager at the initiation of the project covering sampling splitting procedures, work plans, selection of methods, and data recording.

External Performance Audits - Initiated by the principal investigators at the midpoint of the project using reference samples from an external source (where available) to assess the performance of pollutant analyses.

External Systems Audit - May be performed by the ARCS or BOM QA officer.

PREVENTIVE MAINTENANCE

All the required maintenance is performed in-house by the analyst according to the procedure described in the instrument operations manual except for that which can only be performed by a representative of the

instrumentation company. Instrument maintenance logbooks will be kept with each instrument and will be updated by the operator whenever either routine or non-routine maintenance procedures are performed.

ROUTINE DATA ASSESSMENT PROCEDURES

Surrogate Spike Recovery - The percent recovery for each surrogate standard will be calculated and tabulated, and control limits established. A surrogate standard is a compound that when spiked into an environmental sample, behaves in the same manner as the analyte or analytes of interest. The surrogate standard is added prior to extraction and is used to determine chemical recoveries from environmental samples. Surrogate standards for PAHs in sediment are deuterated PAH mixtures. Samples to be analyzed for PCBs will be spiked with an appropriate surrogate (dibutylchlorendate or a specific PCB congener such as congener #151, which is not a major constituent of the analyzed sample). All samples will be spiked with a surrogate standard unless a specific exception is made in the method itself. If a surrogate standard falls beyond the control limit, the data will be regarded as unreliable, corrective action will be taken, and the analysis will be repeated.

Percent recovery for the surrogate spike samples will be calculated as follows:

$$\%P = 100\% \times (Qd/Qa)$$

where:

%P = percent recovery;
Qd = quantity determined by analysis; and
Qa = quantity added to the sample.

A tabulation of percent recoveries will be maintained for each surrogate spike. The tabulation will include the analysis date and the percent recovery.

Precision and Accuracy - Precision will be defined in terms of relative percent difference of the duplicate analysis, where applicable. Precision will be calculated using the following equation for relative percent difference:

$$RPD = \frac{(C_1 - C_2)}{[(C_1 + C_2)/2]} \times 100$$

where:

RPD = relative percent difference;
C_1 = the larger of the two values; and
C_2 = the smaller of the two values.

If calculated from three or more replicates, the relative standard deviation (RSD) will be used rather than RPD. The following equation is applicable for RSD:

$$RSD = (s/y) \times 100\%$$

where:

RSD = relative standard deviation;

s = standard deviation;
y = mean of replicate analyses.

The calculation for accuracy in matrix spikes is as follows:

$$\%R = 100\% \times [(S - U)/C_{sa}]$$

where:

%R = percent recovery;
S = measured concentration in spiked aliquot;
U = measured concentration in unspiked aliquot;
C_{sa} = actual concentration of spike added.

The calculation for accuracy in standard reference materials (SRM) is as follows:

$$\%R = 100\% \times (C_m/C_{srm})$$

where:

%R = percent recovery;
C_m = measured concentration of SRM;
C_{srm} = actual concentration of SRM.

Completeness - Completeness will be defined as followed for all measurements:

$$\%C = 100\% \times (V/n)$$

where:

%C = percent completeness;
V = number of measurements judged valid;
n = total number of measurements.

CORRECTIVE ACTION

Corrective action will be implemented whenever a system is not in compliance. Corrective action schemes are based on data acceptability limits, identification of defects, summarizing defects, and tracing of defects to their source. We will implement measures to correct identified defects, maintain documentation of the results of the corrective process, and monitor the process until each defect is eliminated. In the case of laboratory non-compliance issues, corrective actions are likely to be immediate in nature and most often will be implemented by the analyst. In these cases, corrective actions usually involve recalculation, reanalysis, or repetition of a sample run. Corrective actions may also be started as a result of performance and system audits. These actions may require altering analytical procedures, sampling, or using a different batch of sample containers.

Corrective actions fall into two categories: 1) handling of analytical or equipment malfunctions; and 2) handling of nonconformance or noncompliance with the QA requirements that have been set forth. All corrective measures taken will be included in the Laboratory Record Book.

Corrective action required to conform to the specifications will be recorded by the QC monitor, and reported to the Principal Investigator within five days. Corrective actions will be documented.

Failure of any sample to meet any of the above stated limits for precision and accuracy or failure to fall within limits of detection stated in Section -- QUALITY ASSURANCE OBJECTIVES -- will be considered criteria for re-analysis of the sample or sample set. This may include recalculation, repetition of a sample run, or reconsideration of the appropriateness of the method used. In the case of a change in methodology, all samples of the type in question will be reanalyzed. All corrective action taken will be noted in the Laboratory Record Book.

REFERENCES

1. Test Methods for Evaluating Solid Wastes (SW 846), Third Edition (USEPA 1986).

2. USEPA Athens Environmental Research Laboratory Operations Manual, December 1982.

Table A. Summary of Internal Quality Control Procedures

Procedure	Frequency	Requirement
Calibration Standard	3 per set	Mid-range standard shot at beginning, middle, and end of sample set for pH and TOC.
		Mid-range standard shot at beginning, every 12, and end of set for PAHs and PCBs.
		± 20% of the known value
Method Blanks	1 per set	≤ detection limit (3 per set for TOC at beginning, middle, and end of set)
Matrix Spikes[1]		
contaminated	1 per set	1.0 to 1.5 times the estimated concentration of sample
		± 30% of the spike for PAHs and PCBs ± 30% of the spike for TOC
uncontaminated	1 per set	Spike within a factor of 10 of the instrument detection limits
		± 30% of the spike for PAHs and PCBs ± 30% of the spike for TOC
treated duplicate		20% RPD
Replicates		
triplicate analysis	1 per set	triplicate analysis of one sample
		20% RSD
duplicate test	as applicable	20% RPD
QC Sample	see spikes	
Surrogate Spikes[1]	all samples	applicable to PAHs and PCBs
		30% RSD ± 30% of the spike
Control Charts	postrun analysis	± 3 standard deviations

[1]The use of deuterated PAHs makes matrix and surrogate spikes equivalent.

Table B. Data Quality Objectives for Precision and Accuracy

Measurement Parameter	Analysis Method(s)[a]	Precision	Accuracy
PAHs			
standard	8100 & 8310		± 20%
sample replicates	"	20% RSD	
test duplicates	"	20% RPD	
surrogate spike	"		± 30%
PCBs			
standard	8080 & 8270		± 20%
sample replicates	"	20% RSD	
test duplicates	"	20% RPD	
surrogate spike	"		± 30%
pH			
standard	9040 & 9045	0.1 pH unit	± 0.1 pH unit
sample replicates	"	0.1 pH unit	
TOC			
standard	9060		± 20%
matrix spike	"		± 30%
sample replicates	"	20% RSD	
Surrogate spikes (PAH)			
Matrix spikes (PCBs)			
untreated sample	8080,8270,8100,8310		± 30%
treated sample	"	20% RPD	± 30%

[a]Test Methods for Evaluation Solid Wastes (SW 846), Third Edition (USEPA 1986)

Table C. Constituents of Revised Anaerobic Mineral Medium (RAMM).

Component	per unit volume (liter)		
KH_2PO_4	0.27	g	
K_2HPO_4	0.35	g	
NH_4Cl	0.53	g	
$CaCl_2$-$2H_2O$	75	mg	
$MgCl_2$-$6H_2O$	100	mg	
$FeCl_2$-$4H_2O$	20	mg	
$MnCl_2$-$4H_2O$	0.5	mg	
H_3BO_3	0.05	mg	
$ZnCl_2$	0.05	mg	
$CuCl_2$	0.03	mg	
$NaMoO_4$-$2H_2O$	0.01	mg	
$CoCl_2$-$6H_2O$	0.5	mg	
$NiCl_2$-$6H_2O$	0.05	mg	
Na_2SeO_3	0.05	mg	
$NaHCO_3$	1.2	g	(optional*)
Na_2S-$9H_2O$	0.5	g	(optional*)

*not added in the present study

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