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RESEARCH AND DEVELOPMENT PROGRAM FOR THE DESTRUCTION OF PCBs

Ninth Progress Report
June 1, 1989 -- July 31, 1990

August 1990

Prepared by

General Electric Company
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GENERAL ELECTRIC COMPANY
RESEARCH AND DEVELOPMENT PROGRAM
FOR THE DESTRUCTION OF PCBs

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For the Period

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Submitted by

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EXECUTIVE SUMMARY

The organization of this year's report represents a change from past reports. For the first time, research on anaerobic reductive dechlorination is presented first. This change was made both to reflect the increased amount of research activity in this area over the past several years and because anaerobic dechlorination is the first step in a two-step anaerobic-aerobic process sequence with the potential to degrade completely even the most highly chlorinated Aroclor mixtures. The anaerobic research presented here continues to focus on the characterization and isolation of the individual microorganisms which make up the PCB-dechlorinating consortia that have now been found in many anaerobic environments. Efforts in this area include the characterization of dechlorination patterns of consortia found in different sediments and the development of techniques to transfer activity from one sediment matrix to another. In addition, work on measuring the reduction in toxicity of PCB mixtures effected by reductive dechlorination is also presented. A second goal of this year's research is to prepare for the eventual field application of this technology. In that light, the dechlorination rate of endogenous PCBs is studied and bioavailability issues explored. Preliminary results from a laboratory reactor designed to model the conditions in an actual river are also presented. Laboratory results of sequential anaerobic-aerobic processes are presented and provide a transition back into aerobic biodegradation. Genetic studies of aerobes indicate that engineered organisms may have significant advantages over natural isolates in stability of the genes that encode for PCB-degrading activity. Research on PCB-degrading aerobes that can survive in anaerobic conditions is also presented, along with work aimed at gaining a clearer understanding of the aerobic metabolic pathway. Finally, a progress report is presented on a state-of-the-art analytical method for quantitating PCB congeners present at low levels in Aroclor mixtures which cannot be measured by traditional GC analysis. Of particular significance here is the ability to quantitate the more toxic congeners.

ANAEROBIC

A Systematic Study of Reductive Dechlorination of Trichlorobiphenyls in River Sediments. This report describes a study of the dechlorination patterns of six added trichlorobiphenyls by anaerobic consortia in sediments from the Hudson River, Woods Pond (Lenox, MA) and Silver Lake (Pittsfield, MA). The amount of depletion of added congener, the number of chlorines removed, and the dechlorination pattern observed were shown to vary with both the sediment type and added congener. Anaerobes in Woods Pond sediment were able to dechlorinate even the *ortho* chlorines on 246-CB. The addition of a single congener also stimulated the dechlorination of the endogenous PCBs in Hudson River and Woods Pond sediments. In addition, successful transfer of PCB dechlorination activity to an uncontaminated sediment is demonstrated.

Reductive Dechlorination of Aroclors by Anaerobic Microorganisms. This work focuses on the dechlorination of the more highly chlorinated Aroclors, especially Aroclor 1260. Silver Lake sediments dechlorinated all four Aroclors (1242, 1248, 1254, 1260), but the rate and extent was lowest for 1260. Still, these microorganisms proved more capable of dechlorinating 1260 than did Hudson River consortia. This limited dechlorination ability of Hudson River microorganisms on 1260 is probably not due to inhibition by any of the more heavily chlorinated congeners in that Aroclor. In other experiments, the PCB congeners in Aroclor

1242 with greater dioxin-like toxicity were effectively dechlorinated in laboratory assays, reducing the toxicity of the mixture as measured by EROD induction assays by 75%.

Establishment and Characterization of an Anaerobic Aroclor 1242-Dechlorinating Culture. Anaerobic enrichment cultures were established as a first step in obtaining pure cultures or stable consortia capable of dechlorinating PCBs. Pyruvate was demonstrated to stimulate dechlorination of Aroclor 1242 and greatly improve transferability of dechlorination activity by reducing the lag period before the onset of dechlorination. High sediment loadings and the presence of natural organic carbon in the sediment matrix were shown to be important in supporting the dechlorination consortium.

Reductive Dechlorination of PCBs in Sediments from the Hudson River and New Bedford Harbor. The dechlorination of PCBs in these sediments was studied under both methanogenic and sulfate reducing conditions. Dechlorination of added Aroclor 1242 was observed in both sediments under methanogenic conditions, although it occurred much faster in Hudson River sediment. The addition of a mixture of fatty acids as an auxiliary carbon source greatly enhanced the rate of 1242 dechlorination in Hudson River sediment, but had no impact on the dechlorination rate in New Bedford sediment. No dechlorination activity has been observed to date under sulfate-reducing conditions in either sediment.

Anaerobic Dechlorination of Endogenous PCBs in Woods Pond Sediment. Modest natural dechlorination of the endogenous PCBs in Woods Pond sediment has occurred. The goal of this work was to establish conditions which would stimulate greater dechlorination activity in this sediment. This was accomplished through the addition of single congeners (25-34- or 23456-CB) to the sediment in laboratory assays. After addition of the congeners two distinct dechlorination patterns, designated as Pattern H (*para* dechlorination) and Pattern N (*meta, para* dechlorination) of the endogenous PCBs emerged. Dechlorination of endogenous PCBs could not be stimulated by the addition of nutrients unless a PCB congener was added. These results suggest that the growth and activity of a bacterial population capable of dechlorinating PCBs may be severely limited by poor bioavailability of the endogenous PCBs.

Anaerobic and Aerobic Biodegradation of Endogenous PCBs. A two-fold increase in the rate of anaerobic dechlorination in Hudson River sediments was observed upon the addition of a simple trace metals mixture to the sediment. This result suggests that low levels of a trace metal in the sediment may limit the rate of PCB dechlorination in the environment today. Experiments on several PCB-contaminated soils and sediments indicate that this anaerobic process will effectively attack endogenous PCB contamination at rates comparable to those for spiked samples. Sequential anaerobic-aerobic degradation was demonstrated on sediments that had been extensively dechlorinated both naturally and in the laboratory, resulting in significant reductions in total PCB concentrations.

Sequential Anaerobic-Aerobic Biodegradation of PCBs. A sequential anaerobic-aerobic biodegradation process has the potential to completely degrade even highly chlorinated Aroclor mixtures. This work demonstrates that lack of organic substrate may be a limiting factor for reductive dechlorination in some sediments. Dechlorination activity was observed in sediments with no past history of PCB contamination, indicating that bacterial populations capable of dechlorinating PCBs may be widely dispersed in the environment. Biphenyl-degrading aerobes were also isolated from several sites, indicating they too are ubiquitous. Although

their PCB-degrading competence was not great, PCB levels in sediment samples were significantly reduced in an anaerobic-aerobic laboratory trial using one of these isolated aerobes.

Hudson River Model. *In situ* application is the ultimate aim of most laboratory bioremediation studies. A large-scale (0.8 tons of sediment) model reactor has been set up in an attempt to stimulate dechlorination in PCB-contaminated sediment while at the same time simulating *in situ* conditions. Methanol injection has been able to initiate anaerobic conditions in the reactor without the use of a reducing agent. Active dechlorination is taking place spatially and temporally within the reactor. The similarity of these results to batch incubations suggest it will be possible to translate small-scale work to *in situ* conditions.

Differentiation of Anaerobic Microbial Dechlorination Processes. The PCB congener distribution alteration patterns by PCB-contaminated aquatic sediments or anaerobic cultures inoculated within such sediments have already suggested the operation of some 13-15 different anaerobic microbial dechlorination systems. In order to provide objective criteria for distinguishing among such systems, the specific differences in the range of PCB congeners attacked by each have now been tabulated. Thus far, it would appear that each PCB-contaminated drainage basin exhibits its own distinctive assemblage of PCB-dechlorinating microorganisms, presumably in response to the availability of PCBs as terminal electron acceptors for anaerobic metabolism.

AEROBIC

Genetic Studies of Bacterial PCB Degradation: 1. Bacterial Survival on PCB-Containing Soil 2. Analysis of *BPH* Genes. The ability to develop a practical process for aerobic PCB biodegradation depends largely upon obtaining organisms with the ability to survive and maintain activity in PCB-containing soils. Experiments with both naturally occurring LB400 and recombinant FM4560 organisms showed good survivability (detectable populations after 28 days) on soil in laboratory tests. While instability of the *bph* genes were noted in the LB400, FM4560 was found to be stable if antibiotic selection for the plasmid was maintained. Evidence for at least two separate promoters in the Bbph region suggest that these genes are not organized as a operon.

PCB Biodegradation and Nitrate Reduction. Several organisms which can grow on biphenyl or a biphenyl/Aroclor mixture and use oxygen or nitrate as an electron acceptor have been isolated. One of these organisms, a *Comamonas testosteroni*, grows to high densities on biphenyl/Aroclor 1242 under aerobic conditions, with the formation of chlorobenzoic acids as metabolic products. Efforts are underway to understand the relationship between this metabolic pathway and the one which mediates nitrate reduction.

Availability of PCBs in Soils and Sediments to Surfactant Extraction and Aerobic Biodegradation. An analogy between surfactant extraction and aerobic biodegradation is used to study the effect of natural organic matter (NOM) on PCB availability in soils and sediments. PCB equilibrium partitioning into the NOM phase is demonstrated to have a detrimental effect on the extent and efficiency of PCB removal for both of these processes. Oil and grease also form a secondary phase in soils and sediments, and are shown to have a similar negative impact on biodegradation. A treatment is presented which can enhance PCB

removal by modifying the NOM present in the soil or sediment matrix.

Synthesis of Proposed Intermediates in the Aerobic Degradation of PCBs; Ring-Fission Products. A major pathway for the destruction of PCBs by aerobic microorganisms closely resembles the pathway for oxidation of biphenyl itself, proceeding through a ring-fission intermediate which is further degraded in at least two different ways. Alternatively, the oxidation of 23-3-CB is proposed to proceed through a tri-keto acid intermediate. These intermediates have now been chemically synthesized allowing their reactions under non-enzymic conditions, as well as their transformation by microorganisms, to be explored. To date, non-enzymic hydrolysis of these intermediates has yielded both benzoic acid and acetophenone, which are the major aromatic products in the aerobic degradation of many PCBs, and oxalic acid, which has not previously been isolated as a metabolite of biphenyl or PCBs.

ANALYTICAL

The Quantitation of Polychlorinated Biphenyls by Gas Chromatography and Tandem Mass Spectrometry. Using a selected reaction monitoring mode of operation in a GC/MS/MS experiment and monitoring the exchange reaction of oxygen for chlorine, PCB congeners are able to be quantitated even when not chromatographically resolved. The method couples the high specificity of MS/MS with the excellent detection limits of selected ion monitoring coupled with negative chemical ionization. This ability is critical to the quantitation of toxic congeners present at low levels in PCB mixtures, particularly when other congeners co-elute with these in standard GC/MS methods.

Chapter 1

A SYSTEMATIC STUDY OF REDUCTIVE DECHLORINATION OF TRICHLOROBIPHENYLS IN RIVER SEDIMENTS

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INTRODUCTION

In 1989, Abramowicz et al., reported dechlorination of added polychlorinated biphenyl (PCB) congeners in river sediments containing a background level of a complex PCB mixture [GE Report, 1989]. They determined the pattern of dechlorination for each added congener and quantified the dechlorination rate. They also noted a specific stepwise dechlorination of 2,3,4-3,4-CB to 2-CB within Hudson River sediment.

This report describes a systematic study of the dechlorination of added trichlorobiphenyls with all of the chlorines on one ring by sediments from the Hudson River, Woods Pond and Silver Lake. An earlier study of 2,3,4-CB added to Woods Pond sediment indicated that reductive dechlorination had occurred, and at a rate considerably more rapid than dechlorination of an added Aroclor mixture in Hudson River sediment [H. Van Dort, personal communications]. The goal of this work was to understand the patterns and rates of dechlorination of these single congeners by the microbial populations in the river sediments listed above. The results of the study may help to characterize the microorganisms capable of mediating reductive dechlorination of PCBs and have led to successful passage of PCB-dechlorinating activity.

RESULTS AND DISCUSSION

Experimental Procedure

PCB-contaminated river sediments from the Hudson River downstream of Fort Edward, NY (H7 site), Silver Lake (Pittsfield, MA) and Woods Pond (Lenox, MA) were collected and stored until use in sealed vessels at 4°C. Cultures were prepared and sampled within a glove box containing an oxygen-free atmosphere (95% N_2, 5% H_2). Batches (300-500 mL) of sediment and RAMM mineral salts reduced with 0.1% cysteine hydrochloride were prepared at a 2:3 ratio (volume:volume). The batches were stirred vigorously and 30 mL volumes were removed to 50 mL serum vials. A trichlorobiphenyl stock solution (70 mM in acetone) was added to each serum vial, making the concentration of a single congener addition at 350 μM. The added congeners are shown in Figure 1-1. After congener addition, each vial was

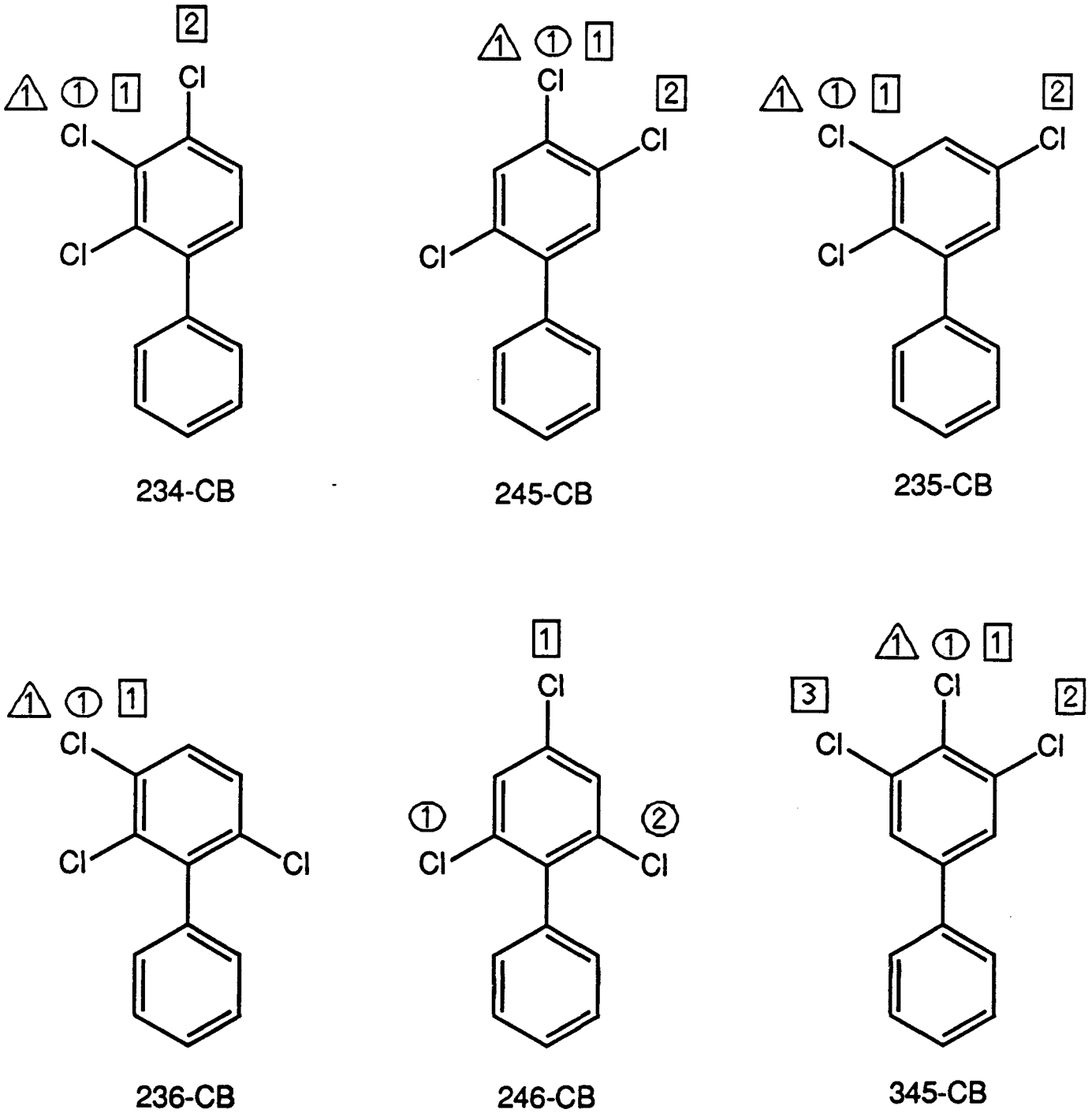

Figure 1-1. Reductive dechlorination of trichlorobiphenyls by river sediments. The number above each chlorine indicates the order of chlorine removal within the particular river sediment. The boxed numbers represent Hudson River sediment; the circled numbers represent Woods Pond sediment; and the triangulated numbers represent Silver Lake sediment.

vortexed for 2 minutes and two 1 mL samples were removed as time zero points and stored at -20°C. All vials were crimp sealed and removed from the glove box.

Four vials were prepared for each added congener and river sediment. Two of each group of four were immediately autoclaved for 3 hours (the autoclaved control samples). All vials were stored stationary at 24°C in the dark. At particular timepoints, vials were placed in the glove box, vortexed for 30 seconds, immediately uncapped and 1 mL samples were placed in 8 mL glass vials. The culture vials were then crimp sealed and returned for further incubation. Each 8 mL glass vial was extracted with 5 mL of diethyl ether and ~0.5 mL mercury, added to precipitate the molecular sulfur in the sediment sample. The ether extract was analyzed by capillary gas chromatography (GC) using a fused silica capillary column (30 m x 0.25 mm ID) coated with a 0.25 μm bonded liquid phase of DB-1 (polydimethylsiloxane, J&W Scientific, Folsom, CA) and an electron capture detector at 300°C.

For five of the six added trichlorobiphenyls, the results are reported as the percent peak area of each congener product relative to the total peak areas for the added congener and its products (no account was made for the response factor of each congener). The results in experiments with 3,4,5-CB were determined using standard solutions of 3,4,5-CB and each of its congener products. This was done because 3-CB has an extraordinarily low response factor relative to the other congeners and would result in a significant underprediction of 3-CB in the mixture if the percent peak area method was used.

Dechlorination of Added Congener Within Hudson River Sediment

Figure 1-1 is a descriptive diagram of the dechlorination patterns for the added congeners by each river sediment. Dechlorination of every added trichlorobiphenyl was observed in Hudson River sediment collected downstream of Fort Edward, NY. However, only *meta* and *para* chlorines were removed. Since 3,4,5-CB does not contain *ortho* chlorines, all of the chlorines were removed in Hudson River sediment resulting in the accumulation of biphenyl (confirmed by mass spectra (MS) analysis after gas chromatography). Figure 1-2 shows the pattern of 3,4,5-CB dechlorination. The figure shows a sequential dechlorination of the trichlorobiphenyl to dichlorobiphenyl, then of the dichlorobiphenyl to monochlorobiphenyl, and finally of the monochlorobiphenyl to biphenyl.

The apparent rate of trichlorobiphenyl dechlorination was very rapid as compared to what has been seen with the addition of the Aroclor mixture [GE Report, 1989]. Dechlorination of every added congener was observed by the first timepoint at 2 weeks (a lag time <2 weeks) and >80% of the added trichlorobiphenyl was converted to product by 5 weeks. The pattern of chlorine removal seemed to be dictated by the spatial arrangement of the chlorines in the PCB molecule, such that the inner chlorine was preferentially removed first (e.g., 2,4,5-CB goes to 2,5-CB, 3,4,5-CB goes to 3,5-CB).

Dechlorination of the endogenous PCBs in Hudson River sediment occurred concomitant with dechlorination of the added congener. 2,3,4-CB, 2,4,5-CB, and 2,4,5-CB all induced a

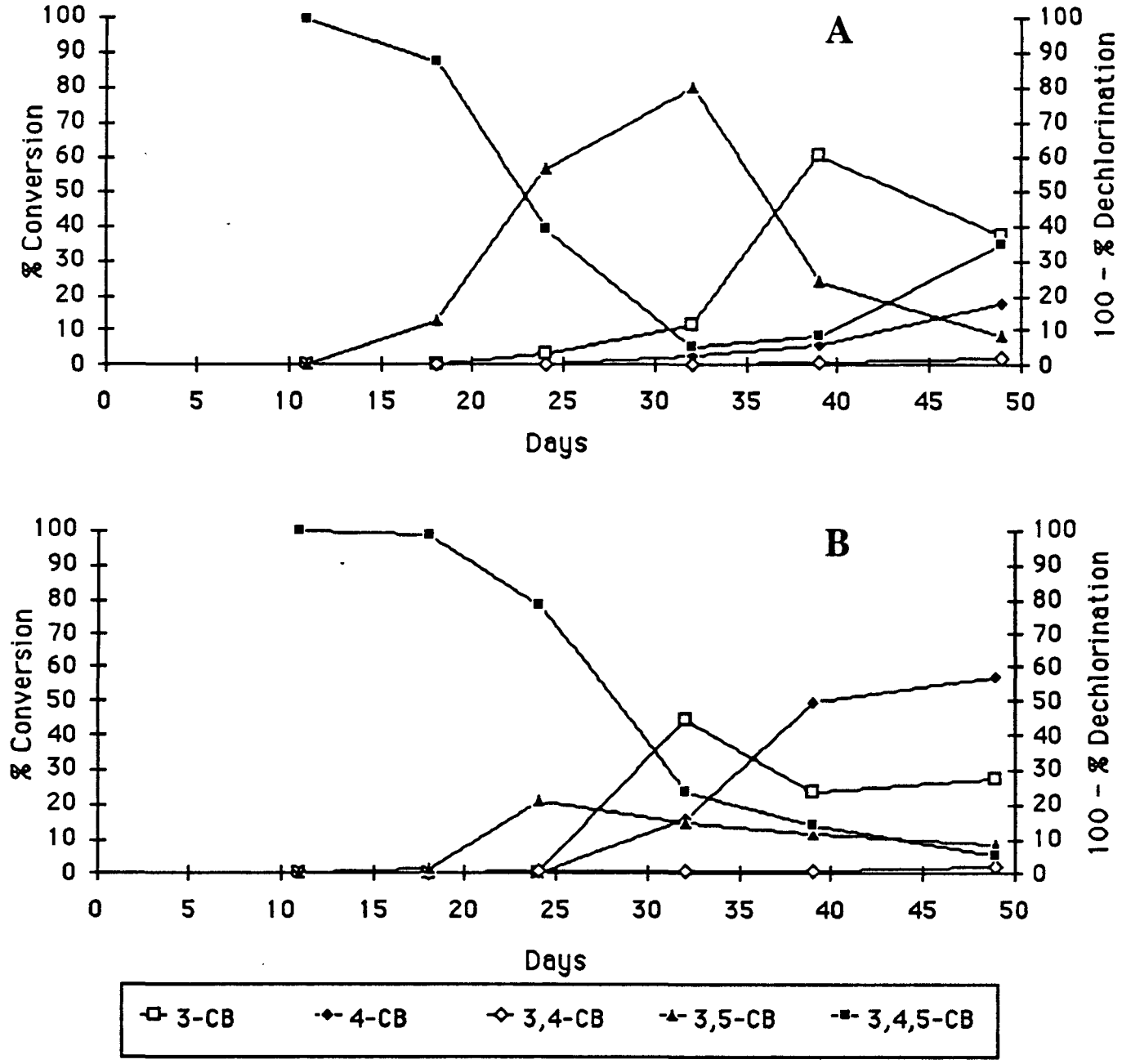

Figure 1-2. Stepwise dechlorination of added 3,4,5-CB in PCB-contaminated Hudson River sediment. Panels A and B are duplicate experiments showing stepwise dechlorination of added 3,4,5-CB in PCB-contaminated Hudson River sediment. The graphs showing the conversion of 3,4,5-CB to the products are defined in the box below Panel B and relate to the left ordinate in each panel. Biphenyl, which is the ultimate dechlorination product, it is not described in this figure because it could not be measured under the GC analysis conditions. The data shown above at 48 days after 3,4,5-CB addition does not take into account the significant amount of biphenyl that has accumulated from dechlorination.

Pattern Q activity [GE Report, 1989], whereas 2,3,6-CB induced a Pattern M activity. Unfortunately, the Hudson River sediment used in the 2,3,5-CB and 3,4,5-CB experiments came from a batch of sediment collected more recently from the H7 site. This sediment contained such a highly dechlorinated endogenous PCB content so as to preclude the observation of any added congener-induced dechlorination pattern.

Two groups of experiments were run using Hudson River sediment collected from a spot upstream of the PCB deposits (Spier Falls, Hudson River). The groups differed by the added congener; one group had 2,3,6-CB and the other group had 2,4,6-CB. After a 3-week lag time, 2,3,6-CB dechlorination to 2,6-CB was observed and dechlorination was >80% complete by 8 weeks. No dechlorination of 2,4,6-CB has been observed after 8 weeks of incubation with Spier Falls sediment. The dechlorination of 2,3,6-CB in Spier Falls sediment is surprising, since no dechlorination activity was observed in this sediment in the past when an Aroclor mixture was added [GE Report, 1989].

Dechlorination of Added Congener Within Silver Lake Sediment

A descriptive diagram of the dechlorination patterns for the added congeners in Silver Lake sediment is shown in Figure 1-1. This sediment can be described as a black mayonnaise with a high content of organic contaminants, including Aroclor 1254 and 1260. Dechlorination of the endogenous PCBs has been noted in the environment [Brown et al., 1987b] as well as in the laboratory [GE Report, 1989]. Dechlorination of five of the six added trichlorobiphenyls was observed in Silver Lake sediment. No dechlorination of 2,4,6-CB has been observed after over 8 months of incubation at room temperature. For each of the added congeners that were dechlorinated, only one chlorine was removed, with accumulation of the dichlorobiphenyl product. As seen in Hudson River sediment, the inner chlorine was preferentially removed (e.g., 2,4,5-CB goes to 2,5-CB). The lag time and rate of the added congener dechlorination varied. Dechlorination of 2,3,4-CB to 2,4-CB and 3,4,5-CB to 3,5-CB was observed by the first timepoint at 2 weeks and was >80% complete by 5 weeks (similar to the results in the Hudson River sediment). Lag times of 4 weeks for 2,4,5-CB dechlorination, 8 weeks for 2,3,6-CB dechlorination, and 14 weeks for 2,3,5-CB dechlorination were observed. For each of these congeners dechlorination was >80% complete by 5-7 weeks after the lag period ended. The dechlorination of the added congeners seemed to have little observable effect on the dechlorination of the endogenous PCBs.

Dechlorination of Added Congener Within Woods Pond Sediment

A descriptive diagram of the dechlorination patterns for the added congeners in Woods Pond sediment is shown in Figure 1-1. In several respects the dechlorination patterns were very similar to those observed within Silver Lake sediment. However, there were some notable exceptions. Most often, as was the case with Silver Lake sediment cultures, added trichlorobiphenyl was only dechlorinated to dichlorobiphenyl. The lag time for dechlorination

of 2,3,4-CB, 2,4,5-CB and 3,4,5-CB was 2-3 weeks, with >80% dechlorination of each congener by 4-5 weeks. Lag times of 5 weeks for 2,3,6-CB dechlorination and 12 weeks for 2,3,5-CB dechlorination were observed, with >80% dechlorination of each congener by 3-4 weeks after the onset of dechlorination. It was noted that 2,3,6-CB addition stimulated significant dechlorination of the endogenous PCBs in Woods Pond sediment. Until very recently, little natural dechlorination of the endogenous PCBs had been observed [GE Report, 1990]. Dechlorination of the endogenous PCBs in Woods Pond sediment has also been observed in the laboratory by Bedard and co-workers after the addition of a single congener.

Recently, it was discovered that 2,4,6-CB is being dechlorinated by Woods Pond sediment after a lag time of ~24 weeks. Most surprising was that both the *ortho* chlorines were being removed. Figure 1-3 shows mass spectra analysis after gas chromatography of the 2,4,6-CB culture at time zero days and after 220 days. *Ortho* dechlorination has previously been noted in Silver Lake sediment [Brown et al., 1987b] and in Woods Pond sediment after the addition of 2,3,5,6-CB [D. Bedard, personal communications].

An experiment was done with Woods Pond sediment to see if heating for a brief period of time (pasteurization) would selectively bring up a PCB-dechlorinating microbial population. Four serum vials containing Woods Pond sediment, RAMM mineral salts reduced with cysteine hydrochloride, and 350 μM 2,3,4-CB were prepared in the same fashion as described above. The four vials were heated in an 80°C water bath for 30 minutes and then kept stationary in the dark at 24°C. Two other serum vials were prepared in the same fashion, but without heating. Two of the four heated vials and the two unheated vials were sampled every 10 days starting at week 2. In the unheated experiments, 2,3,4-CB was dechlorinated to predominantly 2,4-CB with <10% 2,3-CB and >80% 2,4-CB by 5 weeks (lag time of ~2 weeks). In the heated vials, 2,3,4-CB was dechlorinated exclusively to 2,3-CB with >80% 2,3-CB by 10 weeks followed by further dechlorination to 2-CB (lag time of ~5 weeks). Figure 1-4 is a diagram of 2,3,4-CB dechlorination by heat-treated Woods Pond sediment.

Passage of PCB-Dechlorination Activity

Stable PCB dechlorinating cultures have been successfully passed in the laboratory. Dechlorination activity of PCB-contaminated sediments from the Hudson River, Woods Pond, and Silver Lake have been passed to a culture medium of Spier Falls sediment. The cultures were passed at 10% by volume to a medium containing dried river sediment from Spier Falls premixed with RAMM mineral salts reduced with 0.1% cysteine hydrochloride (2:3 ratio, volume:volume) and autoclaved for 3 hours. Passage of PCB-dechlorination activity to a river sediment medium previously uncontaminated with PCBs allows a more accurate quantitation of dechlorination activity. In Figure 1-5, a typical passage of activity from a Woods Pond culture to a medium containing Spier Falls sediment is depicted. Upon passage there was a decrease in the dechlorination lag time of about 1 week. A lag time of ~1-2 weeks for a 10% by volume passage was a typical result for passages from all three

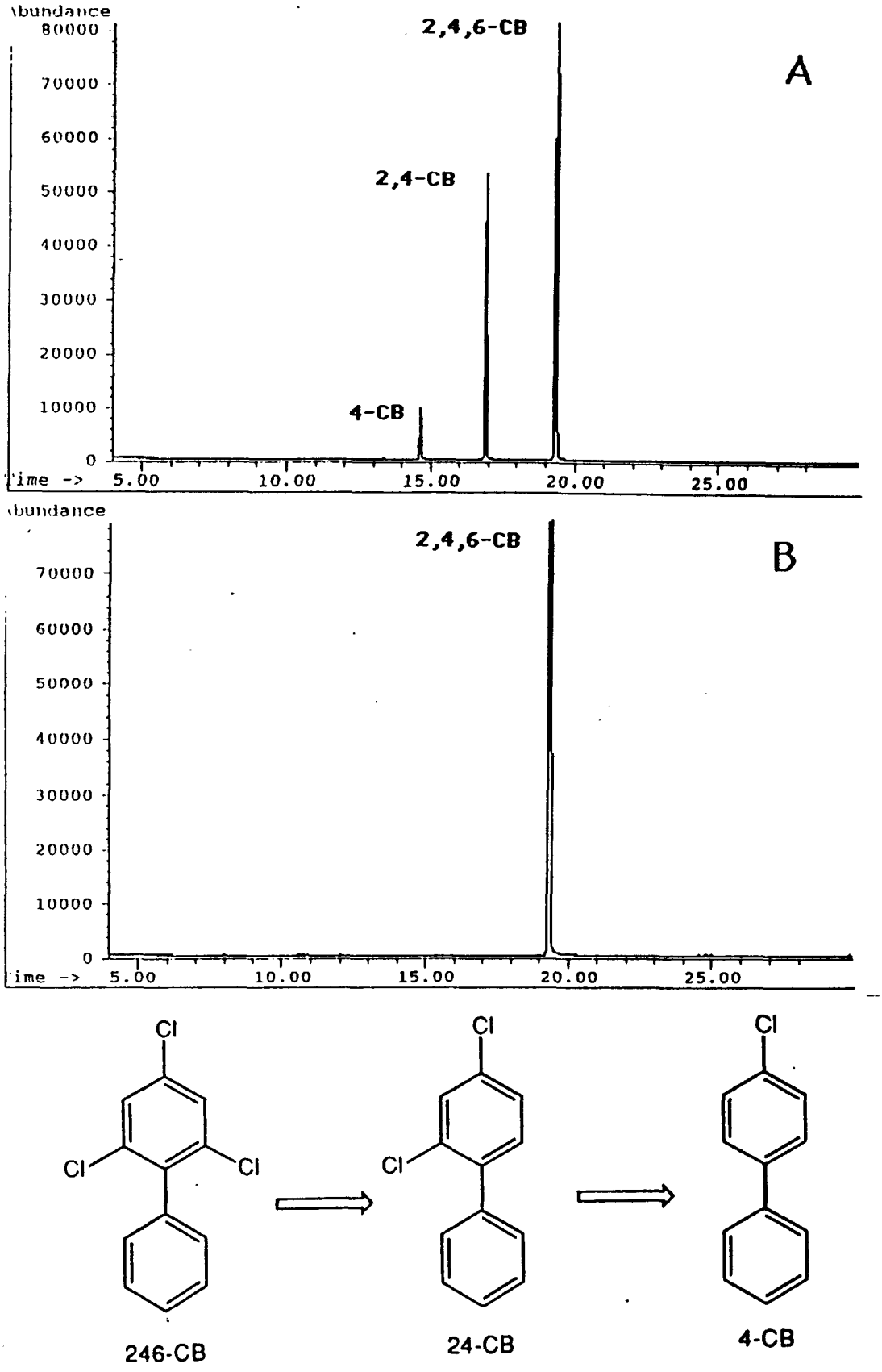

Figure 1-3. *Ortho* dechlorination of 2,4,6-CB added to Woods Pond sediment. Panel A shows ion selective mass spectra analysis after gas chromatography of the dechlorination products of 2,4,6-CB 220 days after addition of Woods Pond sediment. Panel B shows the same analysis of a sample of the experiment taken at time zero days. Below the spectra is a diagram of the dechlorination of 2,4,6-CB.

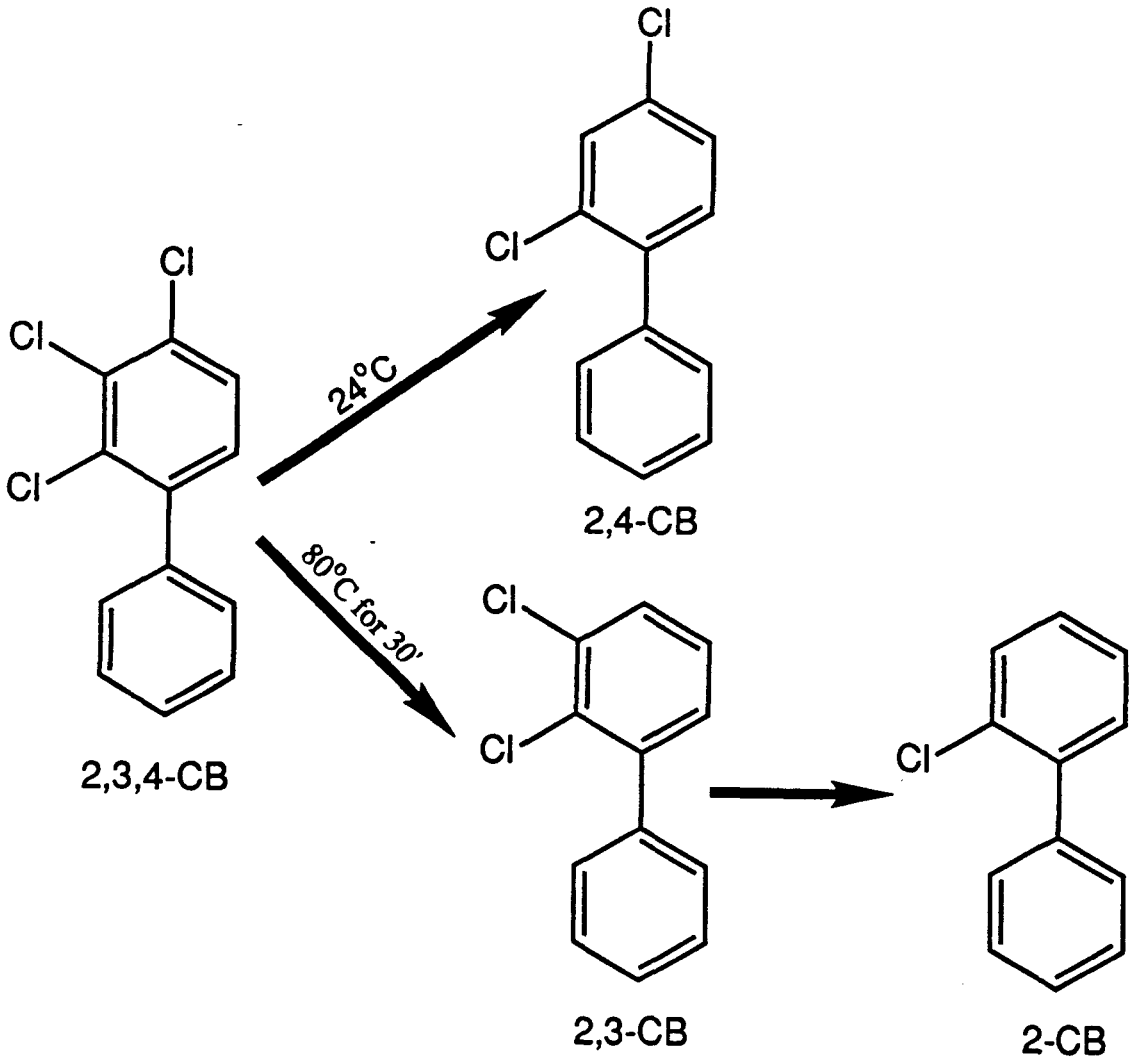

Figure 1-4. Differential dechlorination of added 2,3,4-CB by differential treatment of woods Pond sediment. Dechlorination of 2,3,4-CB added to Woods Pond sediment and incubated at 24°C resulted in conversion to 2,4-CB. A different dechlorination pattern resulted when 2,3,4-CB added to Woods Pond sediment was incubated at 80°C for 30 minutes followed by incubation at 24°C.

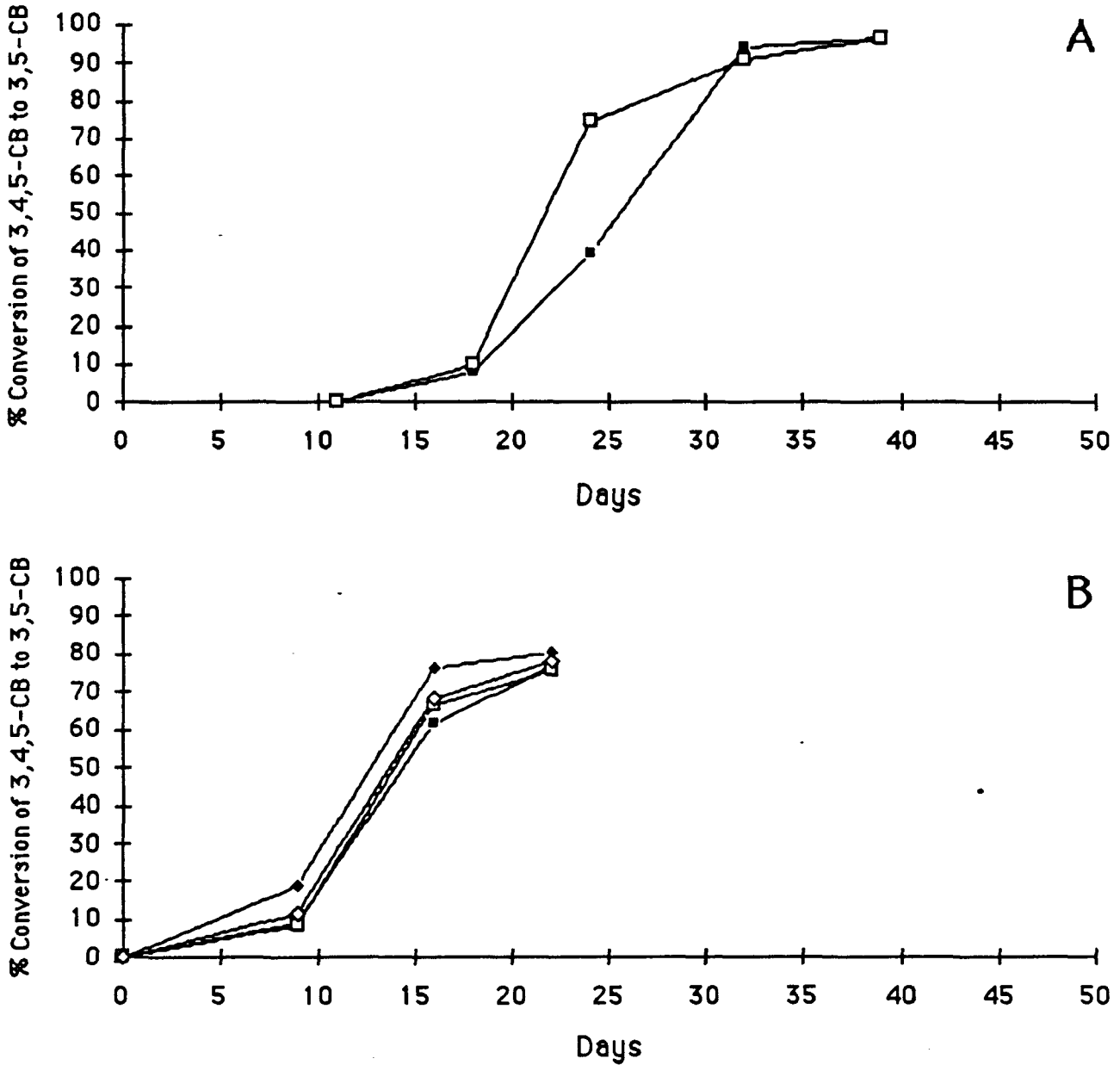

Figure 1-5. Passage of PCB reductive dechlorination activity. Panel A shows dechlorination activity on added 3,4,5- CB in Woods Pond sediment. Panel B describes the same dechlorination activity as shown in Panel A, after a 10% by volume pass from Woods Pond sediment to a Hudson River sediment medium which previously was uncontaminated with PCBs. The multiple curves represent duplicate samples.

sediments. The minimum lag time for passages originating from any of the three PCB-contaminated sediments was ~1 week. Successful multiple passes have been done starting with PCB-contaminated Hudson River sediment and carried with each of the added trichlorobiphenyls. This has resulted in a >1000-fold dilution of the original sediment [M. Brennan, personal communications].

SUMMARY AND CONCLUSIONS

The dechlorination rate of an added trichlorobiphenyl within a river sediment is rapid as compared to the Aroclors. Generally, in cultures incubated at room temperature the pattern of trichlorobiphenyl dechlorination resulted in removal of the inner chlorine first. Microorganisms in the Hudson River which are located in PCB-contaminated sediment can mediate reductive dechlorination of all six trichlorobiphenyls to either biphenyl or *ortho* chlorine-containing biphenyls. The addition of a single congener to a PCB-contaminated river sediment will stimulate dechlorination of the endogenous PCBs, with different congeners stimulating different dechlorination patterns. Microorganisms in Hudson River sediment which is not contaminated with PCBs can mediate dechlorination of 2,3,6-CB to 2,6-CB. This implies that extended exposure of a microbial population to PCBs may not be necessary to bring about microbial PCB-reductive dechlorination.

Microorganisms in Woods Pond and Silver Lake mediate reductive dechlorination of five of the six added trichlorobiphenyls to dichlorobiphenyls. Dechlorination of 2,4,6-CB in Woods Pond sediment has also been observed after long incubation periods. Since Woods Pond and Silver Lake sediments contain more highly chlorinated PCBs (Aroclor 1254 and 1260) than does Hudson River sediment, the active PCB-dechlorinating microbial populations may not have the reducing power necessary to mediate dechlorination of a dichlorobiphenyl to a monochlorobiphenyl. Heat treatment of Woods Pond sediment will bring up different dechlorination patterns of the same trichlorobiphenyl. A PCB-dechlorinating microbial population has been brought up which is either resistant to or activated at a temperature of 80°C. This suggests a spore former is present.

FUTURE PLANS

Since successful passage of PCB-dechlorination activity has been achieved, an effort is currently underway to chemically fractionate the Spier Falls sediment to determine the necessary nutrients for dechlorination. Another effort is underway to distinguish cultures brought up at different temperatures. Considerable interest has been taken in the *ortho* declorinating population brought up on 2,4,6-CB in Woods Pond sediment. Can this activity be passed to PCB-contaminated Hudson River sediment where >90% of the PCBs are *ortho* chlorine containing mono-, di- or trichlorobiphenyls [M. Stephens, personal communications]? All of these efforts are aimed at the enrichment, isolation, and characterization of microorganisms capable of mediating reductive dechlorination of PCBs.

Chapter 2

REDUCTIVE DECHLORINATION OF AROCLORS BY ANAEROBIC MICROORGANISMS

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INTRODUCTION

During the past year our research on PCB dechlorination has advanced on several fronts. We have devoted a major effort to the dechlorination of the more heavily chlorinated Aroclors, especially Aroclor 1260. We have examined the dechlorinating capabilities of Silver Lake microorganisms which were environmentally exposed to Aroclor 1260, and attempted to enhance Aroclor 1260 dechlorination by mixing inocula from different sources. We have also determined that the limited ability of Hudson River microorganisms to dechlorinate Aroclor 1260 is not likely due to inhibition by any Aroclor 1260 components. We have also begun to quantify the toxicity reduction that occurs as a result of anaerobic PCB dechlorination, investigated the bioavailability of PCBs in a contaminated soil, and determined the suitability of alternatives to the natural sediments we normally use in our dechlorination assays.

RESULTS

Aroclor Survey

Microorganisms from site F3 in Silver Lake [Brown et al., 1984] were found to dechlorinate all four Aroclors (1242, 1248, 1254, and 1260) tested. Dechlorination of Aroclors 1242 and 1248 was first evident after 4 weeks of incubation, but leveled off shortly thereafter with approximately one *meta* plus *para* chlorine remaining (Figure 2-1). In this respect dechlorination was inferior to that typically obtained with Hudson River microorganisms, which leave as few as 0.2 *meta* plus *para* chlorines when dechlorinating Aroclor 1242 [GE Report, 1989; Quensen et al., 1990]. This difference in performance can be understood from a comparison of dechlorination patterns. The Hudson River microorganisms removed chlorines from both *meta* and *para* positions with the result that the primary dechlorination products (2-CB, 2-2-CB and/or 26-CB, 26-2-CB and 26-26-CB) were substituted in only the *ortho* position(s). The Silver Lake microorganisms, however, removed chlorines primarily from the *meta* positions with the result that *para* substituted products (especially 2-4-CB, 24-2-CB, 24-4-CB and 24-24-CB) accumulated in addition to *ortho*-only substituted products. The Silver Lake microorganisms, however, dechlorinated Aroclor 1260 sooner and more extensively than had previously been observed for Hudson River microorganisms [GE Report, 1989; Quensen et al., 1990]. Thus they seem capable of more extensively dechlorinating the

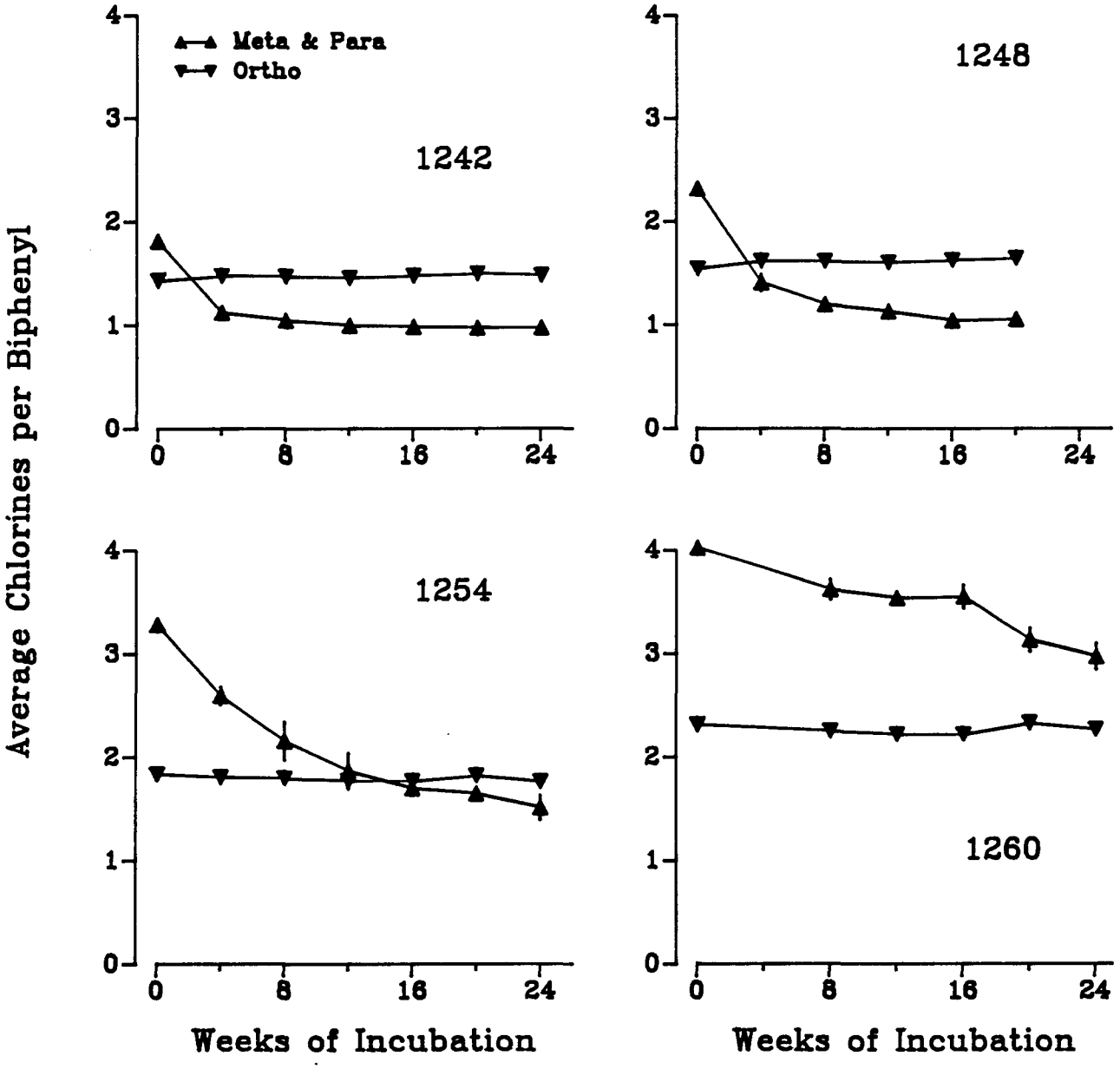

Figure 2-1. Decrease in the average number of chlorines by position for four Aroclors.

more heavily chlorinated PCB congeners. The extent of dechlorination by Silver Lake microorganisms for the congeners represented by each chromatographic peak is given in Table 2-1.

Mixed Inocula

An attempt was made to affect greater overall dechlorination of Aroclor 1260 by using a mixture of Hudson River and Silver Lake microorganisms. The results of the Aroclor surveys [above and GE Report, 1989] demonstrated the complementary nature of the dechlorination activities of microorganisms from these two sources. The Silver Lake organisms more quickly and extensively dechlorinated Aroclor 1260 but left *para* substituted products that should be readily dechlorinated by Hudson River microorganisms. Thus we expected that a mixture of the two organisms should give enhanced dechlorination of Aroclor 1260.

Surprisingly, this did not prove to be the case. The extent of dechlorination of Aroclor 1260 by a mixture of organisms from these two sources was not much greater than for Hudson River microorganisms alone (Table 2-2). An examination of the dechlorination patterns obtained for each treatment suggested that it was primarily the Silver Lake microorganisms that were responsible for dechlorination in the mixed inoculum. These results may be due to competition between the dechlorinating microorganisms from the two sites. Perhaps the Hudson River microorganisms in the mixed inoculum depleted some limiting factor, thereby inhibiting dechlorination by the Silver Lake microorganisms, and at the same time contributed little to overall dechlorination themselves. It was not readily apparent that the Hudson River microorganisms dechlorinated any products from the Silver Lake microorganisms.

TABLE 2-2. Average number of chlorines removed from Aroclor 1260 over time by Hudson River (HR) and Silver Lake (SL) microorganisms alone and in combination.

	Source of Inoculum		
Week	HR	SL	HR & SL
8	0.04	0.14	0.07
16	0.20	0.76	0.19
24	0.24	1.22	0.53

Mixed Aroclors

Because the Hudson River microorganisms dechlorinate Aroclor 1260 so much more slowly than the lesser chlorinated Aroclors, we wondered if any of the PCB congeners present in Aroclor 1260 inhibited dechlorination by these organisms. To investigate this possibility we compared the extent of dechlorination over time for Aroclors 1242 and 1260 added separately and in combination. To allow a determination of quantitative recovery, these experiments

TABLE 2-1. Net decrease (average % for 3 replicates) of each chromatographic peak representing more than 0.1 mole % of each Aroclor after 20 weeks of dechlorination by Silver Lake microorganisms.

		Aroclor			
Peak	Structure	1242	1248	1254	1260
1	2	+[a]	+	+	+
2	4				
3	2-2 26	+	+	+	+
4	24 25	+	+	+	
5	2-3	45	+	+	
6	2-4 23	+	+	+	
7	26-2	+	+	+	+
8	34 3-4	+	+		
9	25-2 4-4	94	86	0	+
10	24-2	+	+	+	+
11	26-3 236	+	+	+	+
12	23-2 26-4	10	+	+	+
13	35-2 235 26-26	+	+	+	
14	245	78	+	+	+
15	25-3	+	+	+	
16	24-3	+	+	+	+
17	25-4	+	+	+	
18	24-4 246-2	+	+	+	+
19	34-2 23-3 234 25-26	91	76	+	+
20	23-4 24-26	87	91		
21	236-2	88	97		
22	23-26	84	76	+	+
23	25-25 26-35	79	82	59	+
24	24-25	68	66	+	+
25	24-24	31	24	+	+
26	245-2 246-4	nr	nr	nr	nr
27	34-3				+
28	23-25	93	96	93	
29	23-24 236-3 34-4	89	90	11	
30	26-34 234-2 236-4 25-35	88	96	53	+
31	236-26		89		
32	23-23	85	85	+	+
33	245-3 246-24 235-3 246-25	92	63	+	+
34	23-35 235-4	+	+	+	
35	245-4 235-26	97	95	81	+
36	25-34 345-2	98	96	94	
37	236-25 245-26 24-34	93	93	89	70
38	236-24 234-3	57	88	85	+
39	23-34 234-4 236-23 235-25	93	91	67	+
40	245-25 235-24	82	82	81	21
41	245-24	95	91	89	+
42	236-246 2356-3 246-34				

[a] + indicates a net increase
nr = not resolved

TABLE 2-1 (Cont'd)

Peak	Structure	Aroclor 1242	1248	1254	1260
43	245-23 2345-2 2356-26	97	91	91	24
44	234-25 2346-4	89	89	86	54
45	234-24	96	94	92	
46	236-236			79	60
47	34-34 236-34	84	92	93	64
48	2356-25	86	86	85	50
49	235-236 345-25 2346-25	66	80	84	57
50	245-34 236-245 2345-3	33	+		75
51	2356-23 2345-26 2345-4			53	49
52	345-23 2346-23 235-235		91	89	65
53	235-245 2356-35	76	80	43	
54	245-245	71	76	78	50
55	234-236 234-34	87	90	90	59
56	2345-25			81	54
57	2356-236			61	40
58	2345-24		91	80	0
59	2346-236			82	47
60	234-245 2356-34	52	67	80	51
61	2346-34		71	81	46
62	2356-235			79	34
63	2346-235			88	52
64	2356-245 2345-246		31	58	29
65	2346-245			67	35
66	245-345			86	53
67	23456-25				42
68	2345-236 23456-24			68	38
69	2356-234			67	39
70	2346-234 2345-34 2356-2356			78	36
71	2346-2356 23456-246 23456-23			80	32
72	2345-235 23456-35			66	33
73	2345-245			62	47
74	2356-345				23
75	2346-345				
76	23456-236				29
77	2345-234			64	34
78	23456-34				19
79	2356-2345			23	15
80	2345-2346 23456-245			44	18
81	2345-345				
82	23456-234				19
83	23456-2356				
84	2345-2345				
85	23456-345				14
86	23456-2345				
87	Internal Standard (Octachloronaphthalene)				
88	23456-23456				

[a] + indicates a net increase

nr = not resolved

were performed using Balch tubes and a tube was sacrificed for each observation. Two μmole equivalents of Aroclor 1242 or 1260 were added per tube to the treatments receiving only one Aroclor. Two μmole equivalents of each Aroclor (1242 and 1260) were added to the treatment receiving both Aroclors.

There was no evidence that the presence of Aroclor 1260 inhibited dechlorination by the Hudson River microorganisms. The amount of chloride released was additive in the treatment receiving both Aroclors (Table 2-3). It appears that the dechlorination of the Aroclor 1260 congeners by these organisms is inherently slower than that of lesser chlorinated congeners. This is the opposite of what is to be expected from a purely thermodynamic standpoint and suggests that rate of congener uptake by the microorganisms and/or enzyme specificity strongly influence(s) the rate of dechlorination of specific PCB congeners.

TABLE 2-3. Comparison of the extent of dechlorination over time of Aroclors 1242 and 1260 separately and in combination by Hudson River microorganisms.

	μgm Atoms Cl^- Removed from Aroclor		
Weeks	1242	1260	1242 & 1260
8	1.50	0.04	1.40
16	1.74	0.30	2.24
24	2.40	0.72	3.19

Toxicity Reduction

The most toxic of the PCB congeners are generally considered to be the coplanar congeners 34-34-CB, 345-34-CB, and 345-345-CB. In a coplanar configuration, these congeners are structurally similar to 2378-tetrachlorodibenzodioxin (TCDD) and exhibit similar toxicity effects. PCB congeners like these but with a single *ortho* chlorine also have similar toxicity effects but are much less potent.

Because these toxicologically important PCB congeners coelute with other PCBs, it is not possible to directly determine the decrease in their concentrations effected by reductive dechlorination using conventional gas chromatography (GC) with an electron capture detector. Recently, Lopshire and Enke have developed a sensitive GC/MS/MS technique to directly quantify these toxic PCB congeners [Chapter 14, this report]. This new method allowed us to determine the percent reduction of each of the toxic congeners after a 16 week incubation of Aroclor 1242 with the Hudson River microorganisms (Table 2-4).

TABLE 2-4. *Percent reduction in concentrations of toxic isomers in Aroclor 1242 as a result of 16 weeks of dechlorination by Hudson River microorganisms under laboratory conditions.* See Lopshire and Enke, Chapter 14, for analytical methodology.

Congener	% Reduction
34-34-CB	>96.5
345-34-CB	83.3
345-345-CB	>90.0
245-34-CB	80.0
234-34-CB	85.8
2345-34-CB	46.6

The dioxin-like toxicity of compounds has been correlated with their potential to induce P_{450} enzymes such as aryl hydrocarbon hydroxylase (AHH) and ethoxy resorufin O-deethylase (EROD) and the toxicities of various PCB congeners have been estimated based on their potential to induce these enzymes [Safe, 1987; Sawyer and Safe, 1982]. Using such toxicity estimates we calculated an 85% reduction in toxicity in 16 weeks as a result of the dechlorination of Aroclor 1242 by Hudson River microorganisms.

EROD induction assays were performed on the PCB extracts from live and autoclaved treatments to directly determine the toxicity reduction resulting from 16 weeks of dechlorination of Aroclor 1242. A 75% reduction was determined by this method, in good agreement with our calculations.

Bioavailability

Bioavailability to microorganisms is of special concern to the implementation of a bioremediation process for PCB destruction. The PCB residues in contaminated soil or sediment may not be as available as the PCBs freshly added in our dechlorination assays. It is commonly believed that over long periods of time PCBs diffuse into soil or sediment particles, that only aqueous phase PCBs can be biotransformed, and that diffusion into the aqueous phase from inside particles is likely to be slow and rate limiting.

We conducted dechlorination assays to assess the availability of PCBs in Aroclor 1242 contaminated Glens Falls dragstrip soil to dechlorinating microorganisms. We compared the amount of dechlorination of selected late eluting congeners in the contaminated soil to the dechlorination of freshly added 23456-CB. A parallel assay in which Aroclor 1242 and 23456-CB were added to clean sediment was also conducted for comparison.

The results did suggest that the PCBs in the contaminated soil were less available than freshly added PCBs. When standardized on the amount of 23456-CB dechlorination that occurred in the two assays, less of the 25-34-CB and/or 345-2-CB (coeluting isomers) in the

dragstrip soil was dechlorinated (Table 2-5). However, overall dechlorination activity was much greater in the dragstrip soil assay with the result that 84% of the 25-34-CB/2-345-CB was dechlorinated compared to only 31% of the 25-34-CB/2-345-CB added to the clean sediments. In this case at least, substantial dechlorination of environmental PCB residues can occur under appropriate conditions and bioavailability does not appear to be a limiting factor.

TABLE 2-5. Dechlorination of selected congeners in a bioavailability experiment with Glens Falls dragstrip soil (GFDS) during 16 weeks of incubation.

Treatment	Congener	Initial Amount (nmoles/tube)	Amount Dechlorinated (nmoles/tube)	% Dechlorinated
GFDS	25-34/2-345-*	51	43	84
	23456-	90	62	69
Clean Sediment	25-34/2-345-*	72	22	31
	23456-	91	12	13

* Present from the historical PCB contamination; the other PCBs were freshly added

Solid Supports

We have investigated the effect of using several alternatives to the sediments normally included in our dechlorination assays. We have found that little or no dechlorination occurs in the absence of sediments. Sand, sawdust, clay, or vermiculite substituted for sediments did not support dechlorination. Dechlorination was observed, however, in the presence of peat and mixtures of peat (20% by weight) with sand or vermiculite or clay. Of these alternative solid supports, mixtures of peat with vermiculite or clay gave the best results, but were inferior to natural sediments. These experiments were repeated with several of the alternative solid supports. In addition, Aroclor 1242 was added in either acetone or as an emulsion with ligno sulfonate [Liu, 1980]. This time the peat/vermiculite mixture gave results comparable to natural sediments (Table 2-6), while the method of PCB addition had no apparent effect.

The difference in the extent of dechlorination obtained with each of these alternative supports was related to the dechlorination pattern that occurred. Three of the dechlorination patterns described by John Brown [GE Report, 1989] are M (predominantly *meta* dechlorination), Q (predominantly *para* dechlorination), and C (both *meta* and *para* dechlorination).

TABLE 2-6. Average number of chlorines removed from Aroclor 1242 by Hudson River microorganisms in 25 weeks.

Solid Support	Aroclor 1242 Added in Acetone	Aroclor 1242 Added in Ligno Sulfonate
Sediments	1.3	1.4
No Support	---	0.0
Peat	0.6	0.6
Peat/Vermiculite	1.2	1.1
Peat/Clay	0.5	0.8

The treatments with sediments and peat/vermiculite mixtures began as Pattern M and became Pattern C. The peat treatment yielded only Pattern Q, and the peat/clay mixture yielded only Pattern M. Patterns were consistent among all replicates within treatments. These alternative supports may select for different subsets of dechlorinating strains initially present in the PCB-contaminated sediments, but the mechanism of selection is unclear.

SUMMARY AND CONCLUSIONS

1. In laboratory assays, microorganisms eluted from Silver Lake sediments dechlorinated all four Aroclors tested (1242, 1248, 1254, and 1260), but the rate and extent of dechlorination was less for Aroclor 1260. Still, these microorganisms proved more capable of dechlorinating Aroclor 1260 than Hudson River microorganisms. In these assays the Silver Lake dechlorinators accumulated *ortho* and *para* substituted products in addition to the *ortho*-only substituted products so typical of the Hudson River dechlorinators.
2. Despite the complementary dechlorination activities of the Hudson River and Silver Lake microorganisms, mixing them together did not enhance overall Aroclor 1260 dechlorination. It is possible that competition between organisms in the two sediments limited dechlorination.
3. The limited ability of Hudson River microorganisms to dechlorinate Aroclor 1260 is probably not due to inhibition by any of the more heavily chlorinated congeners present in Aroclor 1260.
4. The PCB congeners in Aroclor 1242 with greater dioxin-like toxicity were effectively dechlorinated despite their low concentrations in this PCB mixture. EROD induction potency, which correlates with dioxin-like toxicity, was also shown to decrease as a result of PCB dechlorination.
5. Under appropriate environmental conditions, substantial dechlorination of PCB residues in contaminated soils can be achieved despite somewhat decreased bioavailability.

This may be because the generally longer incubation times required for anaerobic dechlorination also allows more time for the PCBs to become available.

6. Of the alternative solid supports investigated, peat mixed with vermiculite gave the best results. The results suggest that both mineral surfaces and organic matter are required for optimal dechlorination activity.

FUTURE PLANS

Our proposed objectives for the coming year are:

1. To compare the toxicity reduction of different Aroclors as a result of dechlorination by inocula giving different dechlorination patterns, and to estimate the extent of detoxification that has occurred as a result of *in situ* dechlorination at different sites. Toxicity reduction will be assessed by quantifying the congeners with dioxin-like toxicity and by EROD induction assays. Additional toxicity assays may also be adopted.
2. To continue to look for microorganisms more capable of Aroclor 1260 dechlorination using different sediments and different incubation conditions. The dechlorination pattern obtained in assays with Silver Lake microorganisms is more limited than has apparently occurred *in situ*, implying only a subset of the organisms present in the sediment is active under our present incubation conditions.
3. To continue our bioavailability experiments using other contaminated soils and sediments. PCB desorption rates will also be determined directly for each.
4. To continue our attempts to identify the physiological group of microorganism(s) responsible for PCB dechlorination and isolate pure cultures.

Chapter 3

ESTABLISHMENT AND CHARACTERIZATION OF AN ANAEROBIC, AROCLOR 1242-DECHLORINATING CULTURE

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INTRODUCTION

Anaerobic enrichment cultures were established as a first step in obtaining pure cultures or stable consortia capable of reductively dechlorinating PCBs. The objective was to determine conditions which allow dechlorination activity as well as growth of the responsible organisms. The bases for conditions tested were previous studies of anaerobic dechlorination of PCBs and of a pure culture which dehalogenates benzoates (strain DCB-1) [Quensen et al., 1988; Mohn and Tiedje, 1990a and b; DeWeerd et al., 1990, in press]. Cosubstrates were included with PCBs to serve several possible functions, including (1) providing reducing potential for dechlorination, (2) providing carbon and energy for dechlorinating organisms, and (3) supporting other organisms which might establish and maintain an environment favorable for dechlorination. Aroclor 1242 was added at a concentration adequate to support significant growth of any organisms capable of utilizing PCBs as catabolic electron acceptors (1 mg/g dry sediment).

RESULTS

Test of Cosubstrates

Initially four conditions were tested (1) no cosubstrate (except any provided by the uncontaminated sediment), (2) formate, (3) formate plus bromoethanesulfonate to prevent electron flow to methanogenesis, and (4) pyruvate. The former three were in bicarbonate-buffered medium to avoid CO_2 as a potential electron acceptor. The cultures with no cosubstrate and those with pyruvate had the highest dechlorination rates. In transfers of those two treatments, those with pyruvate had much higher dechlorination rates (or shorter lag periods) than those with no cosubstrate (Figure 3-1). Pyruvate therefore stimulates dechlorination activity and greatly improves transferability of dechlorination activity.

Serial Transfers

Dechlorination activity has now been maintained through eight monthly transfers on pyruvate medium. This medium contains RAMM mineral salts, Wolin and DeWeerd vitamins,

[DeWeerd et al., 1990, in press], $NaHCO_3$ (30 mM), cysteine (1 mM), titanium citrate (0.1 mM) and pyruvate (20 mM). Aroclor 1242 was added at a concentration of 1 mg/g dry sediment. A 2:1 (v:w) ratio of liquid medium to clean Spier Falls sediment was used in serum bottles. The transfer technique involved shaking the serum bottle, letting the sediment settle for ~ 10 minutes, and removing the supernatant for inoculum. The two month lag before dechlorination in the primary enrichments was not observed in subsequent transfers on pyruvate-containing medium. All inoculum transfers were 20%, but only slightly lower activity was observed with a 1% inoculum transfer. Thus, the dechlorinating microorganisms appear to grow in these enrichment cultures. Dechlorination occurred primarily from the *meta* position, and resembled Pattern M previously observed (Figure 3-2) [GE Report, 1989].

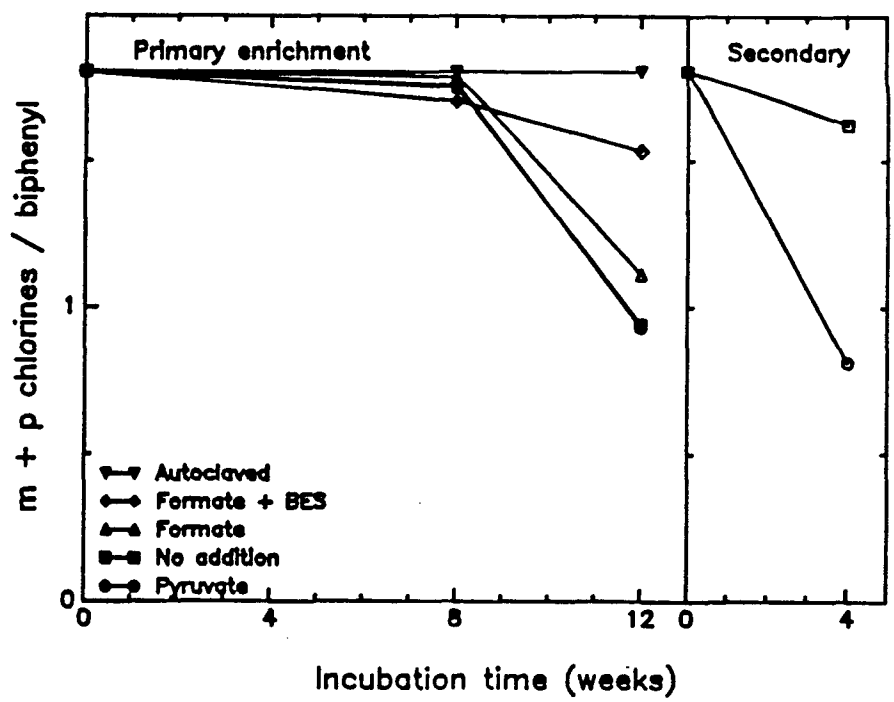

Figure 3-1. Primary and secondary enrichment cultures .

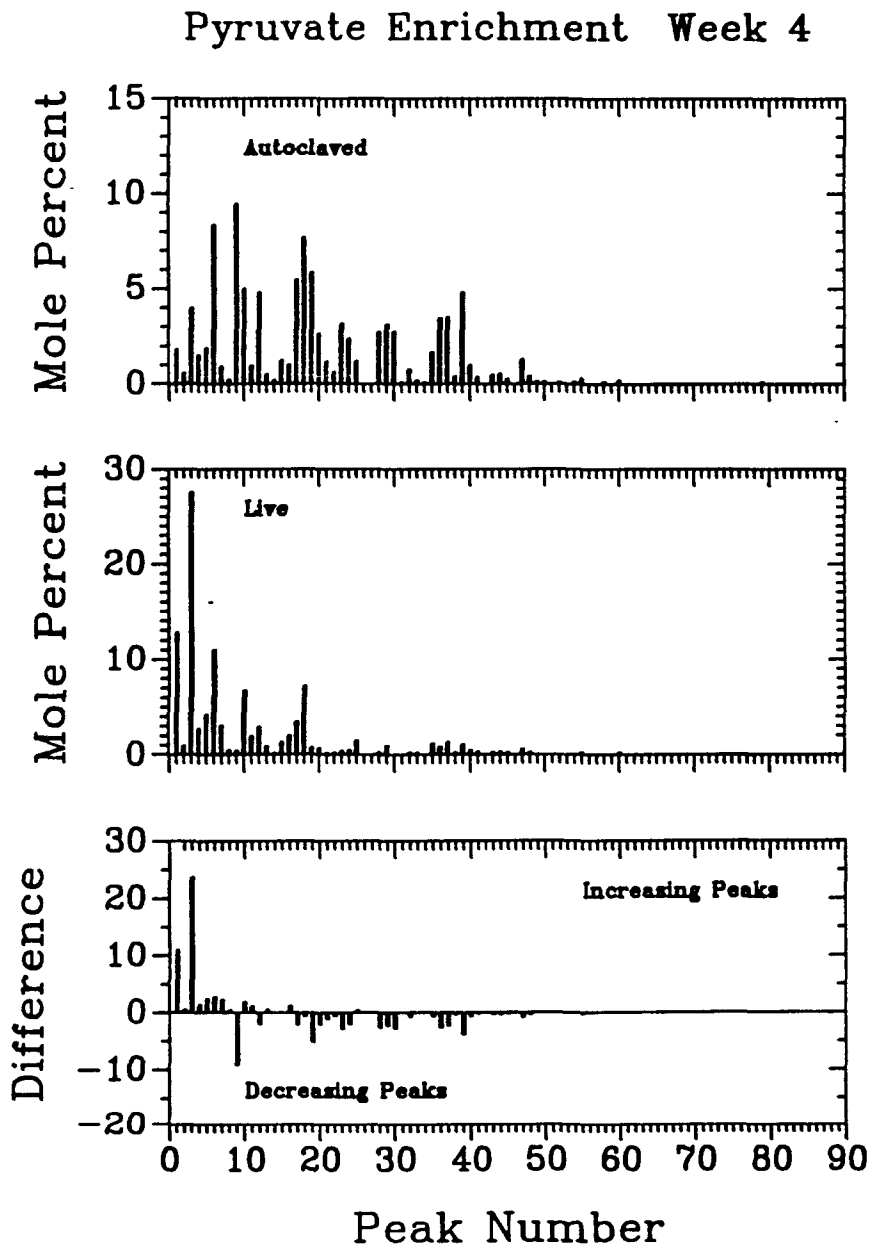

Figure 3-2. Aroclor 1242 dechlorination pattern by pyruvate enrichment at week 4. (Refer to Table 3-1, this report for peak number assignments).

Pyruvate Metabolism

Pyruvate was rapidly consumed by cultures (within 1 week), acetate was a transient product, and methane and CO_2 were final products (Figure 3-3). Dechlorination began simultaneously with the onset of methane production (during week 2 of incubation). Pyruvate was not required for activity but reduced the acclimation period preceding dechlorination. The requirement of the dechlorinating community for reducing potential is apparently not highly specific, as pyruvate could be replaced with H_2 or acetate (the latter supported a somewhat lower dechlorination rate) (Figure 3-4).

Sediment Studies

In all of our studies, uncontaminated Spier Falls sediment was included in the medium, and was required for dechlorination activity. Sediment could not be replaced by defined materials, such as sand and synthetic organoclay, Chromosorb, or a hexadecane phase above the culture. Particle size analysis of the sediment yielded a 90% sand and 10% silt plus clay content. In order to understand which fraction of the sediment was important in supporting the dechlorinating consortium, the sediment was separated into crude fractions. The sediment fractions were separated by sonication and wet-sieving, and the sediment, sediment sand fraction, and sediment silt plus clay fraction were treated with 30% H_2O_2 to remove organic components (Table 3-1). The silt plus clay fraction contained ~ 10 times more organic carbon by weight than the sand fraction. Since 10% of the sediment was silt plus clay, only 2.5 g of this fraction was added to bottles, compared to 22.5 g of the sand fraction. Peroxidized sediment and sediment fractions did not support dechlorination (Figure 3-5). Additionally, varying ratios of sediment to liquid medium were tested (Figure 3-6). A minimal ratio of sediment to liquid medium (>10 g sediment/50 mL medium) was required to support dechlorination.

TABLE 3-1. Total carbon content of Spier Falls sediment and sediment fractions following peroxidation.

Sediment Fraction	% Total Carbon
sediment	1.47
sediment (H_2O_2 treatment)	0.28
sand	0.72
sand (H_2O_2 treatment)	0.21
clay + silt	8.09

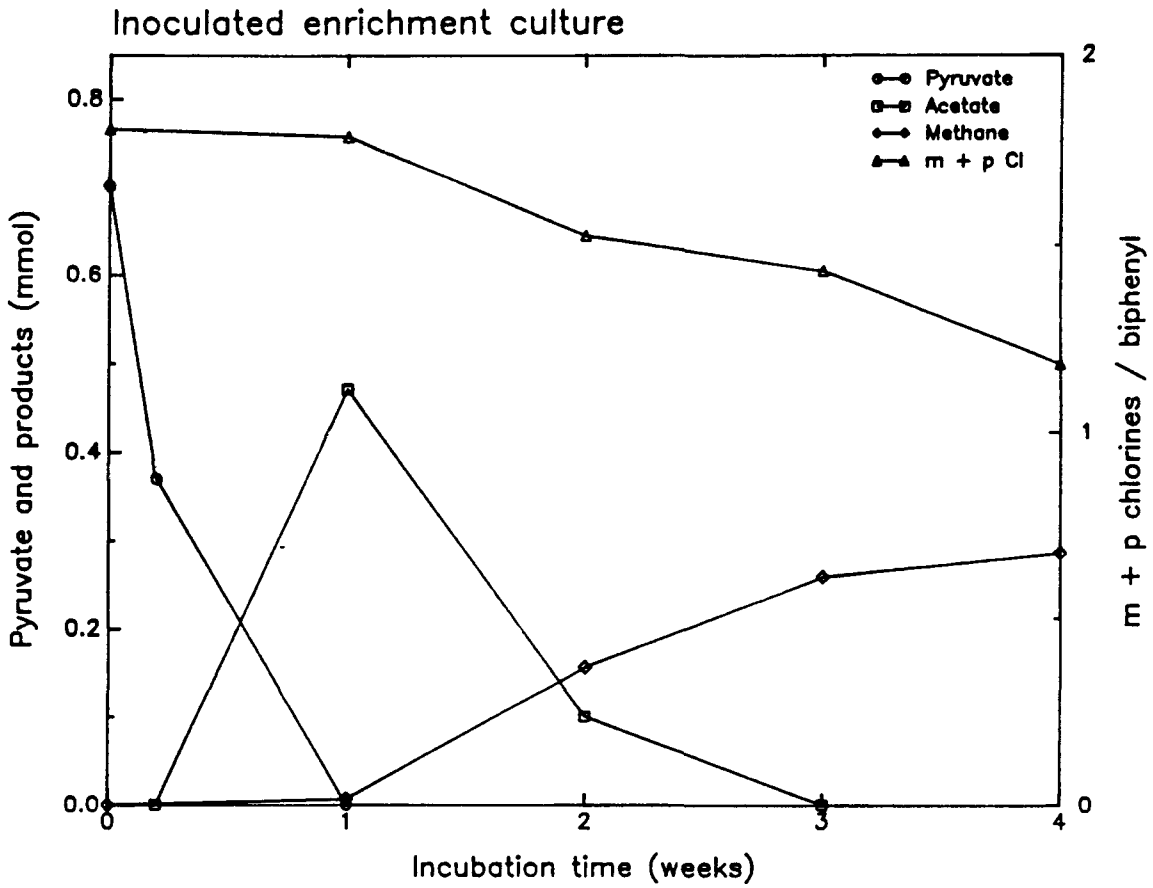

Figure 3-3. Metabolism of pyruvate, methane production, and dechlorination of Aroclor 1242 by anaerobic pyruvate enrichment culture in 4 weeks.

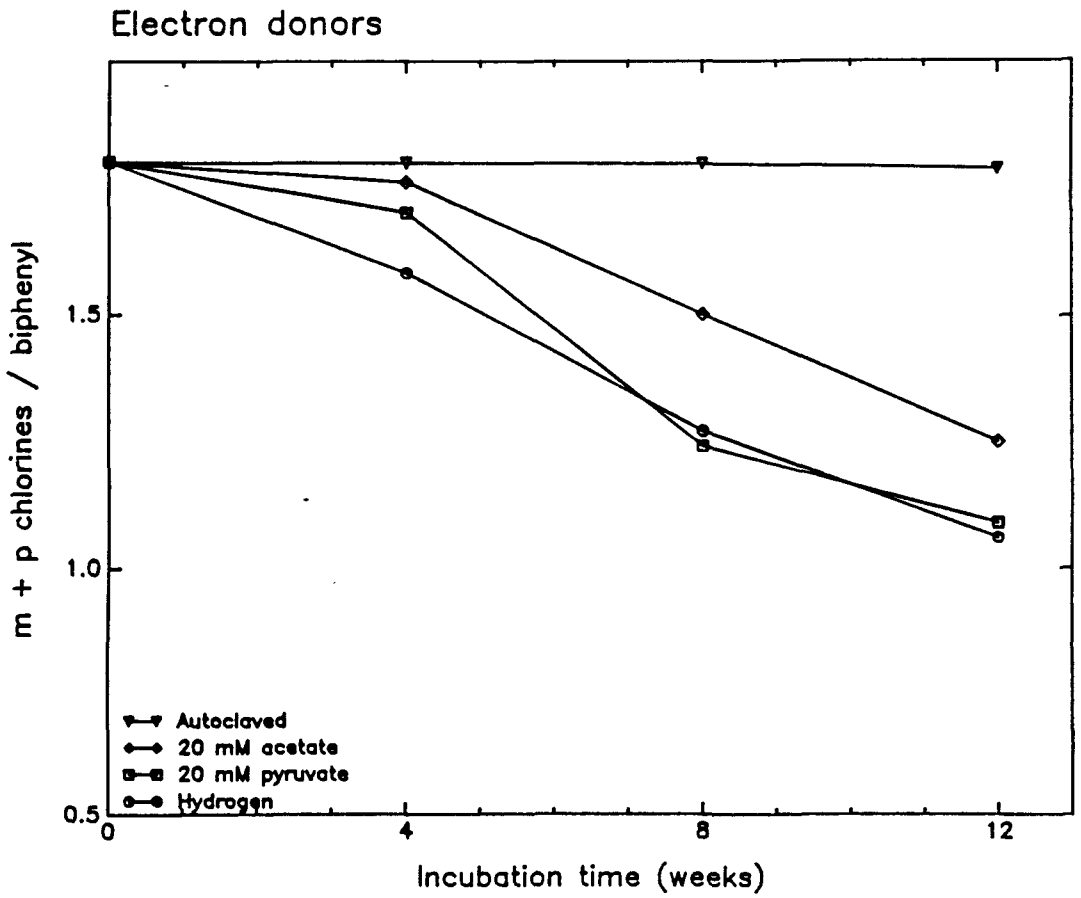

Figure 3-4. Effect of selected electron donors on reductive dechlorination.

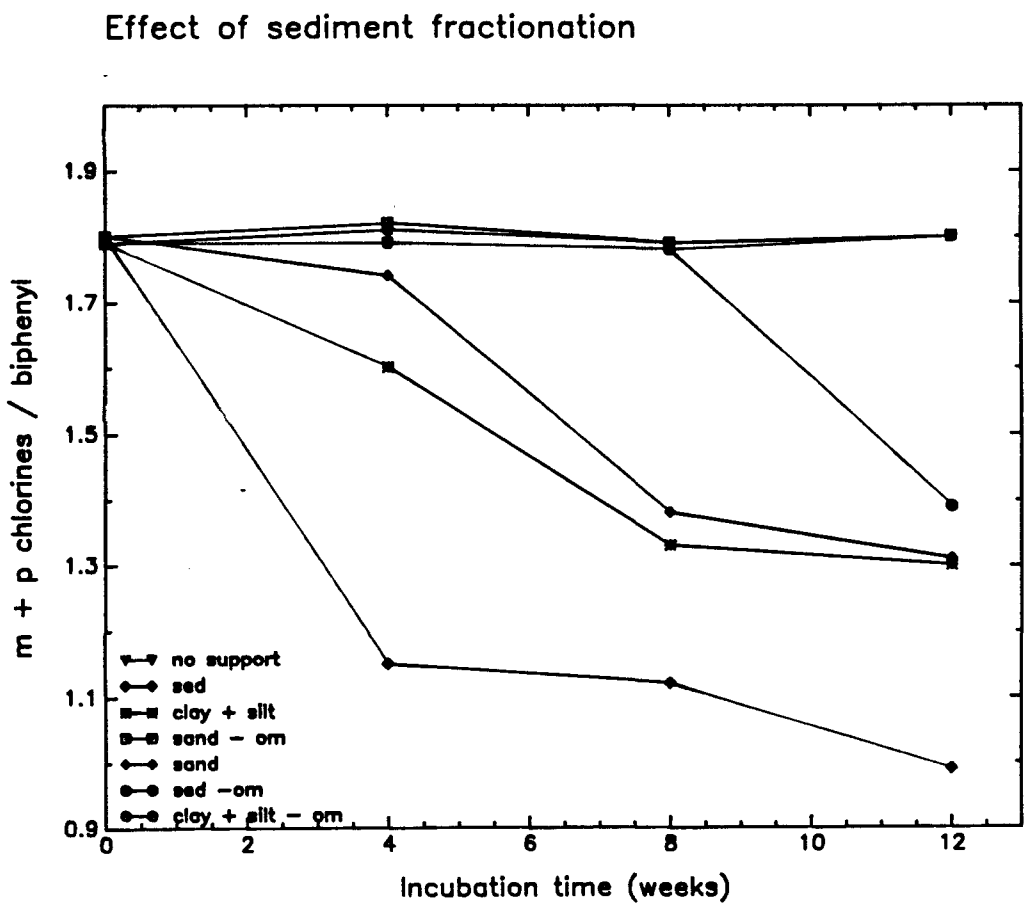

Figure 3-5. Effect of sediment fractionation and peroxidation on dechlorination activity. Treatments included no solid support (50 mL medium), Spier Falls sediment (25 g/50 mL medium), peroxided sediment (25 g/50 mL medium), clay plus silt fraction (2.5 g/50 mL medium), peroxided clay plus silt fraction (2.5 g/50 mL medium), sand fraction (22.5 g/50 mL medium), and peroxided sand fraction (22.5 g/50 mL medium).

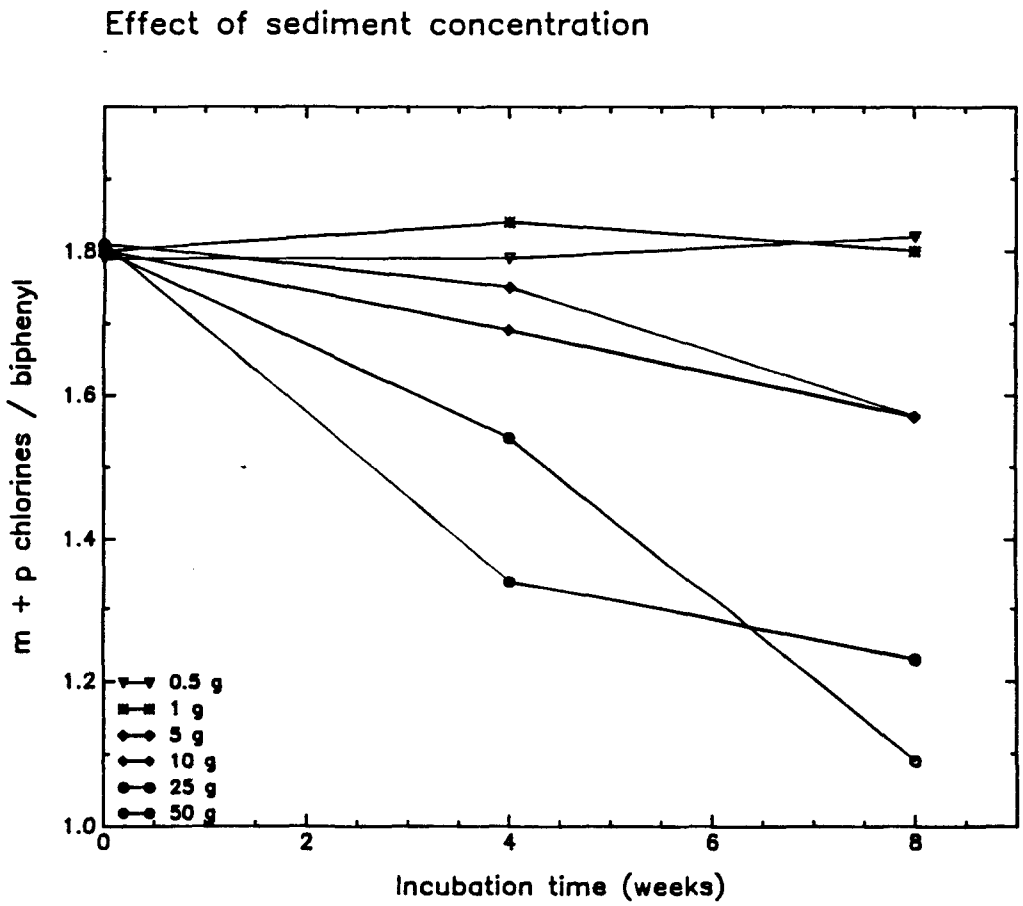

Figure 3-6. Effect of sediment concentration on dechlorination activity. Sediment concentration was varied while the amount of liquid medium remained constant (50 mL).

SUMMARY AND CONCLUSIONS

1. A transferable enrichment culture was established which dechlorinated Aroclor 1242 within 2 weeks of transfer.
2. Pyruvate stimulated the initial dechlorination rate.
3. Hydrogen could replace pyruvate as an electron donor, supporting a higher initial dechlorination rate.
4. High sediment concentrations were optimal.
5. Removal of organic matter by peroxidation inhibited the onset of dechlorination.

FUTURE PLANS

1. To examine the function of the clean sediment in these anaerobic communities, and to find defined materials which can replace the sediments.
2. To further characterize the anaerobic-dechlorinating community, and to attempt isolation of the organism(s) involved.

Chapter 4

REDUCTIVE DECHLORINATION OF PCBs IN SEDIMENTS FROM THE HUDSON RIVER AND NEW BEDFORD HARBOR

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INTRODUCTION

The occurrence and persistence of PCBs in the environment has been a matter of concern over the last few decades. In aquatic systems they can be removed through adsorption to or partitioning into abiotic and biotic settling particles and subsequent sedimentation. Especially for hydrophobic organic chemicals with high affinity for solids, the sediments act as a sink. But through resuspension and mixing by aquatic organisms the surface sediment can also act as a source for these contaminants, increasing the residence time in the ecosystem [Eisenreich et al., 1989].

High concentrations of hydrophobic organic chemicals hint that these compounds may show mobility and bioavailability in the sediments [Capel and Eisenreich, 1990]. PCBs, especially the higher chlorinated congeners, have been shown to undergo reductive microbial dechlorination [Bopp et al., 1984; Brown et al., 1984; Brown et al., 1987a and 1987b; and Quensen et al., 1988]. It has been hypothesized that reductive dechlorination takes place because the high redox potential of these compounds allows anaerobic bacteria to use PCBs as a terminal electron acceptor [Brown et al., 1987b].

To our knowledge, reductive dechlorination of PCBs has only been observed under methanogenic conditions. It has been suggested [Suflita and Miller, 1985; Gibson and Suflita, 1986] that reductive dechlorination of aromatic compounds could not occur under sulfate-reducing conditions because sulfate would be a better electron sink than the chloroaromatics. Chlorophenols, however, can be degraded under such conditions, their mineralization being coupled to sulfate reduction [Häggblom and Young, submitted]. We therefore also chose to study the dechlorination of PCBs under sulfate-reducing conditions.

Objectives

In an attempt to better understand the conditions which control the reductive dechlorination in sediments, we had the following objectives: A) a systematic study of the microbial activity in sediments under two different reducing conditions; B) the study of different sediments; C) the effect of organic substrates on dechlorination; D) the comparison of possible differences in the fate of endogenous and freshly added PCB; and E) studies with a selected congener mixture.

Sulfate reduction and methanogenesis are the major metabolic processes in anaerobic environments. Sulfate reduction is usually the predominant metabolic process in marine sediments or other sulfate-rich habitats, while methanogenesis is predominant in sulfate-poor environments, such as fresh water habitats [Widdel, 1988]. In marine environments a wide variety of haloaliphatic and haloaromatic compounds are produced biologically by marine organisms [King, 1988; Neidleman and Geigert, 1986]. Therefore, anaerobic marine sediments may potentially allow for the selection and enrichment of anaerobic dehalogenating organisms. Different sites, with possible differences in microbial populations, salinity, content of organic matter, porosity of the natural particles, and historical contamination with different Aroclors mixtures, may lead to different dechlorination activities.

Addition of a mixture of fatty acids (acetate, propionate, butyrate and hexanoic acid) was performed in order to support growth and provide accessible energy. Relatively low concentrations were chosen in order to prevent selection for the fast growing organisms and to more closely approximate the situation in the environment. This mixture of fatty acids was chosen because acetate, propionate and butyrate are probably the most important products from the fermentative decomposition of biomass in sediments [Widdel, 1988]. On one hand, further degradation of propionate and butyrate to methane is possible only by syntrophism between methanogens and hydrogen-producing bacteria, since the former do not directly use organic acids higher than acetate [Widdel, 1988]. On the other hand, propionate and butyrate can directly be oxidized by sulfate reducers. Also, a syntrophic association between sulfate reducers and hydrogen-producing acetogens is most likely in sediments of high sulfate concentration, as is probably the case in New Bedford Harbor. In such a situation, methanogenesis can take place in addition to sulfate reduction, suggesting the presence of syntropic acetogenic bacteria [Widdel, 1988].

In addition to studying dechlorination of the endogenous PCBs or added Aroclor mixtures, studies with a specific selection of congeners were performed for a clearer identification of the dechlorination products.

RESULTS AND DISCUSSION

Sediments

In our studies two different sediments were used as inoculum, both with a history of PCB contamination. These were from the Hudson River (NY) and New Bedford Harbor (MA). Samples collected for this study were from the top 20 cm of the sediment. The Hudson River sediment (H7) is a sandy silt with a moderate content of organic carbon [7-8%, M. Harkness, GE, personal communication], and a PCB composition of dechlorinated Aroclor 1242. Much of the organic carbon is present as wood tailings or other poorly biodegradable natural material. The New Bedford Harbor sediment is a marine, silty mud, rich in organic carbon with a PCB contamination consisting of a 300-400 ppm mixture of Aroclor 1242 and 1254 [Alford-Stevens et al., 1988; Brownawell and Farrington, 1986].

Culture Set-up

A heavy sediment inoculum (35% v/v) was added to methanogenic [Healy and Young, 1979] or sulfidogenic [Häggblom and Young, submitted] mineral salts medium supplemented with vitamins to an end volume of 50 mL in 65 mL flasks with CO_2/N_2 (30%/70%) as the head space gas. The flasks were sealed with Teflon-coated butyl rubber stoppers and aluminum crimp sealers. Methanogenic and sulfate-reducing conditions were promoted by addition of excess carbonate or sulfate, respectively. The sediments of Hudson River and New Bedford Harbor were spiked with Aroclor 1242 in acetone (0.26 g/L) to a final concentration of 100 ppm. For the studies with a congener mixture we added 25-4- (66 ppm), 34-34- (30 ppm) and 234-34-CB (6 ppm) in acetone (1.9 g/L).

Fatty acids were added as a carbon source to one set of cultures, initially to 500 mg/L and then monthly to 250 mg/L. All the cultures were incubated statically at 30°C. Controls were prepared in the same way and autoclaved twice. Subsamples were taken after 0, 1, 2, 4 and 7 months by shaking the batches vigorously, removing the stopper and removing 2 mL of sample slurry with an upside-down Pasteur-pipette. The head space was flushed with N_2/CO_2 and the flask recapped. The subsamples were frozen until extraction and analysis. The head space gas was monitored for methane as described [Bossert and Young, 1986; Häggblom and Young, submitted].

Sample Extraction and Analysis

The subsample vial was used as the extraction vessel. The water was replaced first with 1.5 mL acetone, then with 3 mL of 50/50 acetone/hexane with octachloronapthalene as the internal standard. The vial was then shaken overnight on a mechanical shaker. The organic phase was replaced by 2 mL hexane and the sediment was shaken again for 4 hours. The organic and water phases were combined and, after the addition of sodium chloride, back-extracted twice. The organic extract was dried by addition of a few grams of sodium sulfate, concentrated to ~1 mL with a gentle flow of Argon, and cleaned-up in a micro Florisil column. The PCBs were eluted from the Florisil column in the first 4 mL of hexane. Elemental sulfur was removed by the addition of tetrabutylammonium hydrogen sulfate and sodium sulfite [Jensen et al., 1977]. The samples were analyzed by gas chromatography equipped with a ^{63}Ni electron capture detector (DB-5, 30 m x 0.32 mm I.D.).

Over an incubation period of 7 months we observed the following results:

Hudson River:

In Hudson River sediment cultures under methanogenic conditions dechlorination of added Aroclor 1242 was observed after 1 to 2 months. Loss of higher chlorinated congeners was observed, with *meta* and *para* dechlorination and enrichment of *ortho*-substituted mono- (2-CB), di- (2-2- + 26-CB and 2-4- + 23-CB) and tri- (26-2-CB and 236- + 26-3-CB) chlorobiphenyls (Figure 4-1A). The addition of a fatty acid mixture as an auxiliary carbon source greatly enhanced the rate of dechlorination (Figure 4-1B). After 2 months, the

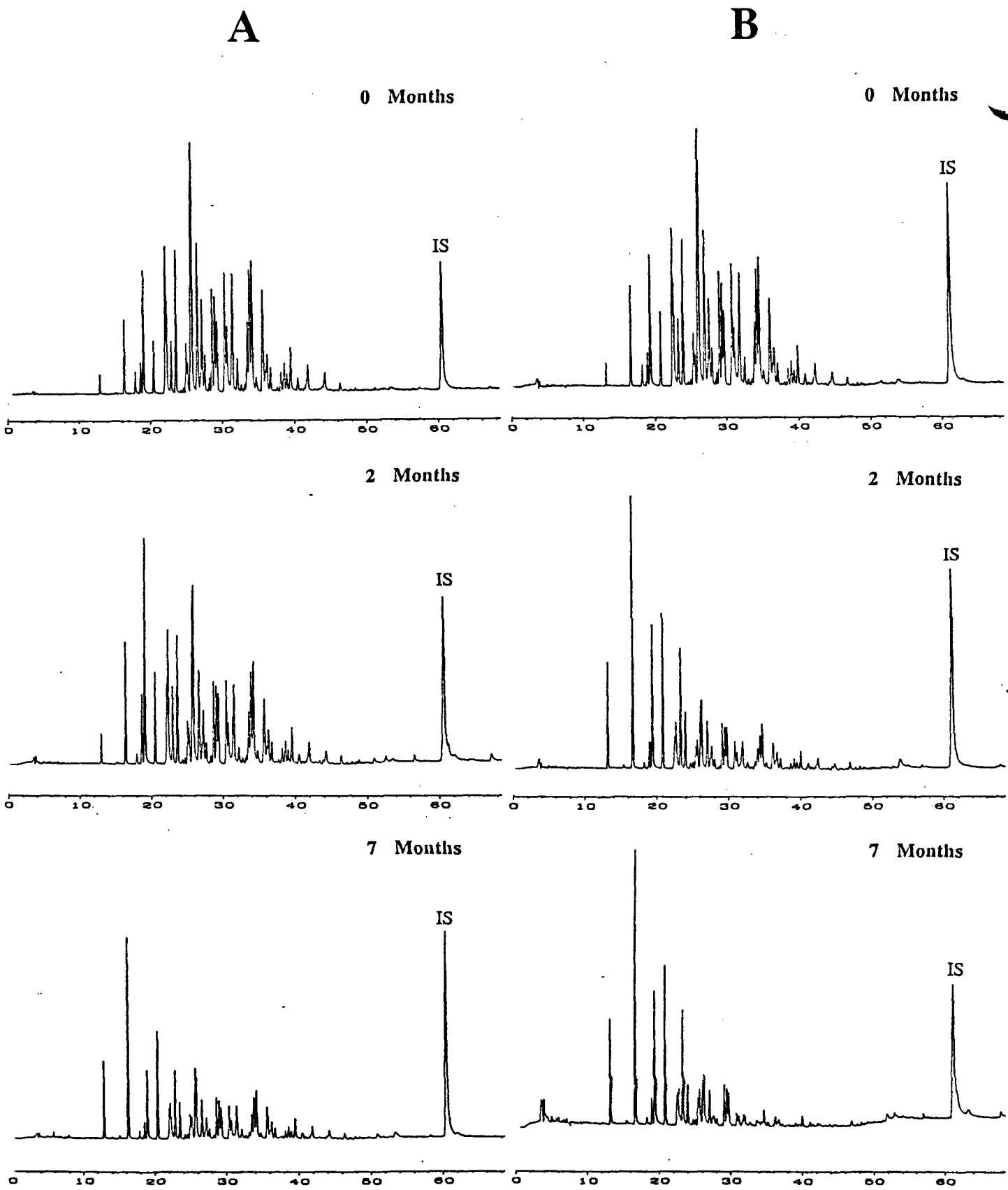

Figure 4-1. Transformation of added Aroclor 1242 in Hudson River sediment: A) no auxiliary substrate added; B) with a fatty acid mixture added monthly. IS: internal standard.

transformation of added Aroclor 1242 generated a pattern of dechlorinated PCBs identical to the endogenous pattern (Figure 4-2). Cultures without the addition of fatty acids showed a significantly longer lag period and slower rates of dechlorination. No transformation of the endogenous PCBs was observed. With the congener mixture the GC-chromatograms show evidence for sequential dechlorination of 25-4-CB to 2-4-CB and 2-CB, and dechlorination of 234-34-CB first, to 24-34-CB and via two possible pathways to 2-CB (Figure 4-3). No transformations have yet been seen of the added Aroclor 1242, endogenous PCBs, or congener mixture under sulfate-reducing conditions.

New Bedford Harbor:

In New Bedford Harbor sediment cultures under methanogenic conditions some trichlorobiphenyls of added Aroclor 1242 were dechlorinated after 2 months of incubation. But only after 4 months did the dechlorination become more pronounced. The overall rate of transformation was much slower than in Hudson River (Figure 4-4). Tetrachlorinated biphenyls were dechlorinated with formation of tri- and dichlorobiphenyls. Transformation of some congeners of tri- and tetrachlorobiphenyls of the endogenous PCBs was observed (Figure 4-4B). The addition of fatty acids as an auxiliary carbon source had no effect on the rate or extent of dechlorination. Under sulfate-reducing conditions no transformation of PCBs was observed.

SUMMARY AND CONCLUSIONS

1. Dechlorination of PCBs in different sediments occurred under methanogenic, but not under sulfate-reducing conditions.
2. Tri- and tetrachlorobiphenyls were transformed in Hudson River sediment with accumulation of *ortho* chlorinated mono-, di- and trichlorobiphenyls. This is consistent with earlier results [Brown et al., 1987a; Quensen et al., 1988].
3. Addition of an auxiliary carbon source enhanced dechlorination of PCBs in Hudson River sediment, a sandy sediment with moderate levels of poorly degradable organic carbon content, but had no effect on dechlorination observed in New Bedford Harbor sediment, a silty sediment which was already rich in organic matter. Lack of an organic substrate (energy and/or reducing power) may thus be limiting PCB-dechlorination in some sediments.
4. Dechlorination of PCBs was much faster in Hudson River than in New Bedford Harbor sediment. This may due to differences in the microbial populations. Another explanation might lie in bioavailability differences caused by differences in the sediment matrices. This could be a mechanism similar to the availability of the fumigant 1,2-dibromoethane (EDB) in soils as reported by Steinberg et al. [1987]. EDB is a volatile, moderately water soluble compound with a low affinity for soils. This compound

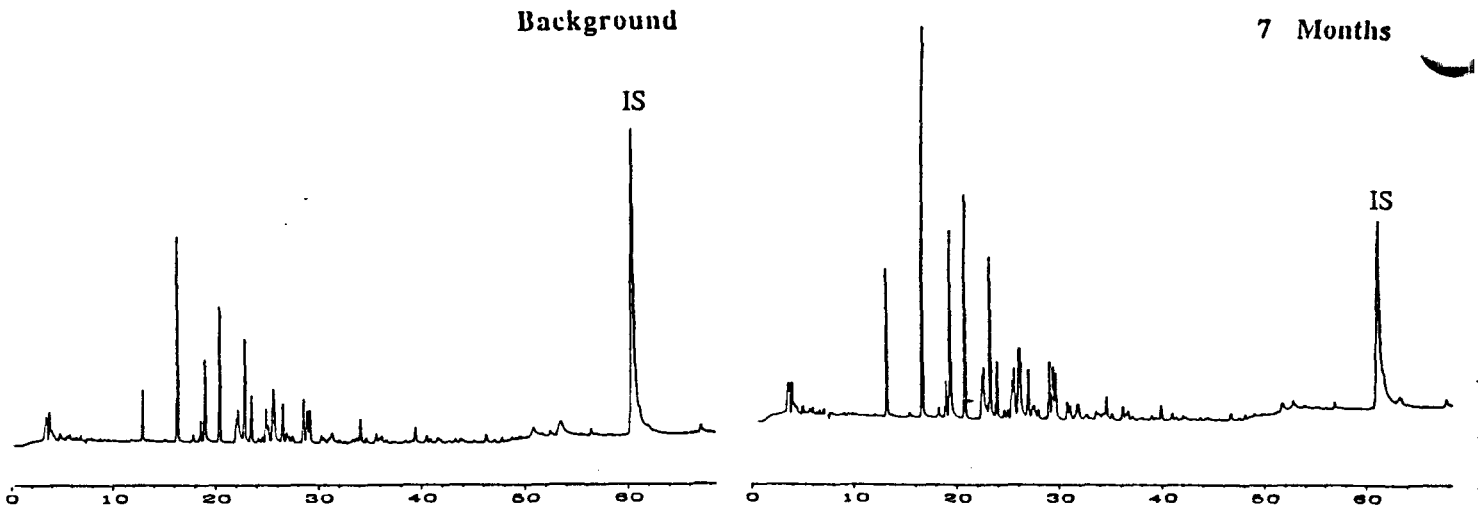

Figure 4-2. Background PCB contamination in Hudson River sediment and sediment with added Aroclor 1242 after 7 months incubation. IS: internal standard.

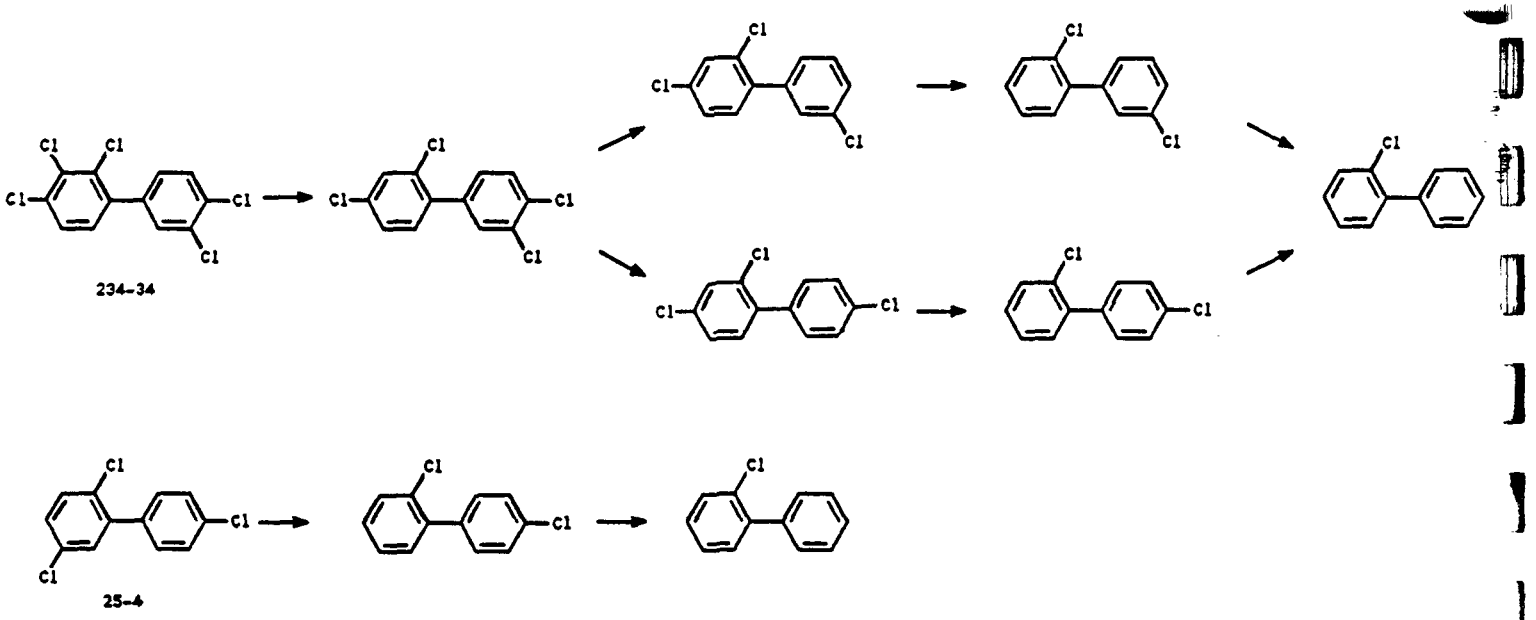

Figure 4-3. Possible pathways for dechlorination of 234-34-CB and 25-4-CB in Hudson River sediment.

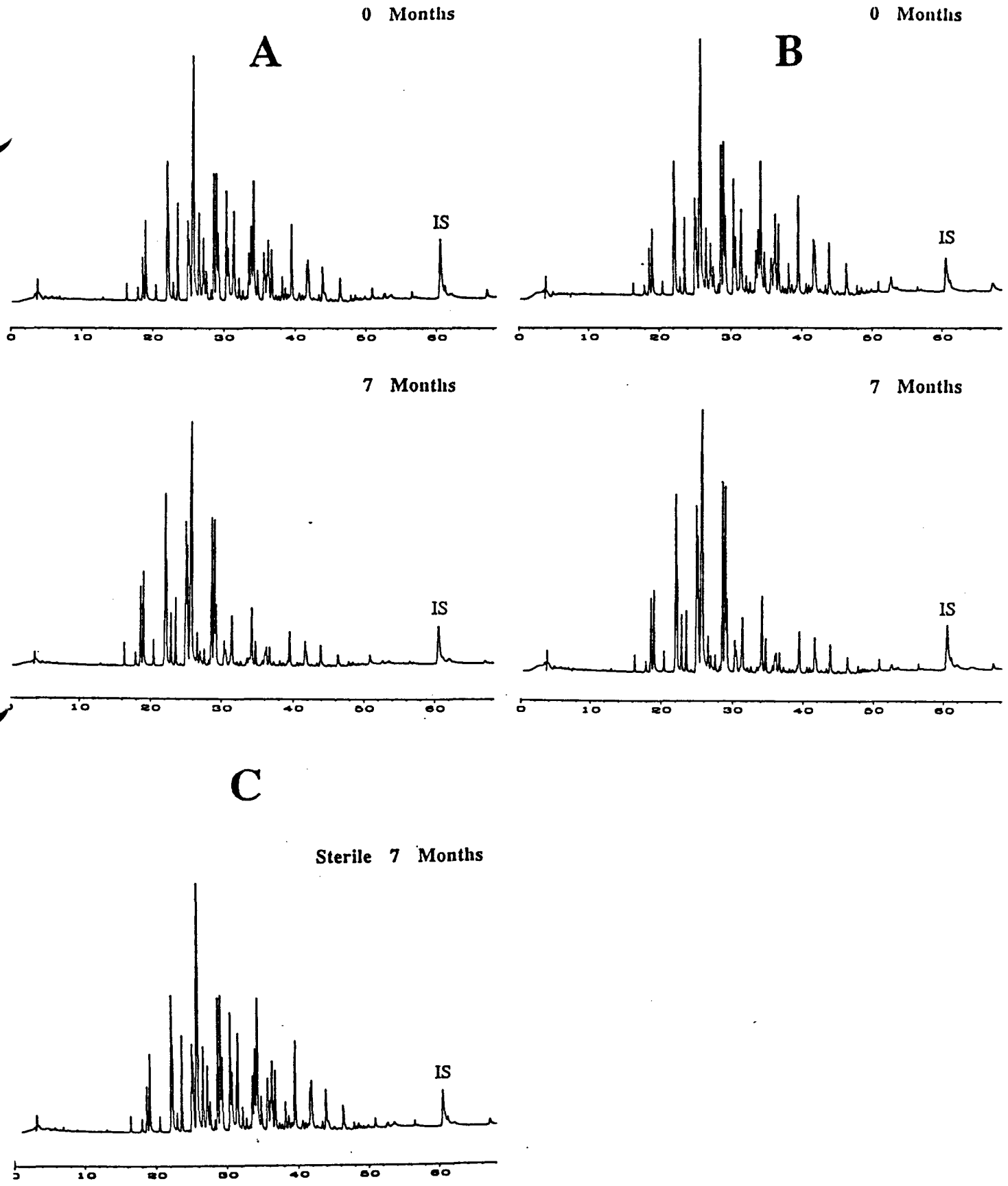

Figure 4-4. Transformations in New Bedford Harbor sediment: A) of added Aroclor 1242, with addition of a fatty acid mixture, B) of background PCB with no addition of an auxiliary substrate, C) sterile control with added Aroclor 1242 after 7 months incubation. IS: internal standard.

was found to persist in surface soil as long as 19 years after its last known application. This residual EDB was extremely resistant to mobilization (desorption into air and water) and biodegradation, in contrast to freshly added [^{14}C]-EDB at comparable concentrations that was rapidly removed and mineralized. Pulverization of the soil greatly enhanced the release of residual EDB. The inertness to biodegradation suggest that native EDB was entrapped in intraparticle micropores, making it inaccessible to bacteria and slow to equilibrate with air or water phases. Similar unavailability to biodegradation was also observed with "old" chlorophenols in some contaminated soils even though freshly added chlorophenols were degraded [Salkinoja-Salonen et al., 1989].

5. The pattern of the dechlorination of added Aroclor 1242 that we observed in the Hudson River sediment is consistent with that found in the environment. The dechlorination in New Bedford Harbor is apparently still in process after 7 months of incubation. It is premature to predict whether the dechlorination pattern is different from that of the Hudson River sediment due to congener specificity of the dechlorination consortia or to dechlorination rate differences.

FUTURE PLANS

Comparison of the anaerobic activity of Hudson River with that of New Bedford Harbor will be made in order to determine whether there are two different anaerobic populations at the two sites. This effort will be extended to examine whether sediments of New Bedford Harbor are inhibitory to PCB transformation. In addition, attempts to propagate active cultures in order to maximize the possibility of isolating pure cultures will be undertaken. Further, the cultures under sulfate-reducing conditions will periodically be monitored to see if a PCB-dechlorinating consortia will evolve under these conditions.

Chapter 5

ANAEROBIC DECHLORINATION OF ENDOGENOUS PCBs IN WOODS POND SEDIMENT

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INTRODUCTION

Woods Pond (Lenox, MA) is a shallow impoundment on the Housatonic River system located 10.5 miles downstream of Silver Lake (Pittsfield, MA). Water depths range from 1-3 feet with the exception of channels created by the flow of the Housatonic River. The sediments are composed of a mixture of black organic matter, sand, and silt and are contaminated with an unidentified hydrocarbon oil (1 to 5%) and with Aroclor 1260 (50 to 150 ppm) to depth of ~18". The Aroclor 1260 in Silver Lake sediments has undergone extensive dechlorination via loss of *ortho, meta*, and *para* chlorines [Brown et al., 1987a, 1987b], but the PCBs in Woods Pond sediments show evidence of only slight dechlorination via loss of *meta* and *para* chlorines. The striking difference in the type and extent of environmental dechlorination at these two sites led us to question the underlying cause(s): Do Woods Pond sediments harbor microorganisms capable of dechlorinating PCBs? Do the Woods Pond sediments lack some nutrient that is essential for the growth and/or activity of these microbes? Does some component of these sediments inhibit the growth of dechlorinating bacteria? Are the PCBs in the Woods Pond sediments accessible to dechlorinating microorganisms? Our objective was to determine whether conditions could be found to stimulate the biological dechlorination of the endogenous PCBs in Woods Pond sediment.

RESULTS AND DISCUSSION

Sediment samples were collected along the eastern and western shores of Woods Pond for analysis of PCBs and assessment of biological dechlorination activity. Typically, core samples were collected to the point of refusal using a Lexan® tube (2" diameter). Samples to be used for cultures were transferred to glass jars, topped with site water, sealed, and stored at 4°C. In all, samples were collected from 88 locations: 28 along the eastern shore, 58 along the western shore, and two at shallow points in the middle. The PCBs were extracted from the sediment samples, analyzed by capillary gas chromatography (GC), and compared with standards of Aroclors 1254 and 1260. A comparison of the congener distribution of the PCBs in the sediment with the Aroclors indicates that the contaminant was virtually all Aroclor 1260. We based this estimate primarily on the proportions of hepta- and octachlorobiphenyls, especially GC peaks 88, 100, 109, 110, and 115 (Figure 5-1). Sediment PCBs from all 88 locations showed evidence of some, albeit only slight, environmental dechlorination. Two

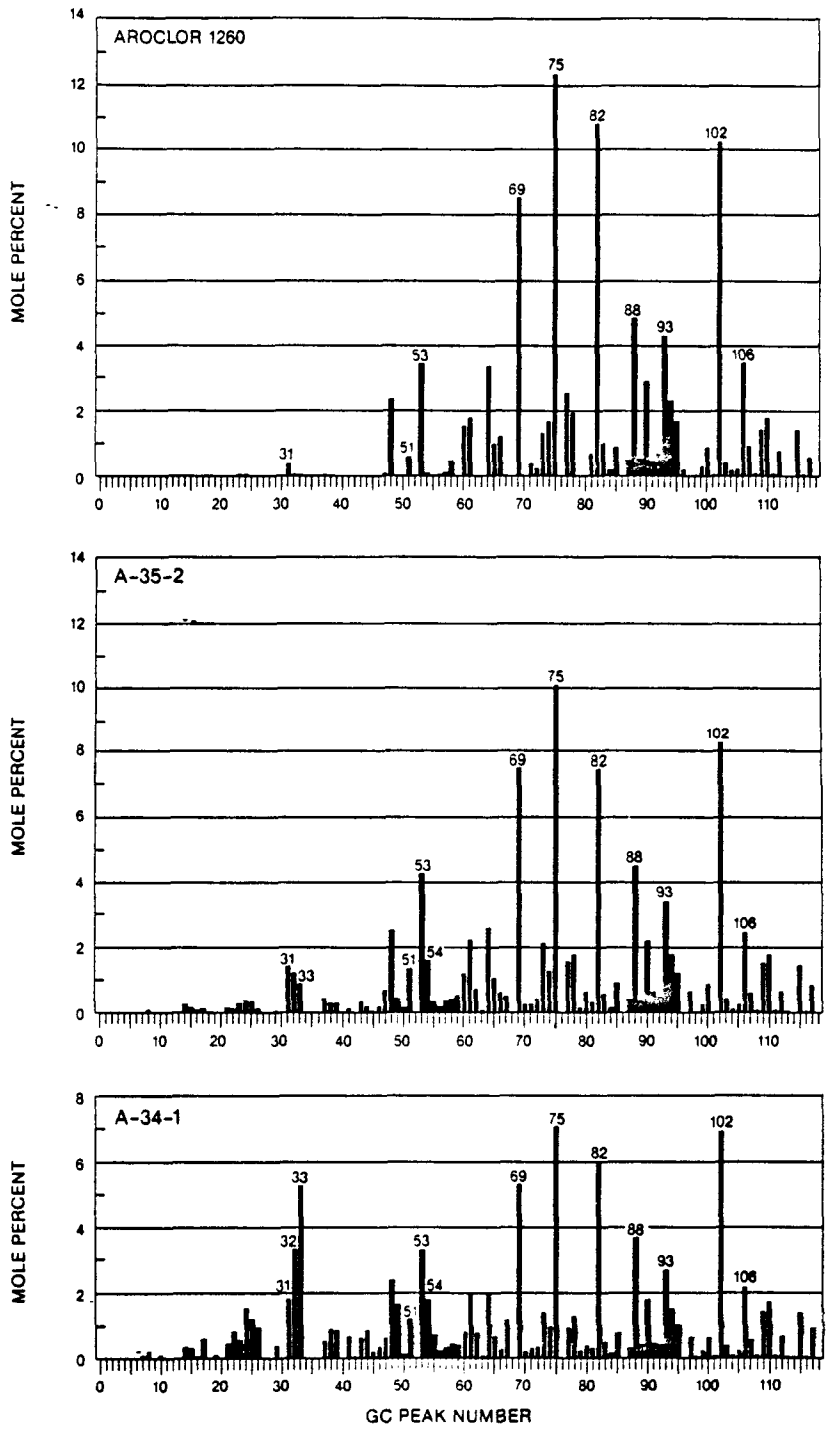

Figure 5-1. Comparison of the proportions of PCB congeners in Aroclor 1260 and in two Woods Pond sediments. Congener peaks were identified and quantified as previously described [Brown et al., 1987b].

sediment samples taken only 15 feet apart on the western shore of Woods Pond illustrate the range of environmental dechlorination seen in the sediments. At site A-35-2 the PCBs showed the least dechlorination, yet four major peaks, 75, 82, 102, and 106 (245-245-, 234-245-, 2345-245-, and 2345-234-CB, respectively) showed decreases of 16 to 33% relative to Aroclor 1260 (Figure 5-1). At the same time, peaks corresponding to expected *meta, para* dechlorination products of these congeners were quite evident: peaks 31, 32, 33, 51, 53, and 54 (25-25-, 24-25-, 24-24-, 235-25-, 245-25-, and 245-24-CB, respectively). At site A-34-1, which shows the most advanced dechlorination we have seen in Woods Pond sediment, peaks 75, 82, 102, and 106 were decreased by 33 to 45% relative to Aroclor 1260, and peaks corresponding to dechlorination products, especially 31, 32, and 33, were quite prominent (Figure 5-1). Table 5-1 compares the proportions of 25-25-, 24-25-, 24-24-, and 245-24-CB (GC peaks 31, 32, 33, and 54, respectively) in Aroclor 1260 and in these two sediment samples. The four congeners are present at only negligible amounts in Aroclor 1260 but are substantial components of the sediment PCBs. These data indicate that the 25-25-, 24-25-, 24-24-, and 245-24-CB in the Woods Pond sediments must have been formed by the dechlorination of components of Aroclor 1260.

TABLE 5-1. Proportions of four PCB congeners in Aroclor 1260 and in two Woods Pond sediments.

Sample	Mole Percent			
	25-25-CB	24-25-CB	24-24-CB	245-24-CB
Aroclor 1260	0.39	0.03	0.05	0.07
A-35-2	1.41	1.12	0.88	1.61
A-34-1	1.79	3.32	5.21	1.75

Although it is clear that some *meta* and *para* dechlorination of the Aroclor 1260 in Woods Pond sediments has occurred, it is not clear when or where this occurred. The dechlorination may have taken place upstream before the sediments were deposited in Woods Pond, or it may have taken place in Woods Pond at some time in the past. The only way to determine if the microoganisms responsible for the dechlorination were still present in Woods Pond sediments was to demonstrate dechlorination in these sediments in controlled laboratory experiments. Our first experiments involved the addition of more Aroclor, either Aroclor 1260 or a 1:1 mixture of Aroclors 1254 and 1260 at two different concentrations (100 and 500 ppm) to slurries of Woods Pond sediment prepared in reduced anaerobic minimal medium (RAMM) [Shelton and Tiedje, 1984]. We detected no dechlorination of the Aroclor over a 2-month period and decided to set up new slurries to which we would add a single PCB congener. The use of a single congener offered two advantages over the use of an Aroclor: (1) Even low levels of dechlorination of a single congener added at high concentration would be easier to detect because only a few products would be formed. (2) We could try a variety of PCB congeners in case some turned out to be better substrates for dechlorination than others.

When 25-34-CB (350 μM) was added to slurries of Woods Pond sediment, we observed loss of the *para* chlorine to yield 25-3-CB after 3 weeks of incubation at 22 to 24°C. This dechlorination continued until ~ 85% of the tetrachlorobiphenyl had been converted to 25-3-CB at 16 weeks. Beginning at 7 weeks, when ~ 30% of the 25-34-CB had been dechlorinated to 25-3-CB, we also began to see changes in the endogenous PCBs. Figure 5-2 compares the proportions of PCB congeners in a Woods Pond sediment at the start of the experiment and after a 12-week incubation. All major peaks except peak 88 were substantially decreased after incubation. At the same time, large increases occurred in some of the tetra- and pentachlorobiphenyl peaks, especially peaks 31, 32, 37, and 51 (25-25-, 24-25-, 23-25-, and 235-25-CB). Table 5-2 identifies the major congeners that have been dechlorinated, while Table 5-3 identifies the major congeners formed as a result of dechlorination. It appears from these data that PCB congeners having 245-, 234-, and 2345-chlorophenyl groups have each lost the chlorine in the *para* position, yielding products carrying 25-, 23-, and 235-chlorophenyl rings. *Para* chlorines were also removed from 34-chlorophenyl groups, but not from 24-chlorophenyl groups.

TABLE 5-2. Major PCB components of Aroclor 1260 decreased by Woods Pond dechlorination Pattern H.

DB-1 GC Peak No.	PCB Congener	Mole Percent			Percent Decrease	Proposed Dechlorination Products
		T_0	T_{12}	Net Loss		
53	245-25	3.97	2.12	1.85	47	25-25
54	245-24	1.97	0.33	1.64	83	24-25
69	245-34 236-245 }	8.31	6.38	1.93	23	{ 25-34[a] → 25-3[a] 236-25
75	245-245	9.15	5.32	3.84	42	245-25 → 25-25
82	234-245	6.81	4.90	1.92	28	234-25 → 23-25
93	2345-236	3.11	2.19	0.91	29	235-236
102	2345-245	6.73	5.66	1.07	16	2345-25 → 235-25
106	2345-234	2.16	1.75	0.42	19	235-234 → 235-23

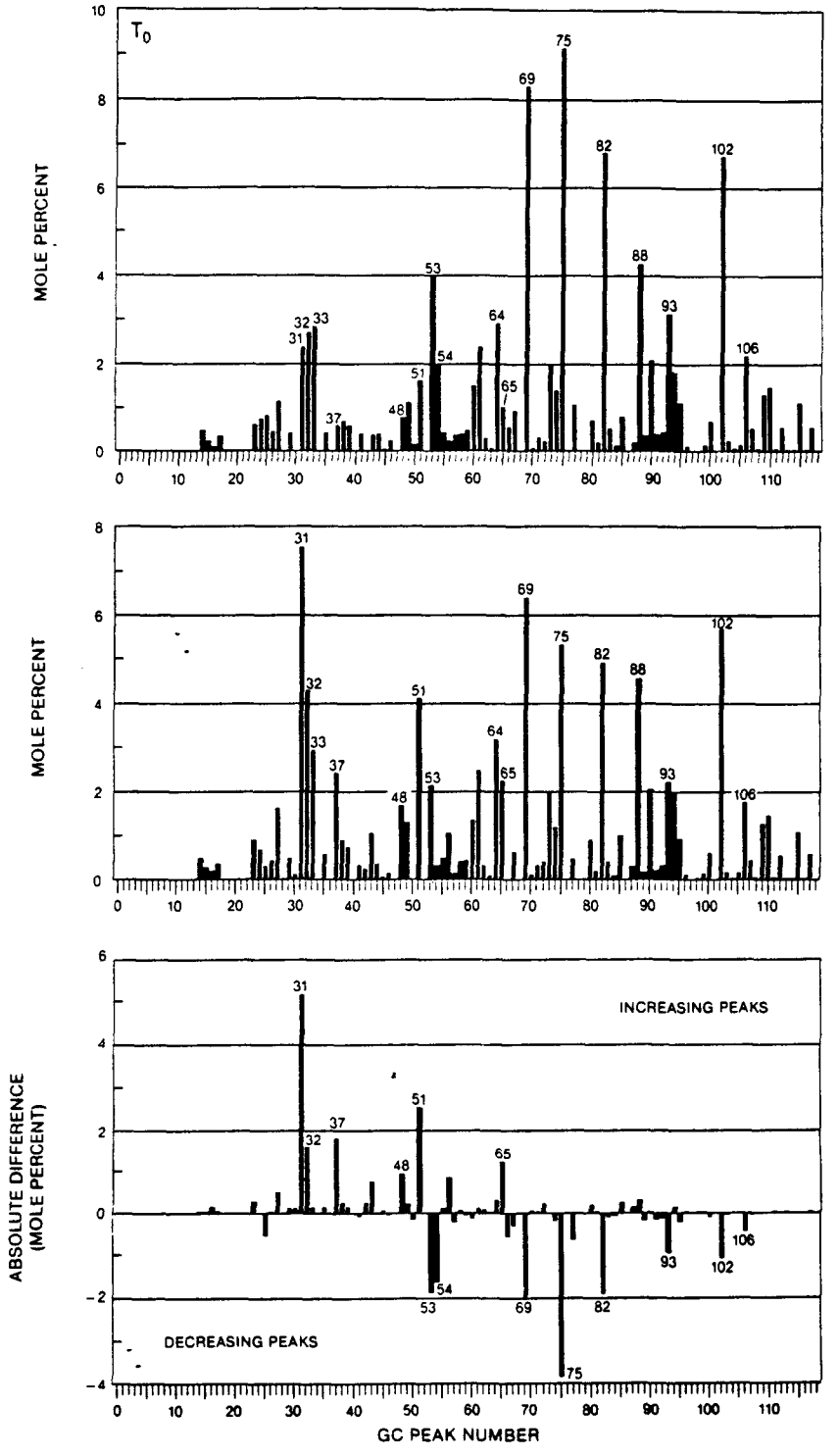

Figure 5-2. Pattern H dechlorination. Comparison of the PCB Congener distribution of PCBs from Woods Pond sediment before and after dechlorination. Peaks corresponding to 25-34-CB (peak 47) and its dechlorination product 25-3-CB (peak 21) were edited out of the chromatogram before calculating the mole percentages of the sediment PCBs. The data presented represents the average of duplicate samples. Top panel: Sediment PCBs at the beginning of the experiment. Center panel: Sediment PCBs after 12 weeks incubation. Bottom panel: Net change in individual PCB peaks after 12 weeks incubation.

TABLE 5-3: Major dechlorination products formed from Aroclor 1260 by Woods Pond Dechlorination Pattern H.

DB-1 GC Peak Number	PCB Congener	Mole Percent		
		T_0	T_{12}	Net Increase
31	25-25	2.36	7.52	5.17
32	24-25	2.70	4.30	1.60
37	23-25	0.58	2.39	1.81
48	236-25, 245-26	0.75	1.66	0.91
51	235-25, 236-23	1.57	4.10	2.53
56	235-23	0.24	1.07	0.83
65	235-236	0.99	2.21	1.22

This type of dechlorination is known as Pattern H. Pattern H dechlorination of Aroclor 1260 is most easily recognized on chromatograms by a large increase in peak 31 (25-25-CB), and moderate increases in peaks 32 and 51 (24-25-, and 235-25-CB) (Figure 5-2). Pattern H dechlorination of Aroclors 1242 and 1254 has been observed in environmental samples from New Bedford Harbor (Massachusetts), Escambia Bay (Pensacola, Florida), and some regions of the Hudson River [Brown and Wagner, 1990] and in laboratory incubations of Aroclor 1260 using inocula from the Hudson River [Quensen et al., 1990].

We subsequently incubated slurries of Woods Pond sediment with various other PCB congeners to determine whether microorganisms capable of removing chlorines from the *meta* positions were also present in Woods Pond sediments. When 23456-CB was added, it was dechlorinated primarily by loss of both *meta* chlorines to yield 246-CB (86%), and to a lesser extent by loss of the *para* chlorine to yield 2356-CB (13%). Small amounts of 236- (0.5%) and 26-CB (0.5%) were also formed. The endogenous Aroclor 1260 began to dechlorinate after most of the 23456-CB was dechlorinated. This time we observed extensive and almost indiscriminate decreases in hexa-, hepta-, and octachlorobiphenyls (peaks 69 through 115) and large increases in tri-, tetra-, and pentachlorobiphenyls (peaks 14 through 63). A single dechlorination product, 24-24-CB (peak 33), accounted for 21.3% of the total PCB (Figure 5-3). Table 5-4 lists the major PCB congeners in the sediment and shows the extent to which they were removed by dechlorination. Table 5-5 lists the major products formed by dechlorination.

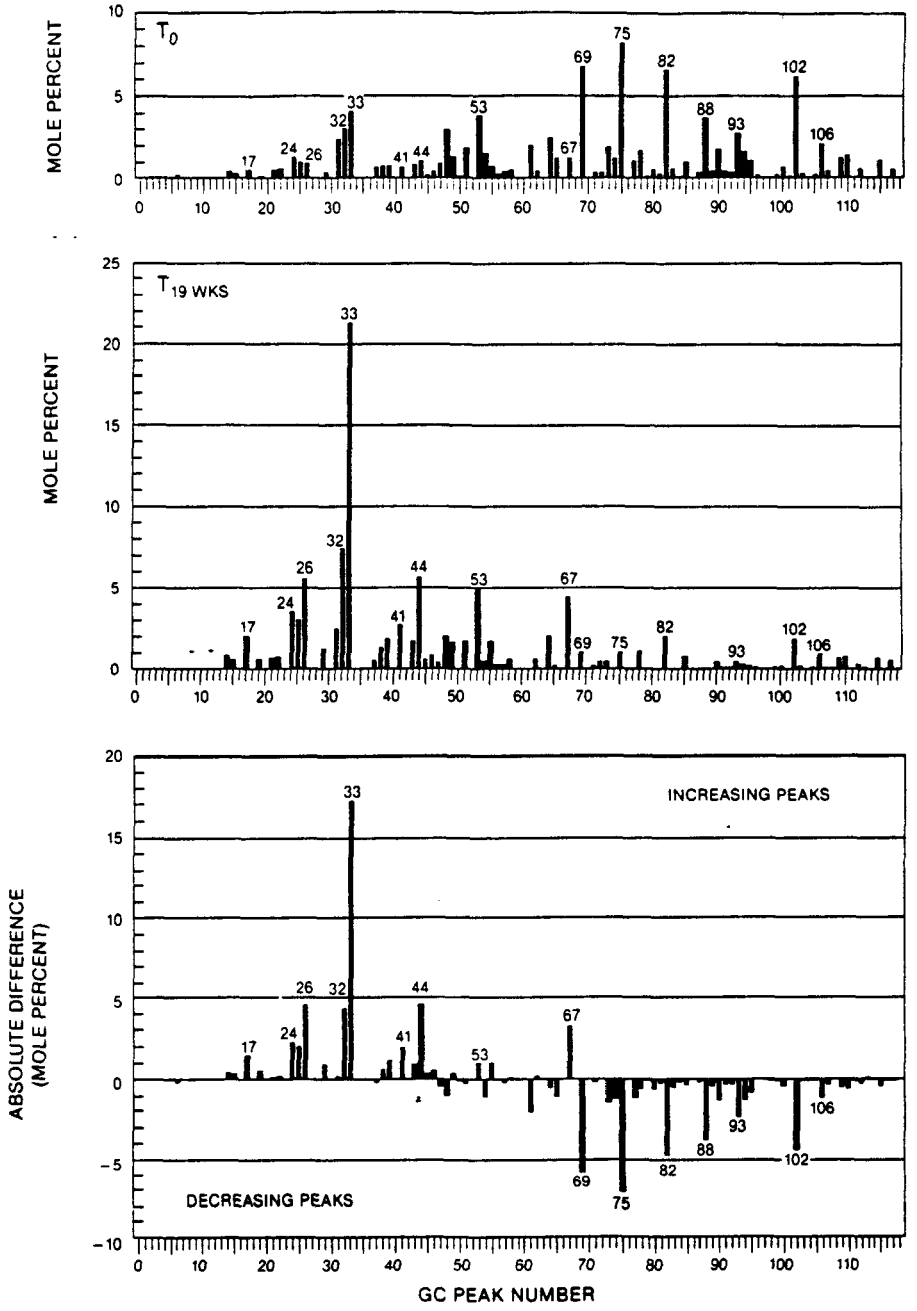

Figure 5-3. Pattern N dechlorination. Comparison of the PCB Congener distribution of PCBs from Woods Pond sediment before and after dechlorination. Peaks corresponding to 23456-CB (peak 60) and its dechlorination products were edited out of the chromatogram before calculating the mole percentages of the sediment PCBs. The peaks edited out were: 2356-CB (peak 52), 236-CB (peak 16), 246-CB (peak 11), and 26-CB (peak 5). Top panel: Sediment PCBs at the beginning of the experiment. Center panel: Sediment PCBs after 19 weeks incubation. Bottom panel: Net change in individual PCB peaks after 19 weeks incubation.

TABLE 5-4. Major PCB components of Aroclor 1260 decreased by Woods Pond dechlorination Pattern N.

DB-1 GC Peak Number	PCB Congener	Mole Percent		Net Loss	Percent Decrease	Proposed Dechlorination Products
		T_0	T_{19}			
54	245-24[a]	1.53	0.49	1.04	68	24-25
61	236-34	2.04	0	2.04	100	26-34 → 26-4
65	235-236	1.19	0.18	1.01	85	235-26 → 25-26
69	236-245 245-34	6.72	0.91	5.81	86	236-24 → 24-26 24-34 → 24-4
73	235-245	1.93	0.45	1.48	77	235-24 → 24-25
75	245-245	8.09	0.98	7.11	88	245-24 → 24-24
82	234-245	6.61	1.94	4.67	71	234-24 → 24-24
88	2356-245 2345-246	3.66	0	3.66	100	2356-24 245-246 → 246-24
90	2346-245	1.74	0.44	1.30	74	245-246 → 246-24
93	2345-236 23456-24	2.69	0.40	2.29	85	236-245 → 236-24 → 24-26 2346-24 → 246-24
94	2356-234	1.63	0.29	1.34	82	2356-24
102	2345-245	6.16	1.81	4.35	71	245-245 → 245-25 → 24-24 235-245 → 235-24 → 24-25
106	2345-234	2.05	0.91	1.14	55	245-234 → 234-24 → 24-24 235-234 → 235-24 → 24-25
TOTAL		46.04	8.80	37.24	81	

[a] 245-24-CB is not present in Aroclor 1260 but transiently accumulates in Woods Pond sediments, most likely as an intermediate in the dechlorination of 245-245-CB (peak 75) and 234-245-CB (peak 82).

TABLE 5-5. Major dechlorination products formed from Aroclor 1260 by Woods Pond dechlorination Pattern N.

DB-1 GC Peak Number	PCB Congener	Mole Percent		
		T_0	T_{19}	Net Increase
17	26-4	0.49	1.93	1.44
24	24-4[a]	1.32	3.52	2.20
25	25-26[a]	1.01	3.02	2.01
26	24-26[a]	0.93	5.52	4.59
32	24-25	2.96	7.30	4.34
33	24-24	4.03	21.28	17.25
41	236-26[ab], 24-35[a]	0.68	2.64	1.96
44	246-24[a]	1.05	5.66	4.61
67	2356-24[a]	1.18	4.34	3.16
TOTAL		13.65	55.21	41.56

[a] The identity of these products was confirmed by GC-MS.

[b] This product was probably derived primarily from 236-236-CB (peak 60) which coelutes with 23456-CB and was therefore edited out of the chromatogram.

The endogenous PCBs appear to have lost almost exclusively *meta* chlorines: 2345-, 234-, and 245-chlorophenyl groups were all dechlorinated to 24-chlorophenyl groups, and 2346-, 236-, and 34-chlorophenyl groups were dechlorinated to 246-, 26-, and 4-chlorophenyl, respectively. The 24-24-CB appears to have been almost totally derived from the dechlorination of four major peaks: 75, 82, 102, and 106 (245-245-, 234-245-, 2345-245-, and 2345-234-CB, respectively). The moderate increase in 24-25-CB (4.34 mole percent), however, indicates that some of the 2345-chlorophenyl group in 2345-245- and 2345-234-CB (peaks 102 and 106) must undergo *para* dechlorination to 235-chlorophenyl and subsequently *meta* dechlorination to 25-chlorophenyl (see Tables 5-4 and 5-5). Analysis of some of the smaller peaks (data not shown) also indicate that 235- and 2356-chlorophenyl groups were slowly dechlorinated, whereas 25-chlorophenyl groups were not. This type of dechlorination has been designated Pattern N and closely resembles the Pattern N previously seen in laboratory cultures of sediment incubated with Aroclor 1260 and an inoculum from Silver Lake [Quensen et al., 1990]. Pattern N dechlorination of Aroclor 1260 is most easily recognized on chromatograms by a large increase in peak 33 (24-24-CB), and moderate increases in peaks 32 and 67 (24-25- and 2356-24-CB, respectively) (Figure 5-3).

Figure 5-4 shows the shift in the PCB homolog distribution of the endogenous PCBs that occurred and underscores the effectiveness of the Pattern N dechlorination system in eliminating hexa-, hepta-, and octachlorobiphenyls. These three homolog classes were decreased by 70, 72, and 44%, respectively, while tri-, tetra-, and pentachlorobiphenyls increased to 82% of the total PCB content.

Our data demonstrate that microbial populations from Woods Pond sediments can effect two different types of dechlorination of Aroclor 1260: Pattern H (*para* dechlorination), and Pattern N (*meta, para* dechlorination). The major dechlorination products of both of these dechlorination systems, 25-25-CB for Pattern H, and 24-24-, and 24-25-CB for Pattern N, were found at all 88 locations sampled in Woods Pond, albeit in varying proportions. Pattern H dominates in sediments taken from the eastern shore. Along the western shore, sediment PCBs randomly display both Patterns H and N in various combinations ranging from predominantly Pattern H (25-25- >24-25- >>24-24-CB), to predominantly Pattern N (24-24- >24-25- >>25-25-CB). Sample A-35-2 (Figure 5-1) is an example of slight dechlorination by a mixture of Patterns H (dominant) and N, whereas sample A-34-1 illustrates moderate Pattern N dechlorination mixed with some Pattern H dechlorination (evidenced by peaks 31 and 51). Laboratory incubations with sediments taken from 30 different locations along the entire eastern and western edges of the pond all showed dechlorination activity, thus demonstrating that the microorganisms capable of dechlorination are widely distributed.

SUMMARY AND CONCLUSIONS

It is apparent from our data that two different dechlorination systems, Pattern H, *para* dechlorination, and Pattern N, *meta, para* dechlorination, are active throughout the sediments of Woods Pond. Given this information, it is not clear why only modest dechlorination of the

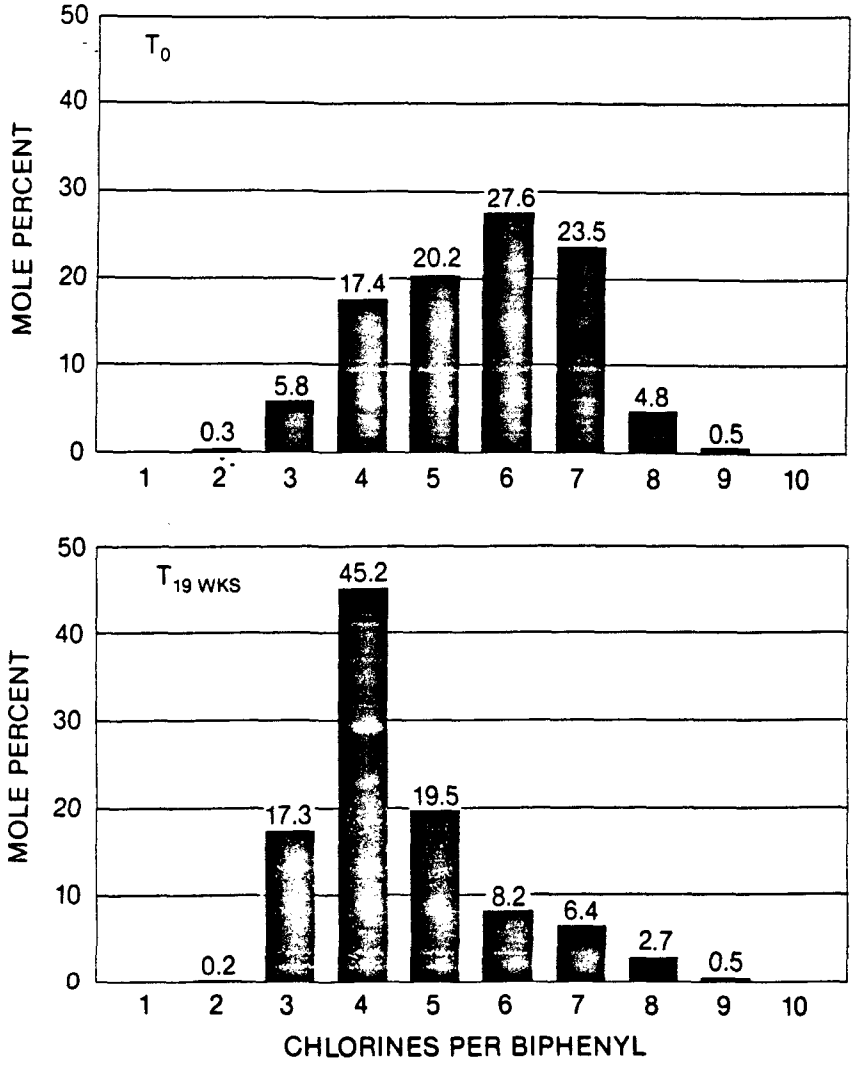

Figure 5-4. Homolog distribution of PCBs in Woods Pond sediment before and after dechlorination.

sediment PCBs has occurred. Even in the laboratory we have only been able to stimulate the dechlorination of endogenous PCBs by adding a high concentration (350 μM) of PCB. The addition of medium (RAMM) with or without various carbon sources, but without the addition of PCBs, stimulated methanogenesis but did not stimulate dechlorination of the endogenous PCBs. Collectively these data imply that the slow dechlorination in Woods Pond cannot be attributed to the lack of an essential nutrient or to the presence of a toxic inhibitor. A more likely explanation is that limited bioavailability of the Woods Pond sediment PCBs, caused by partitioning of the PCBs into both the highly organic sediment and the oil associated with the sediment, may severely limit the growth and activity of the appropriate microbial population(s). It has been proposed that the anaerobic bacteria responsible for dechlorination of PCBs may use the PCB as an electron acceptor and may therefore derive energy from the dechlorination of PCBs [Brown et al., 1987b; Quensen et al., 1988]. If this is the case, then the addition of a single congener in high concentration may stimulate the growth of these microorganisms by providing them with a readily accessible electron acceptor. The resulting high numbers of dechlorinating bacteria could then dechlorinate the endogenous PCBs. However, regardless of the reason, our laboratory experiments (Figures 5-3 and 5-4) clearly demonstrate that it *is possible* to extensively dechlorinate the hexa-, hepta-, and octachlorobiphenyls in the Woods Pond sediments.

FUTURE PLANS

Our objectives for the next year are:

1. To learn how to grow the microbial population responsible for Pattern N dechlorination.
2. To learn how to accelerate PCB dechlorination of endogenous PCBs without the addition of more PCBs.
3. To find means of further dechlorinating the major dechlorination products of Pattern N (24-24-, and 24-25-CB).

Chapter 6

ANAEROBIC AND AEROBIC BIODEGRADATION OF ENDOGENOUS PCBs

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INTRODUCTION

The altered distribution of residual PCB contamination observed in several aquatic sediments is evidence of widespread microbial reductive dechlorination of PCBs in the environment [Brown et al., 1984; 1987a; 1987b]. This process effects the specific removal of *meta* and *para* chlorines, resulting in the depletion of highly chlorinated PCB congeners with corresponding increases in lower chlorinated, *ortho* substituted PCBs. In addition, microbial anaerobic dechlorination of PCBs has been observed in the laboratory [Quensen et al., 1988; GE Report, 1989; Abramowicz et al., 1990], and has recently been reviewed [Bedard, 1990; Abramowicz, 1990]. This report will focus on recent findings involving the acceleration of dechlorination in Hudson River sediments, the dechlorination of endogenous PCB contamination, as well as the sequential anaerobic/aerobic treatment of contaminated sediments.

The acceleration of anaerobic dechlorination in Hudson River sediments was observed upon the addition of a complex nutrient mixture, surfactants, or a simple trace metal mixture. The latter result may indicate that low levels of a trace metal in the sediment may limit the rate of PCB dechlorination occurring in the environment today. Dechlorination of endogenous PCB contamination has been observed in three different soils and sediments. This result indicates that anaerobic microorganisms have access to PCBs in even "aged" soil environments. In addition, sequential microbial treatment via anaerobic dechlorination and aerobic biodegradation has been demonstrated on such endogenous PCB contamination.

RESULTS AND DISCUSSION

Rate Enhancement

The methods utilized in these experiments will be briefly discussed. The sediments were collected and stored until use in sealed vessels at 4°C. All subsequent operations to investigate anaerobic dechlorination were performed in an oxygen-free atmosphere (95% N_2, 5% H_2). The sediment was mixed with medium (35 g soil/ 50 mL medium) to produce a sediment slurry from which aliquots were removed while mixing to obtain reproducible sampling. Typically 10 μL of a concentrated PCB stock solution in acetone was added to spiked samples. Samples were crimp sealed, vigorously vortexed, and stored stationary in the dark at 23°C, unless otherwise indicated. Aerobic studies were performed by initially growing

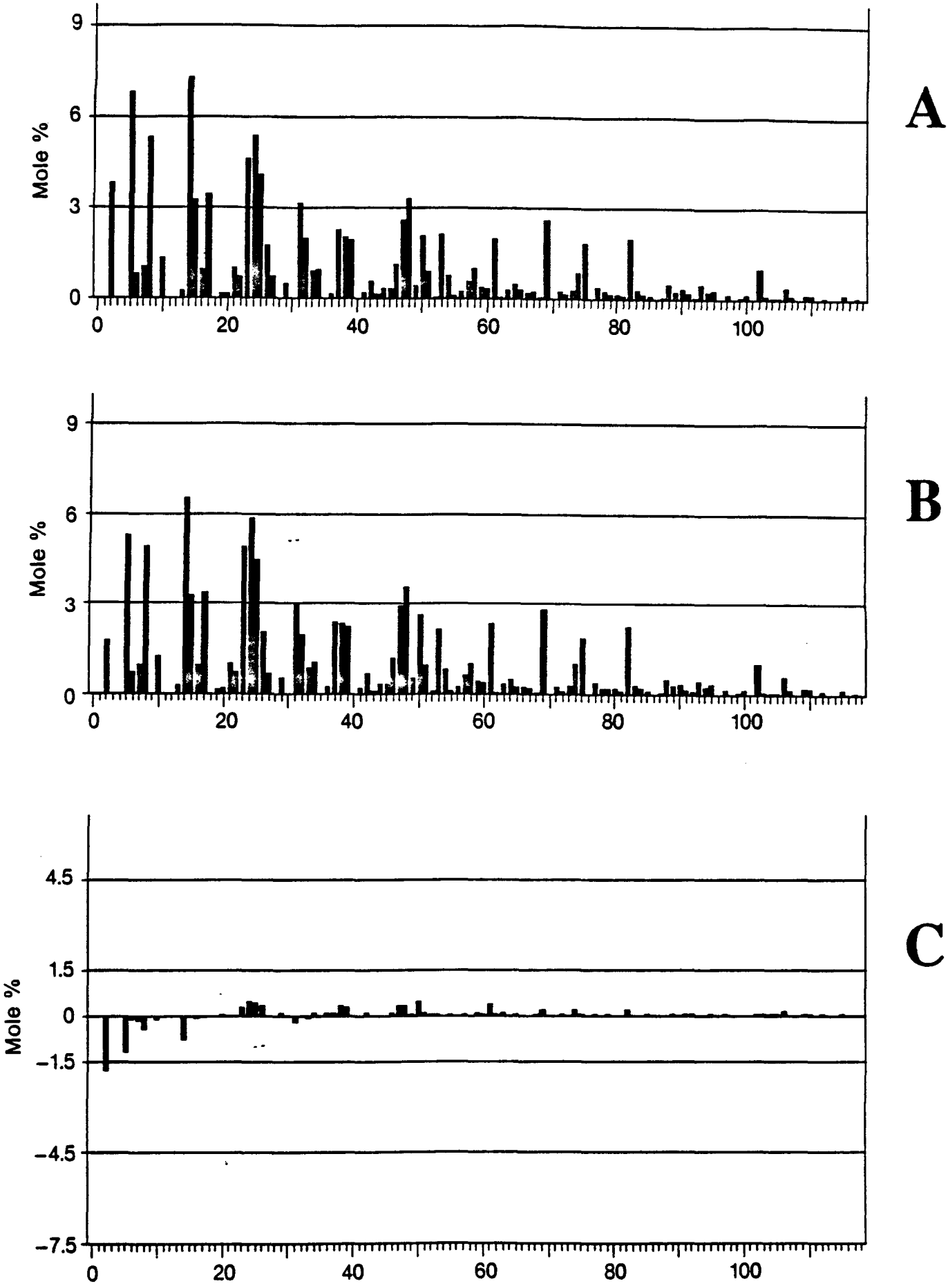
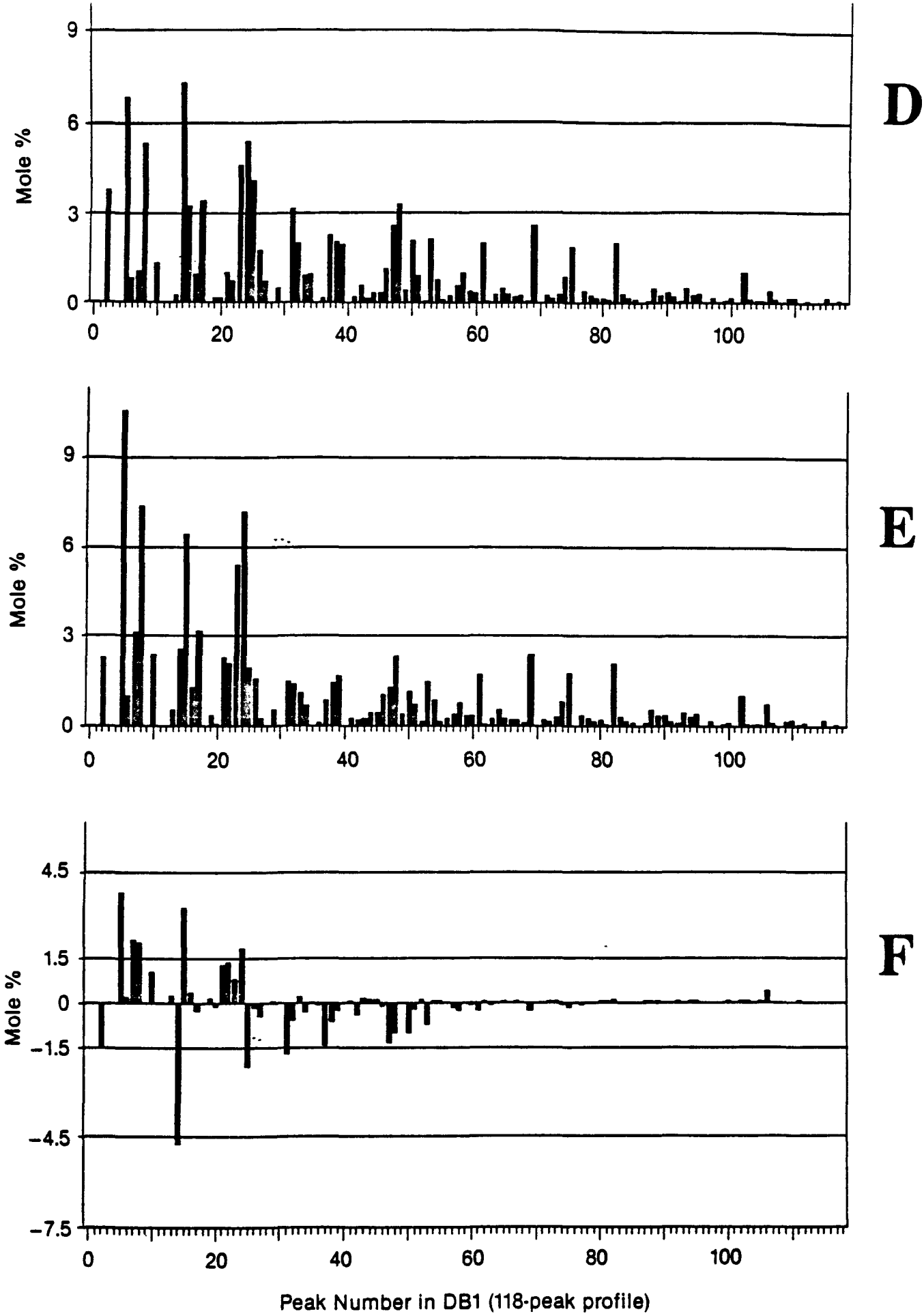


Figure 6-1: Acceleration of the reductive dechlorination of PCBs upon addition of nutrients (8 week timepoint). Panel A and D, autoclaved control; Panel B, includes distilled water; Panel C, difference (B-A); Panel E, includes RAMM; Panel F, difference (E-D). All samples contain 500 ppm PCB mixture.


D
E
F
Mole %
9
6
3
0
4.5
1.5
-1.5
-4.5
-7.5
0
20
40
60
80
100
Peak Number in DB1 (118-peak profile)

cultures of *Pseudomonas* strain LB400 and *Corynebacterium* strain MB1 on biphenyl as the sole carbon source. Cells were harvested, washed, and added to contaminated sediments at 1 OD with a 20% soil loading by weight. Individual vials were sacrificed for timepoints by extraction of the entire contents with three volumes of diethyl ether. Samples were then analyzed by high resolution gas chromatography with an electron capture detector on a fused silica capillary column (30 m by 0.25 mm ID) coated with a 0.25 μm bonded liquid phase of DB-1.

The addition of a minimal medium to the sediment slurry resulted in a dramatic increase in the observed rate of anaerobic dechlorination after 8 weeks (see Figure 6-1). The RAMM minimal medium contained nutrients, trace minerals, and bicarbonate [Shelton and Tiedje, 1984]. The control was autoclaved and incubated along with the samples; no change was observed in any of the heat-treated controls during the experiments. The control (Figure 6-1A and 6-1D), therefore, represents the PCB distribution in the original mixture added to the sediment (70% Aroclor 1242, 20% Aroclor 1254, 10% Aroclor 1260).

The sample mixed with distilled water in place of the minimal medium is shown in Figure 6-1B. Only slight activity was observed in this experimental sample after an 8-week incubation. This is contrasted by the significant change observed in the sample to which RAMM minimal medium had been added (Figure 6-1E). A direct comparison can be found by comparing the difference plots in Figure 6-1C and 6-1F. The control and water-only sample contains nearly the same average number of chlorines per biphenyl (Cl/BP; 3.75 and 3.78, respectively), indicating that no significant dechlorination had occurred at this timepoint. The sample with RAMM nutrients added contains 3.52 Cl/BP, indicating a loss of approximately 0.25 Cl/BP during the initial 2 months of the experiment. The selective *meta* and *para* dechlorination observed in this sample continued at later timepoints, and is consistent with the environmental changes observed in the Hudson River [Brown et al., 1987].

This result suggests that a limiting nutrient present in RAMM medium may be restricting the rate of dechlorination in upper Hudson River sediments. It should be noted that at later timepoints significant dechlorination was also observed in the sediment to which no nutrients were added. These changes were similar although less extensive than the sample to which nutrients were added. Therefore, nutrient addition can decrease the lag time before activity is initiated, as well as increase the extent of dechlorination observed.

The RAMM medium was subdivided into four different components to further investigate nutrient stimulation of this PCB dechlorination activity. Individual components were added in various combinations and concentrations; results are shown in Table 6-1. Note that the addition of the trace metals (Zn^{+2}, Cu^{+2}, Ni^{+2}, Se^{+2}, B^{+3}) correlates with nearly a two-fold increase in the rate of dechlorination of 234-34-CB. This effect suggests that one of these trace metals, added at less than the 0.02 ppm level, may represent the component that limits the PCB dechlorination rate in Hudson River sediments. Other agents that have been demonstrated to increase the rate and/or extent of PCB dechlorination in Hudson River sediments include non-ionic high molecular weight surfactants (e.g., Triton X-705) and the addition of a complex carbon source (e.g., yeast extract or fluid thioglycollate medium

TABLE 6-1. Effect of RAMM components on PCB dechlorination rate (500 ppm 234-34-CB).

Relative Dechlorination Rate	A	B	C	D
105%	+			
90	+	+		
105	+	+	+	
171	+	+	+	+
210	+	++	+	+
171	++	++	+	+
202	+	+		+
210	+	+	++	+
191	+	+		++

A = phosphate salts, cysteine, HCO_3^-
B = nitrogen + minerals (Ca^{+2}, Mg^{+2}, Fe^{+2})
C = Mn^{+2}, Mo^{+6}, Co^{+2}
D = trace metals (Zn^{+2}, Cu^{+2}, Ni^{+2}, Se^{+2}, B^{+3})

with beef extract). In addition, PCB dechlorination has been observed over a broad range of PCB concentrations (20-1500 ppm) and on Aroclor 1260 [Abramowicz et al., 1990].

It has been reported that corrinoids, including cobalamin vitamin B_{12}, can catalyze the reductive dechlorination of chlorinated methanes in the presence of an electron donor [Krone et al., 1989]. It is possible that these cobalt-heme coenzymes may play a role in anaerobic microbial dehalogenations of PCBs as well. Therefore, the effect of vitamin B_{12} addition on the rate of dechlorination was investigated at various concentrations, and the results are shown in Figure 6-2. The unexpected results display a classic inhibition curve for the dechlorination of PCBs upon vitamin B_{12} addition. The decreased dechlorination activity observed in these experiments can be explained by the known inactivation of enzymes with vitamin B_{12} in the presence of small molecular weight thiols [Harada et al., 1975a, 1975b, 1975c]. Harada and coworkers showed that μM concentrations of corrinoids like cyanocobinamide (Factor B) and cobalamin (Vitamin B_{12}) inactivate papain and yeast alcohol dehydrogenase via the formation of mixed disulfides between small molecular weight thiols and the enzymes. Our RAMM medium contains cysteine and the observed inhibition of PCB dechlorination may be explained by a similar mechanism. An alternate explanation could involve naturally produced thiols as the nucleophile responsible for the *in vivo* dechlorination. Thiols are known to be involved in the dechlorination of chlorobenzenes [Stiles, personal communication]. Therefore, the coenzyme could be inhibiting dechlorination by inactivating the natural thiol nucleophile via disulfide formation.

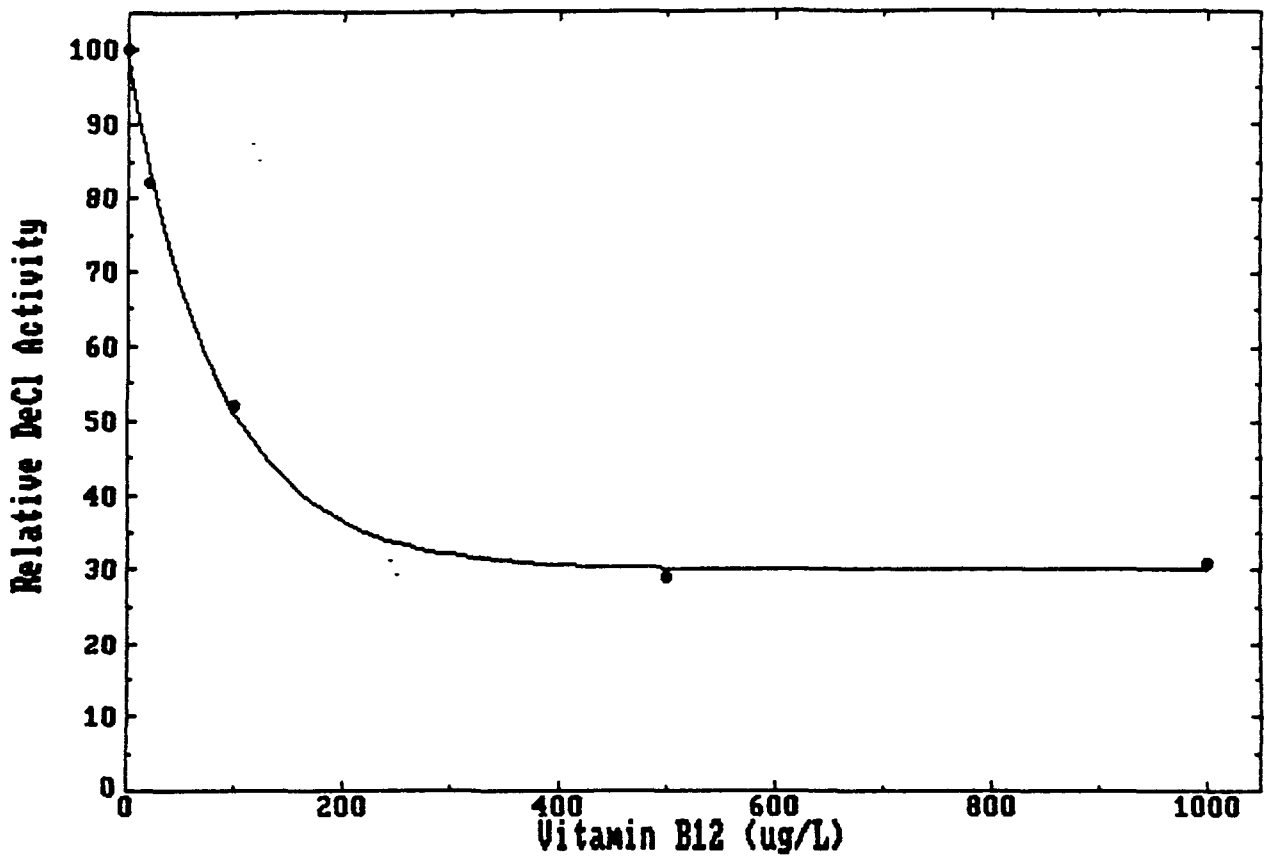


Figure 6-2: Effect of Vitamin B_{12} addition on the rate of dechlorination (100 ppm 234-34-CB).

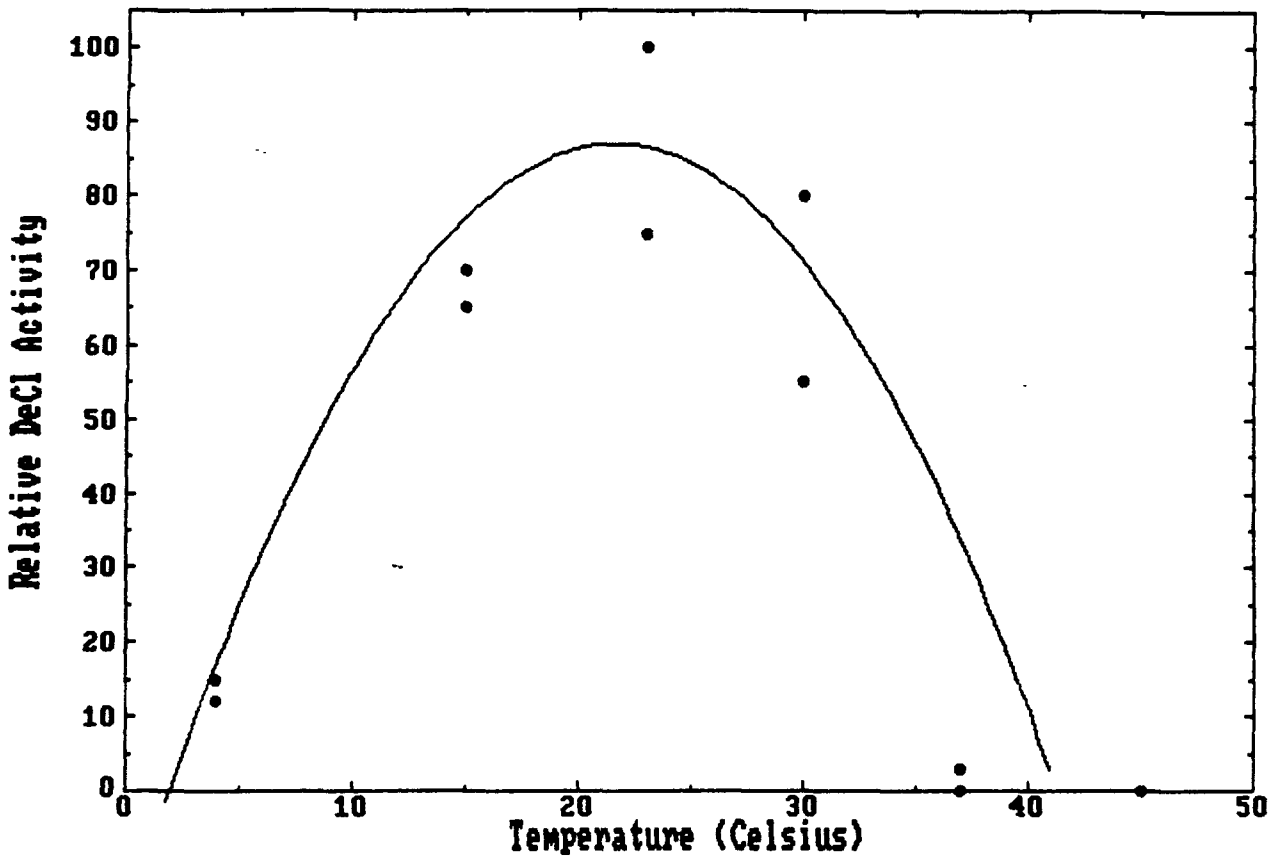


Figure 6-3: Temperature profile of dechlorination activity with Hudson River sediments. All samples contain 500 ppm PCB mixture.

Initial experiments to investigate the effect of temperature on dechlorination activity are shown in Figure 6-3. Note that the dechlorination occurred over a broad range of temperatures (4-30°C), indicating that this process may occur year-round in river sediments. The optimal dechlorination rate was found at 15-30°C.

Endogenous PCBs

It is possible that biodegradation studies on soils spiked with PCBs may not provide accurate kinetic data for similar experiments on endogenous, aged PCB contamination. It has been observed with South Glens Falls dragstrip soil that aerobic biodegradation rates can be limited due to bioavailability issues [GE Report, 1990]. Therefore, several different contaminated soils and sediments were investigated to directly monitor the PCB dechlorination rate of the endogenous contamination.

Upper Hudson River sediments contaminated >15 years ago have already been extensively dechlorinated in the environment [Brown et al., 1987]. Such sediments can be even further dechlorinated by the addition of RAMM nutrients (see Figure 6-4). Note that the environmental sample (Figure 6-4A) contains ~ 41% 2-CB (peak 2) and 2-2/26-CB (peak 5). The mole fraction represented by these congeners increases to >63% after RAMM addition (Figure 6-4B). This dechlorination activity attacks all of the more highly chlorinated congeners (difference plot in Figure 6-4C), and is similar to environmental Pattern C. The dechlorination rate is comparable to that observed in samples spiked with PCB. Endogenous PCB contamination can also be dechlorinated by anaerobic bacteria in Woods Pond sediments (Aroclor 1260, data not shown).

The endogenous PCBs bound to dragstrip soil were also available for dechlorination via anaerobic microorganisms (see Figure 6-5). In this experiment, 25% by weight Hudson River sediments were added to the dragstrip soil containing RAMM medium. Again, this microbial process successfully attacked the endogenous PCB contamination. The control sample contains on average 3.86 Cl/BP. This value has decreased to 3.64 in the live sample (Figure 6-5B). The difference plot (Figure 6-5C) displays strong losses of the more highly chlorinated congeners (5 or more Cl), with the production of tri- and di-CBs. This result is particularly encouraging since this same soil demonstrated bioavailability limitations upon aerobic treatment [Chapter 12, GE Report 1990].

Anaerobic microorganisms capable of PCB reductive dechlorination are widespread and exist in even uncontaminated river sediments, as shown in Table 6-2. The Spier Falls sediments are located >10 miles upstream of the source of contamination (river mile 205) and contain <1 ppm PCB. This is compared to the H7 site, located downstream of the source (river mile 193.5) and containing 45 ppm PCB. The dechlorinating organisms are found in such uncontaminated sediments, although they appear to be present at lower concentrations and dechlorination rates are about four-fold slower. This PCB dechlorination activity has been observed in a number of other aquatic sediments, including Woods Pond (Lenox, MA), Silver Lake (Pittsfield, MA), New Bedford Harbor (MA), Escambia Bay (Pensacola, FL), the

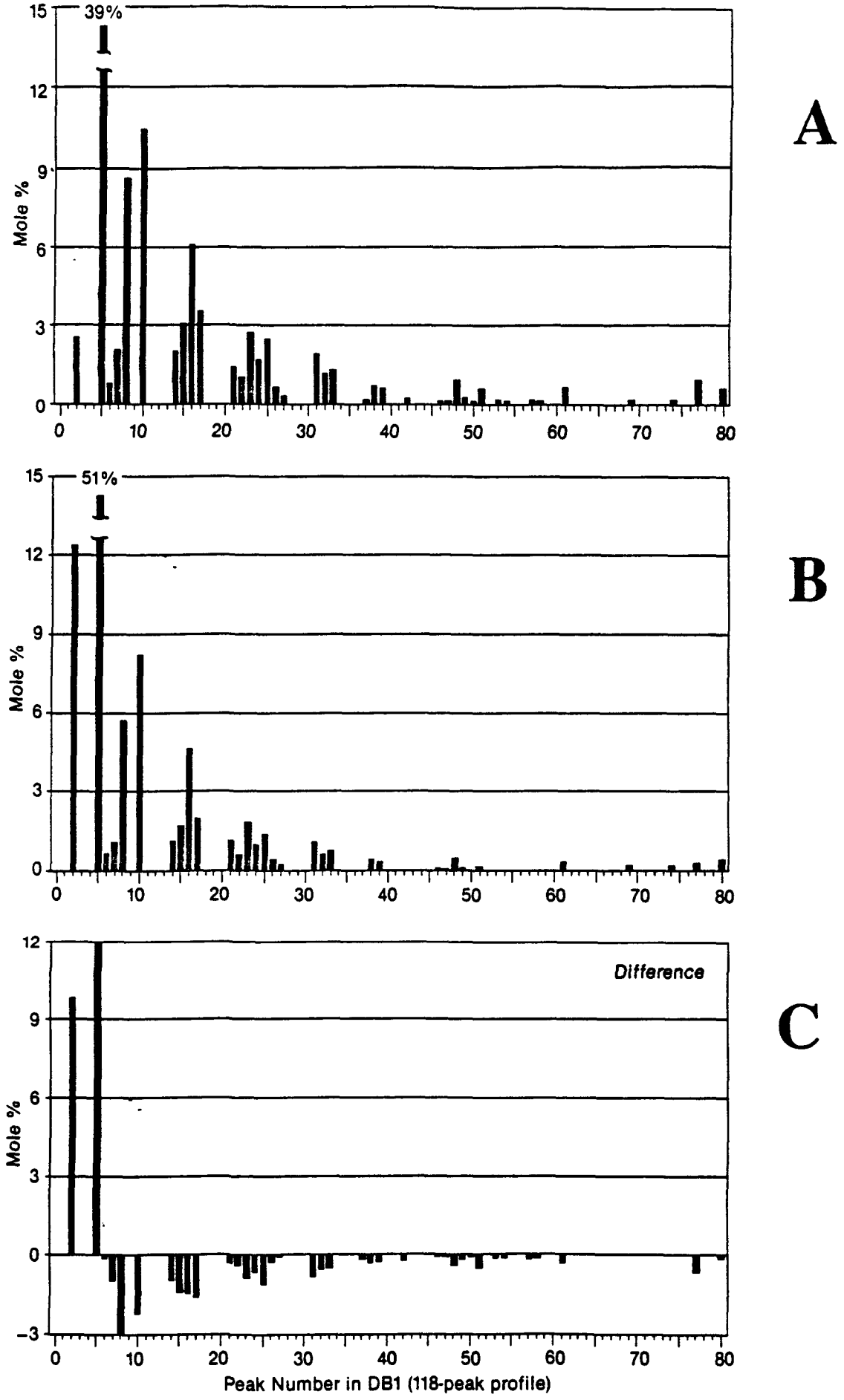


Figure 6-4: Dechlorination of endogenous PCB contamination in Hudson River sediments (24 week timepoint, H7 site). Panel A, autoclaved control; Panel B, experimental; Panel C, difference.

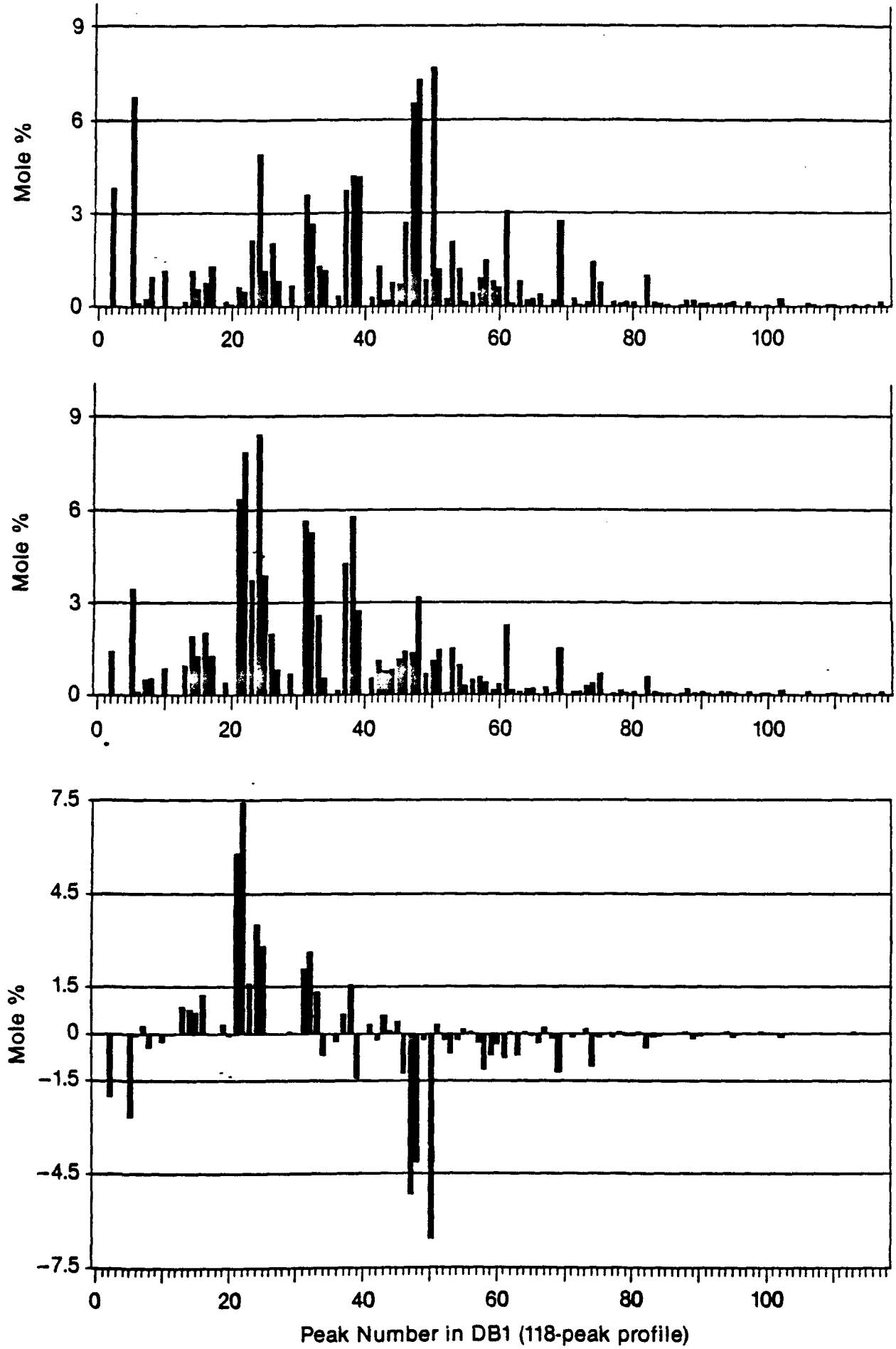


Figure 6-5: Dechlorination of endogenous PCB contamination in South Glens Falls soil with 25% Hudson River sediment (24 week timepoint). Panel A, autoclaved control; Panel B, experimental; Panel C, difference.

Housatonic River (CT), the Sheboygan River (WI), Waukegan Harbor (IL), the Hoosic River (North Adams, MA), and many different locations in the Hudson River (NY). This widespread occurrence of natural anaerobic dechlorination in different sediments indicates that it is a general phenomenon that occurs in environments that differ dramatically in their geography, seasonal temperature, PCB concentration, PCB composition, and organic content.

TABLE 6-2. Comparison of PCB dechlorination rates observed with PCB-contaminated (H7) and uncontaminated (Spier Falls) Hudson River sediments. (500 ppm 234-34-CB)

Time	% Dechlorination	
(wk)	Contaminated	Uncontaminated
0	0.0	0.0
4	5.0	0.0
8	19.7	0.0
12	23.6	0.0
18	ND	9.0
32	90.0	18.2

This natural dechlorination of endogenous PCB contamination is still occurring in the environment. The H7 Hudson River site has been repeatedly sampled over the last 10 years. A timecourse displaying the observed PCB distribution is shown in Figure 6-6. Note that even the early sample (Figure 6-6A) displays a significant amount of 2-CB and 2-2/26-CB (see arrows), indicating that dechlorination is already well advanced. In our most recent sampling (Figure 6-6C), the 2-CB and 2-2/26-CB now account for >85% of the total PCB in the sample. Six samples taken at this time at 20-foot intervals all displayed similar PCB distributions, indicating that nearly all of the *meta* and *para* chlorines have been removed to date (1.63 Cl/BP, including <0.1 non-*ortho* chlorines).

Sequential Anaerobic/Aerobic Treatment

The *ortho* substituted, lower chlorinated products of anaerobic dechlorination should be good substrates for subsequent aerobic biodegradation. Therefore, Hudson River sediments dechlorinated in the laboratory (as described above) were then subjected to aerobic treatment, and the results are shown in Figure 6-7. The control bars indicate the original PCB mixture added to the sample. Anaerobic treatment results in the classic redistribution previously described, and the total PCB concentration has not been significantly decreased. In contrast, subsequent aerobic treatment now reduces the PCB concentration 85% (mole percent values have been reduced by 85% for these bars to reflect this decrease).

Similar experiments have also been performed on endogenous PCB contamination. Hudson River sediments that had previously undergone environmental dechlorination were

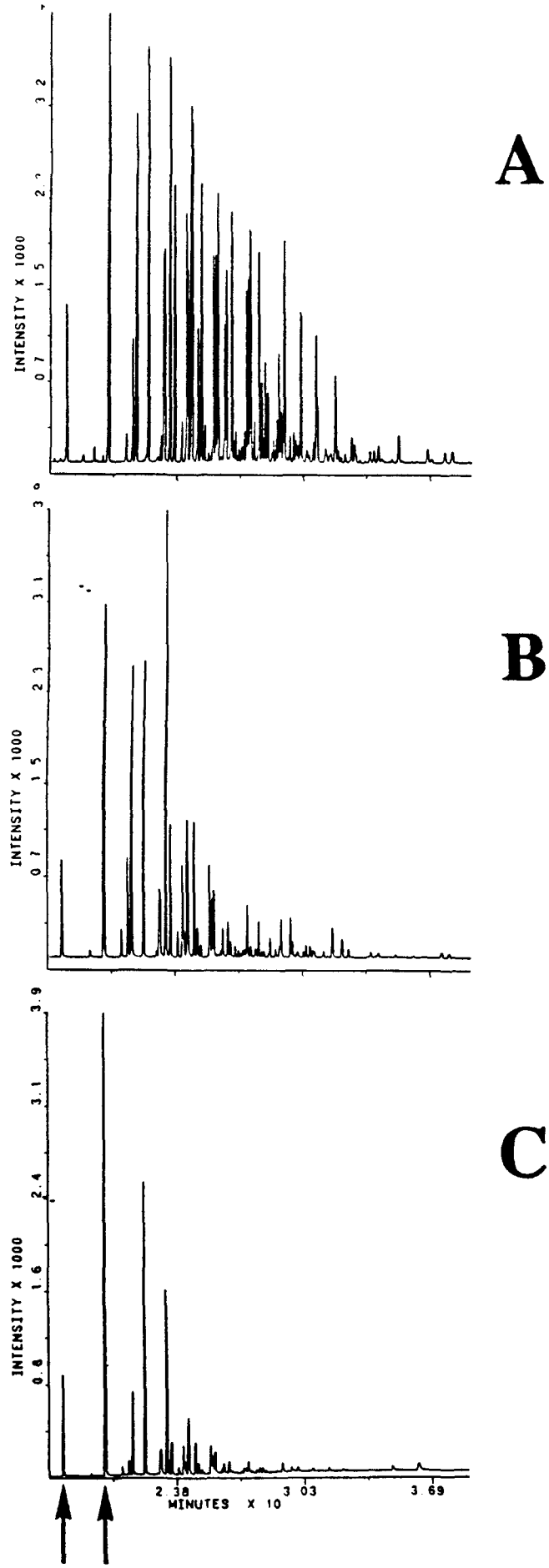


Figure 6-6: PCB distribution at H7 site (Hudson River). Panel A, year 1982; Panel B, year 1985; Panel C, year 1990.

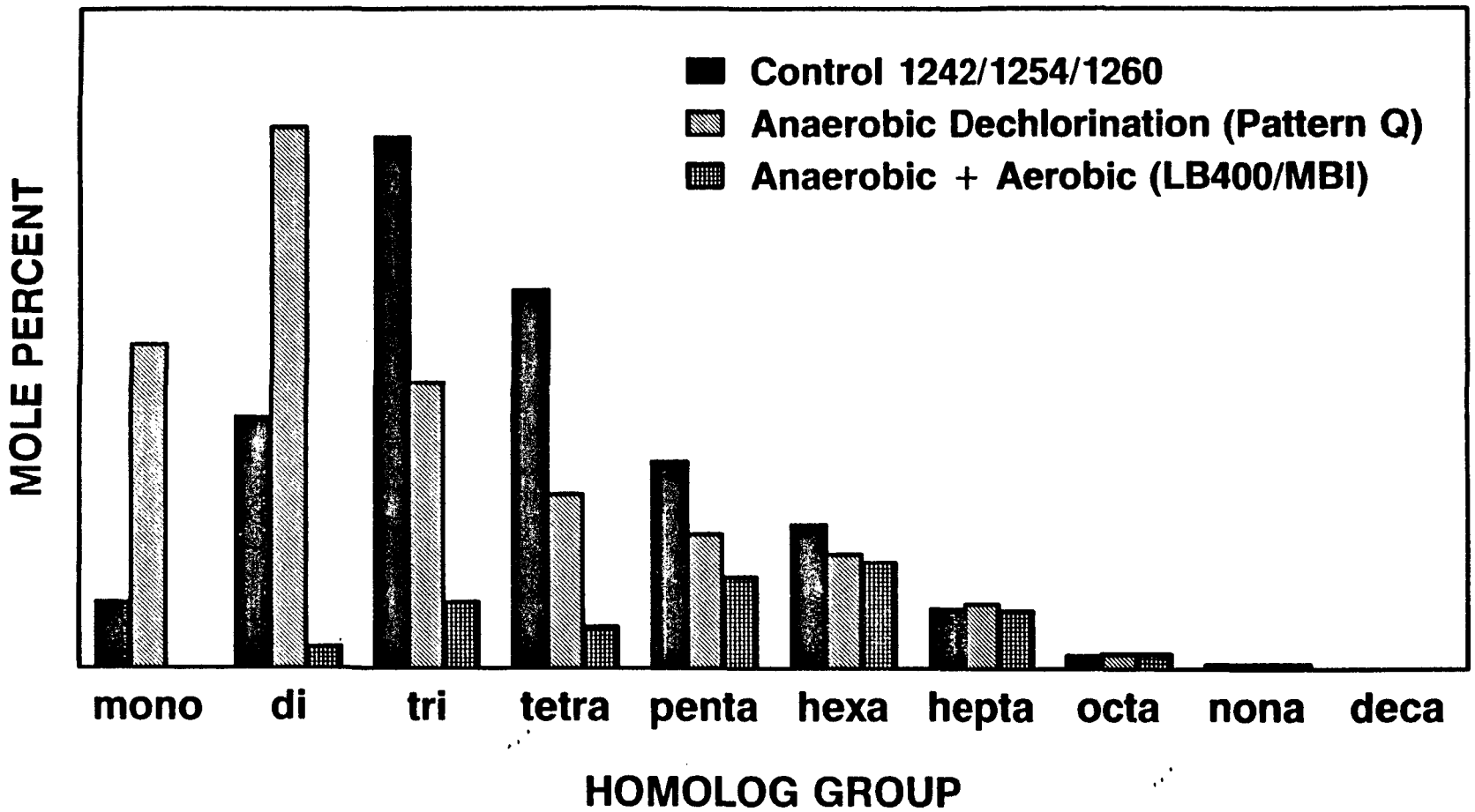


Figure 6-7: Sequential anaerobic/aerobic treatment of spiked PCB contamination in Hudson River sediments

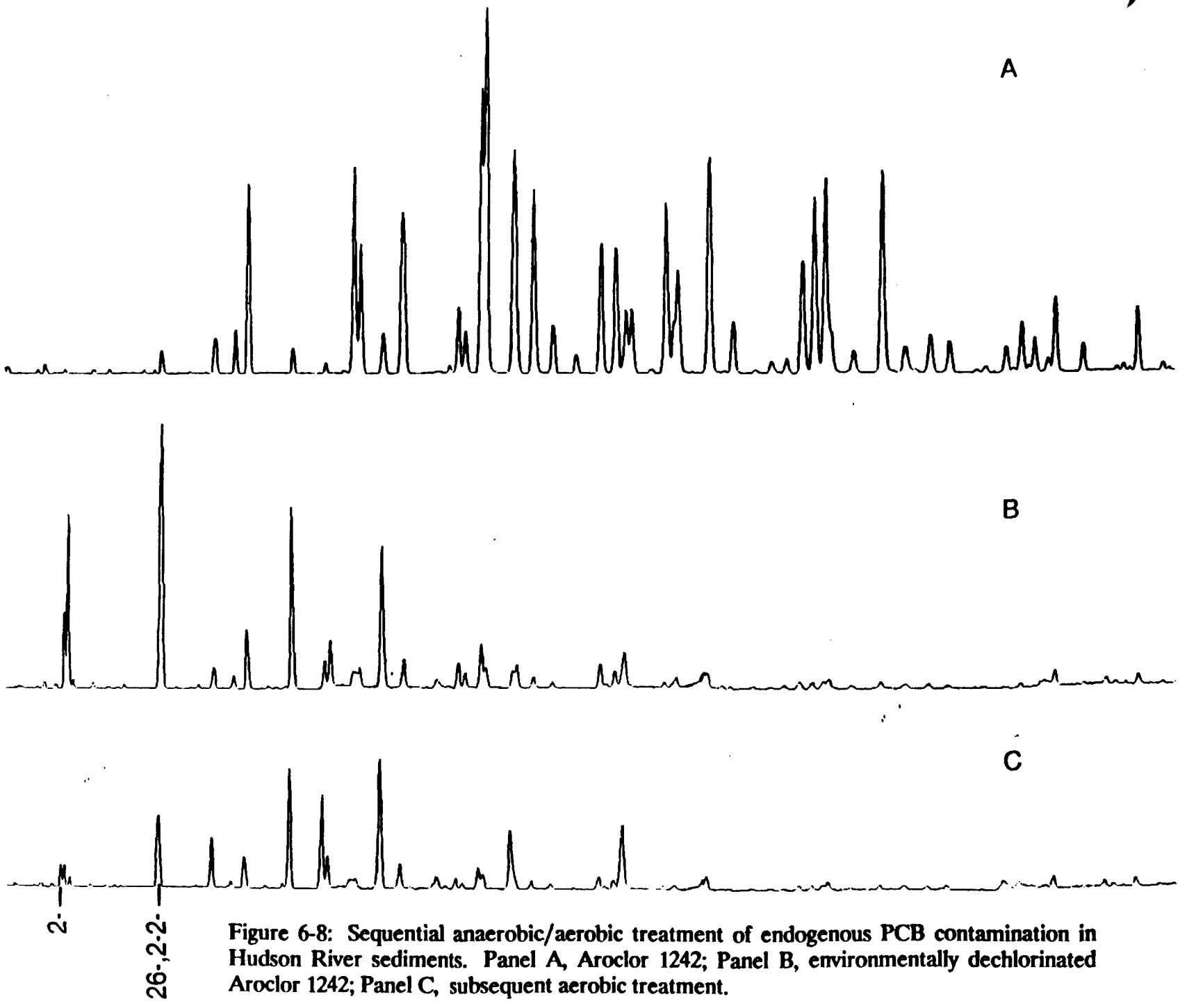


Figure 6-8: Sequential anaerobic/aerobic treatment of endogenous PCB contamination in Hudson River sediments. Panel A, Aroclor 1242; Panel B, environmentally dechlorinated Aroclor 1242; Panel C, subsequent aerobic treatment.

then treated by aerobic PCB-degrading organisms to demonstrate the effect of this combined process (see Figure 6-8). Figure 6-8A represents the original contamination (Aroclor 1242); Figure 6-8B displays a recently obtained sediment sample from the Hudson River that has been environmentally dechlorinated (>85% mono- and di-CB); Figure 6-8C displays the resulting chromatogram after aerobic treatment. This initial trial demonstrated ~80% reduction in the PCB concentration after 1 day of aerobic treatment. Therefore, this two-stage process has resulted in a dramatic change in the PCB distribution to lower chlorinated products, as well as a marked reduction in the PCB concentration.

SUMMARY AND CONCLUSIONS

Hudson River sediments contain anaerobic microorganisms capable of extensively dechlorinating PCB mixtures. The observed rate of laboratory dechlorination can be stimulated by the addition of a trace metal mixture or detergents at low concentrations. This dechlorination occurs over a broad range of temperatures (4-30°C), and the organisms are even capable of attacking the highly chlorinated Aroclor 1260.

Experiments on a variety of PCB-contaminated soils have demonstrated that this anaerobic process will effectively attack endogenous PCB contamination. No significant rate difference was observable for endogenous or spiked PCB samples. In addition, sequential anaerobic/aerobic treatment of the PCB contamination present in Hudson River sediments have resulted in a dramatic shift in PCB distribution to lower chlorinated, *ortho* substituted products and an 80% reduction in total PCB concentrations.

Chapter 7

SEQUENTIAL ANAEROBIC-AEROBIC BIODEGRADATION OF PCBs

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INTRODUCTION

Evidence for the reductive dechlorination of PCBs under anaerobic conditions has accumulated from studies of PCB congener pattern changes of commercial Aroclors in natural sediments [Brown et al., 1984, 1987a,b; GE Reports, 1987, 1988]. Recent laboratory studies confirm that the anaerobic dechlorination of PCBs is due to biological activity [Quensen, 1988]. Several laboratory studies have shown that a broad range of PCB congeners can be aerobically degraded by microorganisms [Bedard et al., 1986, 1987a,b]. Aerobic degradation is usually faster for congeners with fewer chlorine substituents. Although sequential anaerobic dechlorination and aerobic degradation of PCBs have not been observed in natural sediments, extensive aerobic degradation of anaerobically transformed Aroclors has been shown [Bedard et al., 1987b]. Initial attempts at a sequential anaerobic-aerobic biodegradation of PCBs in our laboratory have also been relatively successful. These results provide evidence for the potential of complete *in situ* degradation of PCBs.

Last year we reported on the effects of substrate amendments for enhancing the anaerobic reductive dechlorination of Aroclor 1242 in Hudson River sediments. Acetone and methanol, compounds often used as solvents for the addition of PCBs in laboratory studies, and glucose were all shown to have a beneficial effect on the rate of dechlorination relative to incubations receiving no substrate amendments. These incubations were done in non-autoclaved sediments [GE Report, 1989; Nies and Vogel, 1990]. In addition, Fluid Thioglycollate Medium with Beef Extract (FTMBE) or sediment extract (organic solubilized by autoclaving) were also shown to enhance dechlorination activity [GE Report, 1989].

Since last year, we have completed a more extensive study of substrate amendments by varying the concentration of the different substrates added. These more recent anaerobic studies have all been done without the use of any oxygen scavenging reducing agents, in order to model more closely the environmental conditions that would be encountered during *in situ* bioremediation. We have demonstrated that a sequential anaerobic-aerobic biodegradation scheme is possible in the laboratory by inducing anaerobic conditions in sediments where PCBs were reductively dechlorinated, and then aerobically degrading many of the less chlorinated PCB congeners in those sediments. We have transferred anaerobic Hudson River organisms from sediments to incubations with sterile sand or glass beads, while maintaining dechlorinating activity. We have also shown that anaerobic river sediments that had not previously been contaminated with PCBs can dechlorinate Aroclor 1242 without long acclimation times.

RESULTS AND DISCUSSION

Anaerobic Substrate Effects

Anaerobic batch incubations of Hudson River sediments were started to begin the first phase of the anaerobic-aerobic biodegradation sequence. In order to test independently the effects of different organic substrates on the dechlorinating microorganisms, the sediments were respiked with Aroclor 1242 without the addition of a solvent [GE Report, 1989; Nies and Vogel, 1990]. The batches were inoculated with microorganisms that had been acclimated to the particular substrate being tested. The organic substrates tested were acetate, acetone, butanol, glucose, and methanol. Each substrate was tested at three different concentrations (high, medium, low) across three orders of magnitude (Table 7-1). The sediments were not autoclaved prior to inoculation, as this would solubilize large amounts of organic carbon and potentially mask differences between the substrates being tested. Control batches were autoclaved. Anaerobic conditions were induced by substrate addition and subsequent microbial consumption of oxygen. Batches that had been started previously, without the addition of any organic substrate, had sodium sulfide added to maintain anaerobic conditions.

TABLE 7-1. Substrate amounts added to anaerobic incubations[a]

	Acetate	Acetone	Butanol
H	0.78%	0.67%	0.54%
M	0.078%	0.067%	0.054%
L	0.0078%	0.0067%	0.0054%
	Glucose	Methanol	
H	0.93%	0.67%	
M	0.093%	0.067%	
L	0.0093%	0.0067%	

[a] - Substrate amount given as weight percent of substrate per wet weight of sediment.

H-High level M-Medium level L-Low level

High levels of acetone and methanol, and medium levels of glucose, all enhanced reductive dechlorination with the extent and pattern being nearly identical (Figure 7-1a). High

TABLE 7-2: Congener assignments for chromatographic peak numbers in this report.

Peak number	Congeners in peak		
1	2		
2	2-2	2,6-	
3	2,5-		2,4-
4	2-3		
5	2-4	2,3-	
6	2,6-2		
7	2,5-2	4-4	
8	2,4-2		
9	2,6-3	2,3,6-	
10	2,3-2	2,6-4	
11	2,5-3		
12	2,4-3		
13	2,5-4		
14	2,4-4	2,4,6-2	
15	2-3,4	2,3-3	2,3,4-
16	2,5-2,6		
17	2,4-2,6		
18	2,3-4		
19	2,3,6-2		
20	2,3-2,6		
21	2,5-2,5	3,5-2,6	
22	2,4,-2,5		
23	2,4-2,4		
24	2,4,5-2	2,4,6-4	
25	2,3-2,5		
26	3,4-4		
27	2,3-2,4	2,3,6-3	
28	2,6-3,4	2,3,6-4	2,3,4-2
29	2,3-2,3		
30	2,4,5-4	2,3,5-2,6	
31	2,5-3,4	2-3,4,5	
32	2,4-3,4	2,4,5-2,6	
33	2,3,6-2,5		
34	2,3,6-2,4		
35	2,3-3,4	2,3,4-4	
36	2,4,5-2,5	2,3,5-2,4	
37	2,3,6-3,5		
38	2,4,5-2,3	2,3,4,5-2	2,3,5,6-2,6
39	2,3,4-2,5	2,3,4,6-4	
40	3,4-3,4	2,3,6-3,4	
41	2,3,5,6-2,5		
42	2,4,5-3,4	2,3,4,5-3	2,3,6-2,4,5
43	2,4,5-2,4,5		
44	2,3,4-3,4	2,3,4-2,3,6	

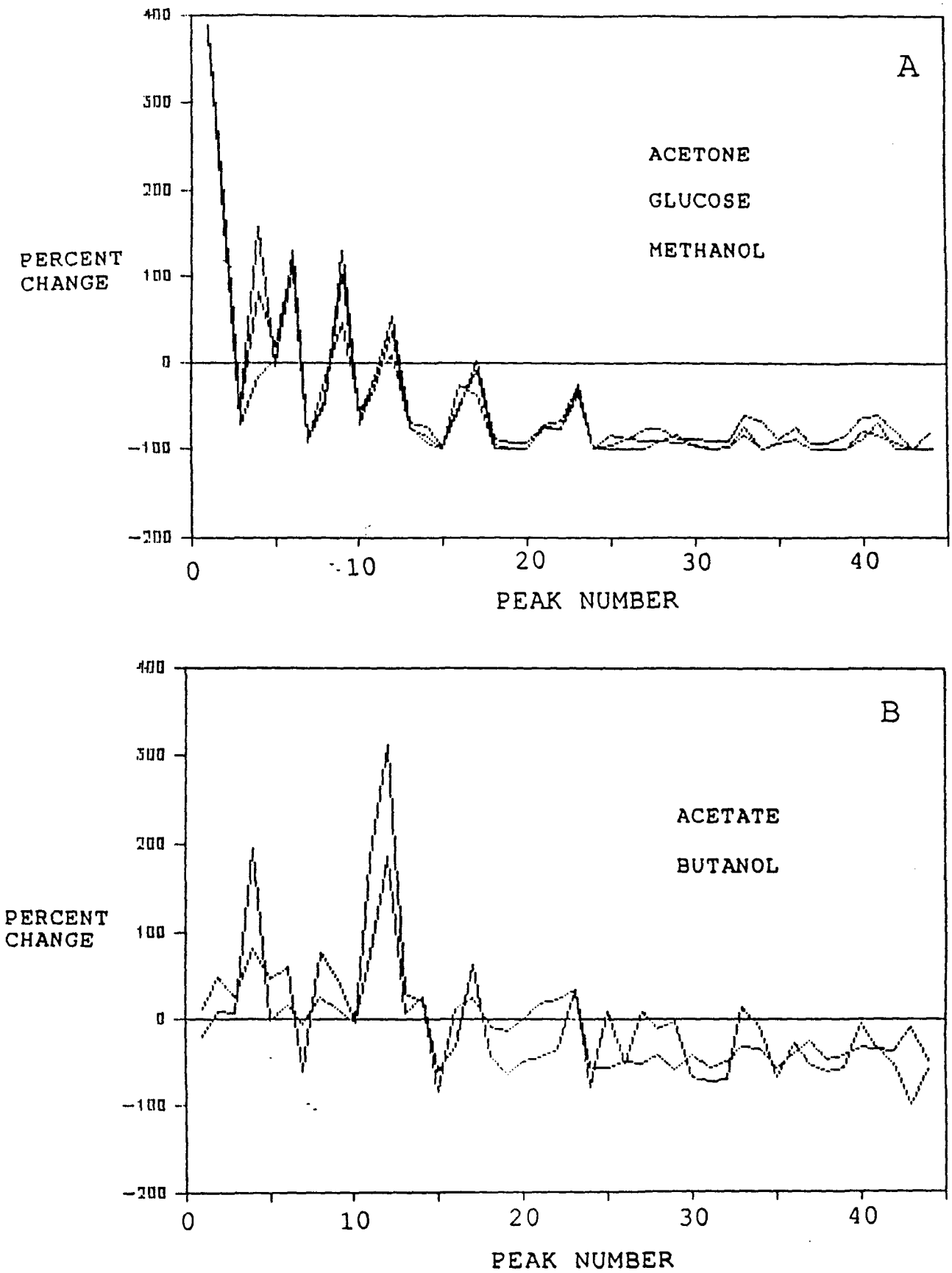

Figure 7-1. Difference plot of anaerobic substrate-fed batches at 20 weeks: a) acetone-, glucose-, and methanol-fed batches; b) acetate- and butanol-fed batches. Note: congener peaks are discrete points, lines are drawn between points for ease of comparison.

levels of glucose caused a drop in pH in those batches, which inhibited dechlorination activity. Acetate and butanol also enhanced dechlorination, relative to batches receiving no substrate, although the patterns were different and dechlorination was not as extensive as the other substrates tested, as indicated by the accumulation of peak number 12 (2,4-3-CB) relative to acetone-, glucose-, and methanol-fed batches (Figure 7-1b). The congener assignments for the peak numbers in Figures 7-1 and 7-3, are given in Table 7-2. After more than 54 weeks incubation, slight dechlorination has been observed in batches receiving no additional organic substrate.

All substrate-fed batches showed an increase in relative rates and extent of reductive dechlorination with an increase in substrate concentration from low to medium levels. The transformation of Aroclor 1242 by reductive dechlorination is illustrated in Figure 7-2 by the decrease in tri-, tetra-, penta-, and hexachlorobiphenyl homologs, and the increase in the mono- and dichlorobiphenyl homologs. After 20 weeks incubation, the mono- and dichlorobiphenyls account for ~ .80% of the total PCBs present. Dechlorination in acetone- and methanol-fed batches also increased when substrate was increased from medium to high levels. An example of the increase in rate and extent of dechlorination with increased substrate levels is shown in Figure 7-3. Results from acetate-, butanol-, and glucose-fed batches indicate that the high substrate levels may have some slight inhibitory effect on dechlorination relative to medium substrate levels.

Microbial Enrichments

Serum bottles with sterile sand or glass beads (4 mm) and mineral medium were spiked with Aroclor 1242 dissolved in acetone. Dechlorinating Hudson River microbial enrichments, acclimated to acetone, were used as an inoculum. Reductive dechlorination has been observed in all batches, roughly coinciding with the onset of methanogenesis. These consortia of microorganisms are only one transfer removed from Hudson River sediments. If successive attempts to transfer these dechlorinating enrichments to inert substratum are successful, this will provide a unique opportunity to study the microorganisms responsible for reductive dechlorination of PCBs in a defined environment.

Dechlorination in Non-PCB-Contaminated Sediments

Aroclor 1242 was added to anaerobic sediments taken from the Saline River, Michigan. Acetone, glucose, and methanol were added to different batches as substrate. After 8 weeks, dechlorination activity was observed in all batches. This sediment has no detectable background levels of PCBs, and no history of previous PCB-contamination. It appears that dechlorination of PCBs by microorganisms does not require long (years) acclimation times. There may be PCB dechlorinating capability across broad classes of microorganisms, rather than adaptation to dechlorination of PCBs by unique organisms.

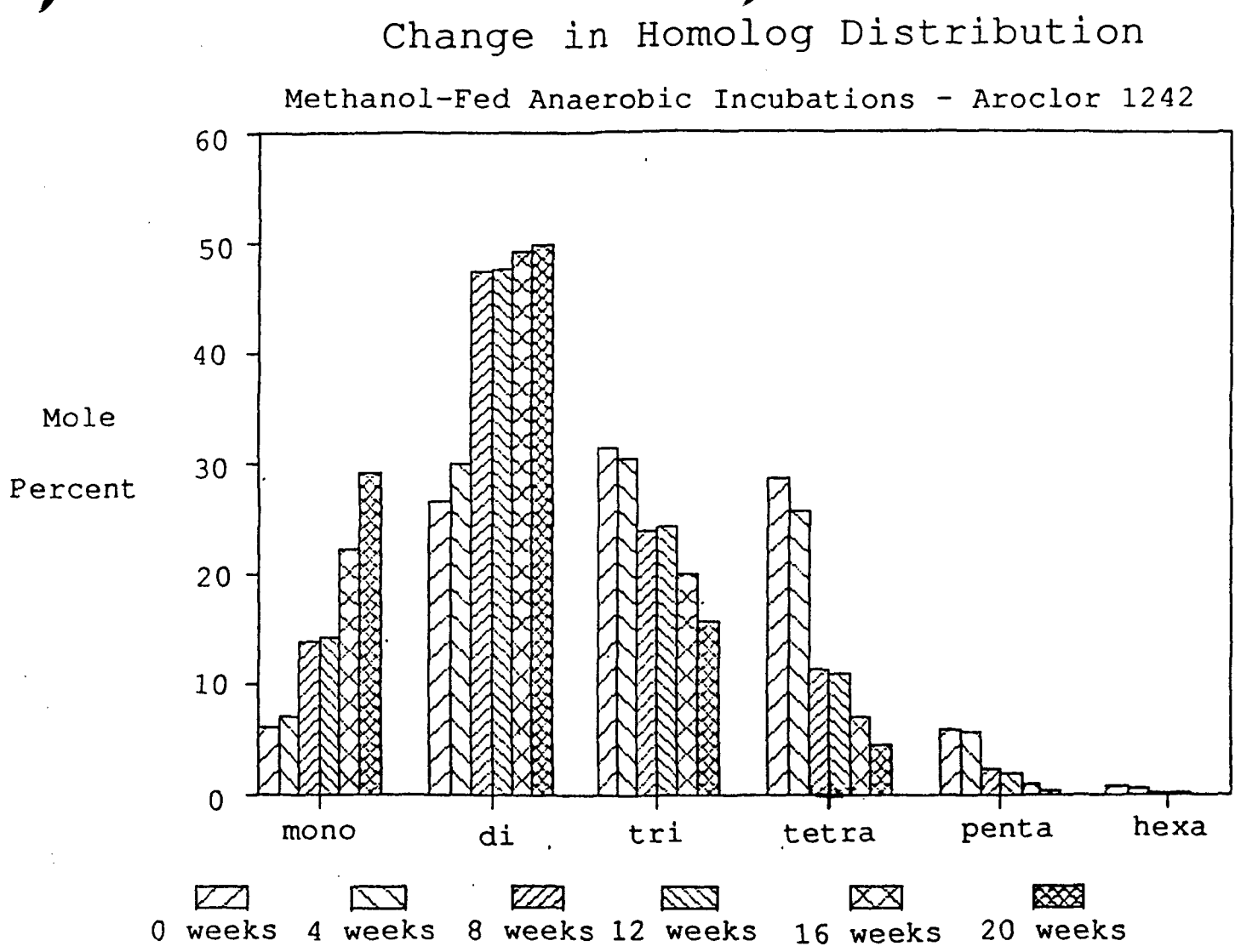

Figure 7-2. Change in homolog distribution of Aroclor 1242 in methanol-fed (high level) batches through 20 weeks.

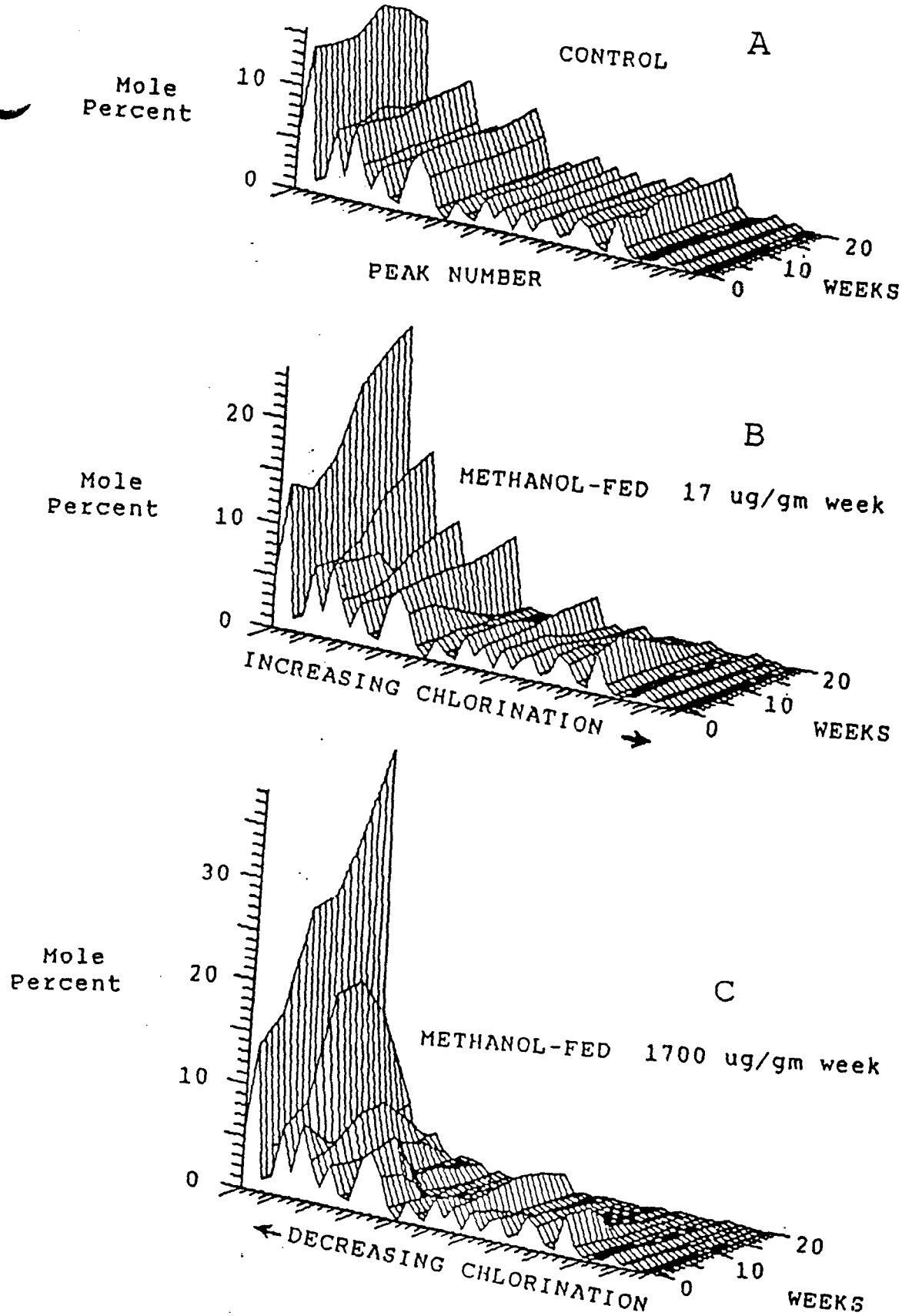

Figure 7-3. Change in congener distribution of Aroclor 1242 in methanol-fed batches through 20 weeks: a) autoclaved controls; b) low methanol level; c) high methanol level.

AEROBIC DEGRADATION

Rapid Assay

Biphenyl-degrading microorganisms were enriched from more than 20 sediment and soil samples. Although some of the samples had no detectable PCBs present, all samples yielded cultures of biphenyl degraders. These cultures were then screened for PCB degradative competence using the rapid assay procedure [Bedard et al., 1986] with the congener mixture shown in Table 7-3 at a concentration of 5 μM/congener. The enrichments varied in their ability to degrade the dichlorobiphenyl congeners, and none of the isolates showed any ability to degrade tetrachlorobiphenyl congeners. The rapid assay results of the two most competent isolates are shown in Table 7-3, along with the results using the pure culture LB400 for comparison. Note that significantly better degradation has been observed with LB400 under similar conditions by others [Mondello, 1989; Bedard et al., 1986]. The Hudson River isolate, S3, was selected to test for aerobic degradation of PCB congeners on anaerobically dechlorinated sediment.

TABLE 7-3. Rapid assay degradation of PCBs by biphenyl grown microorganisms (percent degraded 96 hours)[a]

	Culture		
Congener Mix	16	S3	LB400
2-2	100	100	100
2,3-	100	100	100
4-4	100	100	0
2,5-2,6	0	0	17
2,5-2,5	0	0	75
2,4-2,5	0	0	43
2,4-2,4	0	0	0
3,4-3,4	0	0	0
3,5-3,5[b]	0	0	0
2,4,5-2,4,5	0	0	0

[a] - Degradation of less than 10% is not considered significant and is shown as 0%.

[b] - Internal standard.

Aerobic PCB Degradation in Sediments

Anaerobic incubations (dechlorinated sediments and mineral medium) were purged with pure oxygen for 5 minutes. Approximately 4 mLs of freshly grown S3 was then added to the incubations. After 96 hours, live and autoclaved control samples were extracted. Significant degradation of all mono- and dichlorobiphenyl congeners occurred, except for the di*ortho* substituted 2-2:2,6-CB (Table 7-4). The total PCB concentration in the sediment was reduced from 300 ppm to less than 50 ppm by the sequential anaerobic-aerobic treatment. No trichlorobiphenyls were significantly degraded aerobically. These preliminary results can be improved through longer aerobic treatment, use of a more competent organism, or more extensive dechlorination during the anaerobic phase.

TABLE 7-4. Aerobic degradation of anaerobically dechlorinated PCBs on sediment

Congener	Percent Degraded
2-	85
4-	100
2-2 : 2,6-	18
2,5- : 2,4-	94
2-3	72
2-4 : 2,3-	75

SUMMARY AND CONCLUSIONS

Organic substrate may be a limiting factor for anaerobic reductive dechlorination in some sediments. Anaerobic batches with non-autoclaved sediments, receiving inorganic nutrients and vitamins, showed only slight dechlorination after more than 1 year incubation.

Aerobic biphenyl-degrading microorganisms are extremely common in soils and sediments, although their PCB degradative competence varies considerably. However, it is possible that more discriminatory enrichment techniques could select for more competent PCB degraders.

Using aerobic organisms with only "average" PCB-degrading ability, it is possible to significantly reduce PCB levels on sediments using a sequential anaerobic-aerobic biodegradation process.

FUTURE PLANS

The proposed objectives for the coming year will focus on the transition between the anaerobic and aerobic phases of PCB biodegradation. Anaerobic sediments are relatively

high in organic matter and very reduced, therefore, the sediments would be expected to have a large oxygen demand. The use of pure oxygen or hydrogen peroxide for inducing aerobic conditions will be tested. Methods that are potentially applicable to *in situ* bioremediations will be modeled in the laboratory.

Once a suitable aerobic environment has been established, methods to initiate aerobic PCB degradation will be examined. Ideally, there would be indigenous bacteria throughout the sediment that survived the anaerobic phase. Enrichment techniques that select for the most competent PCB degraders will be tested. In addition, the reintroduction of previously enriched PCB-degrading organisms will be tested in order to determine the optimum conditions for effective aerobic PCB degradation *in situ*.

Chapter 8

HUDSON RIVER MODEL

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INTRODUCTION

In situ application is the ultimate aim of most laboratory bioremediation studies. The goal of these studies is to better understand the naturally occurring processes that might aid in the remediation of a contaminated site. This can help in developing strategies that enhance the degradative abilities of the indigenous microorganisms. Laboratory studies have shown that PCBs can be dechlorinated under both anaerobic and aerobic conditions [GE Report,1988; Brown et al., 1987; Quensen et al., 1988] and aerobically oxidized by mixed cultures and pure cultures of microorganisms [Bedard et al., 1987; Bedard et al., 1986; Bopp, 1986; GE Report, 1987]. Generally, anaerobic microbes reduce the chlorine number and aerobic microbes degrade the lightly chlorinated PCBs that remain.

Anaerobic dechlorination and aerobic degradation have not been shown to occur in sequence within the same natural system. One experiment on a PCB-contaminated soil has been attempted that clearly showed the potential of *in situ* methods to remove PCBs using aerobic organisms, although the degradation efficiency was higher under laboratory conditions [GE Report, 1988]. Unfortunately, an *in situ* method using both anaerobic and aerobic processes has not been attempted yet. However, illustration of the sequential anaerobic-aerobic degradation of PCBs in batch reactors using river sediments is well underway and might provide evidence for the potential of *in situ* biodegradation. Determining whether this technique is likely to succeed depends on results from bench-scale experiments that simulate river conditions.

Because of its past history of PCB contamination, the Hudson River sediments represent an ideal site for testing the feasibility of this *in situ* bioremediation technique. Using both anaerobic and aerobic processes, the objectives of this research are: (1) To model the Hudson River bottom sediment environment in order to determine whether PCBs can be degraded under sequential anaerobic-aerobic conditions using substrate addition to enhance that degradation. (2) To examine the design parameters that influence the degradation process and to relate those parameters to real *in situ* conditions.

RESULTS AND DISCUSSION

The river model consists of a closed glass reactor (1.8 x 0.6 x 0.6 m) (Figure 8-1), filled with 0.8 tons of a lightly PCB-contaminated Hudson River sediment (<5 ppm of mono-, di-, and trichlorobiphenyl congeners). Additional Aroclor 1242 was added and mixed into the sediment to a final concentration of ~ 300 ppm on a wet basis. Anaerobic conditions were created in the sediment by adding methanol in two increments of 6.67 mg/L/week, along with a mineral media, through eight injection wells connected to a multichannel pump. In order to accurately model the flow in the river bed, a flowing stream of water on top of the sediment was permanently recirculated. The flow rate (0.15 L/second) was low enough so that no sediment refluidization occurred. The liquid was circulated for the duration of the experiment, which to date has been 8 weeks. The reactor was kept at room temperature.

In order to monitor dechlorination, eight samples of sediments were collected every 4 weeks using a special coring device through eight top access ports and analyzed. Samples were taken from the bottom, middle, and top of the sediment. Batch incubations were prepared in parallel by adding 15 g of the same sediment (after Aroclor 1242 was added) to 120-mL serum bottles. Methanol was added in one increment to all bottles except three, along with the anaerobic media. Triplicate bottles were kept at 12°C, 25°C and 30°C, respectively. Control bottles were prepared identically and autoclaved twice at 24-hour intervals. All bottles were sealed with Teflon-coated stoppers and aluminum crimp caps. Gas production by the batch incubations was measured by inserting the needle of a wetted, glass-barreled syringe through the stopper of the serum bottle and measuring gas volume. Sample extraction, PCB analysis, and Aroclor peak identification are described elsewhere [Nies and Vogel, 1990].

In order to study the transport and distribution of the substrate into the sediment, a study using bromide as a conservative tracer was also performed on a smaller, 5-gallon model reactor. Water with bromide tracer at 600 ppm was injected into the reactor through a central injection well and the bromide monitored as it moved with the flow past multilevel sampling wells located at varying distances from the injection well. The pumping rate was such that the reactor fluid volume was replaced every 6 hours. The bromide concentration was measured by ion chromatography (IC). The distribution of the bromide tracer into the smaller reactor varied with time and distance from the injection port. Early after pumping began, bromide was found in locations close to the injection well. Total breakthrough was quickly achieved in the bottom of the reactor, whereas it was only after 24 hours of pumping that bromide reached near maximal concentration in the top sand.

Methanol addition to the river model quickly initiated anaerobic conditions within the reactor, as evidenced by the intense methane bubbling at the top of the flowing water. However, considerably less gas was observed whenever the methanol was apparently depleted. After 4 weeks of operation, a shift of the congener pattern from the highly chlorinated congeners to the less chlorinated PCBs was already observed, indicating that a dechlorination process had started. The extent of dechlorination is the removal of chlorine relative to the control. Table 8-1 gives the homolog distribution for Aroclor 1242 at different levels in the

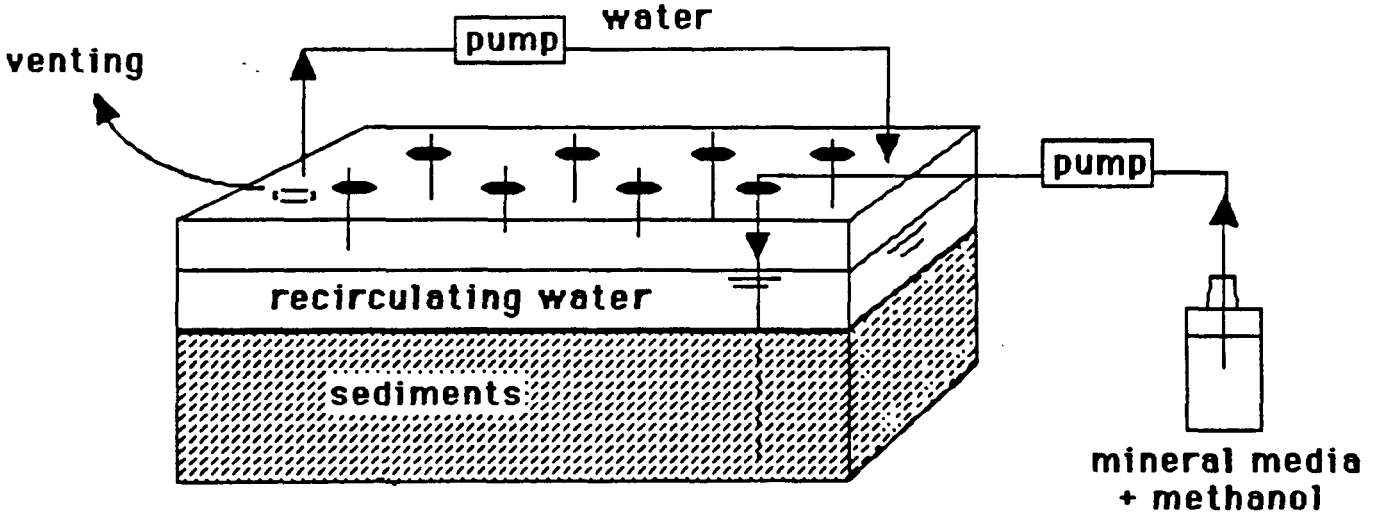

Figure 8-1. Schematic description of the Hudson River model.

TABLE 8-1. Homolog distribution (mol%) and percent change for Aroclor 1242 in the Hudson River model sediment.

Homolog	Week 0[a]	Top Week 4	Top Week 8	Middle Week 4	Middle Week 8	Bottom Week 4	Bottom Week 8
Mono-	2.55	2.94 (+15.1%)	4.13 (+61.6%)	6.94 (+171%)	2.2.76 (+8.0%)	4.02 (+57.4%)	3.52 (+38.1%)
Di	19.50	23.06 (+18.2%)	24.28 (+24.4%)	28.38 (+45.4%)	24.80 (+27.1%)	24.96 (+27.9%)	27.31 (+40.0%)
Tri-	34.31	33.14 (-3.4%)	31.27 (-8.8%)	31.20 (-9.0%)	33.34 (-2.8%)	32.30 (-5.8%)	32.25 (-6.0%)
Tetra-	35.08	32.54 (-7.2%)	31.78 (-9.4%)	26.97 (-23.0%)	30.84 (-12.0%)	30.74 (-12.3%)	29.37 (-16.2%)
Penta	7.57	7.32 (-3.2%)	7.57 (+0.07%)	5.79 (-24.4%)	7.34 (-3.0%)	6.97 (-7.8%)	6.69 (-11.6%)
Hexa-	0.96	0.97 (+1.35%)	0.94 (-1.96%)	0.68 (-28.6%)	0.90 (-6.5%)	0.98 (+2.4%)	0.80 (-13.5%)

[a] The homolog distribution in controls without methanol addition remained unchanged after 8 weeks.

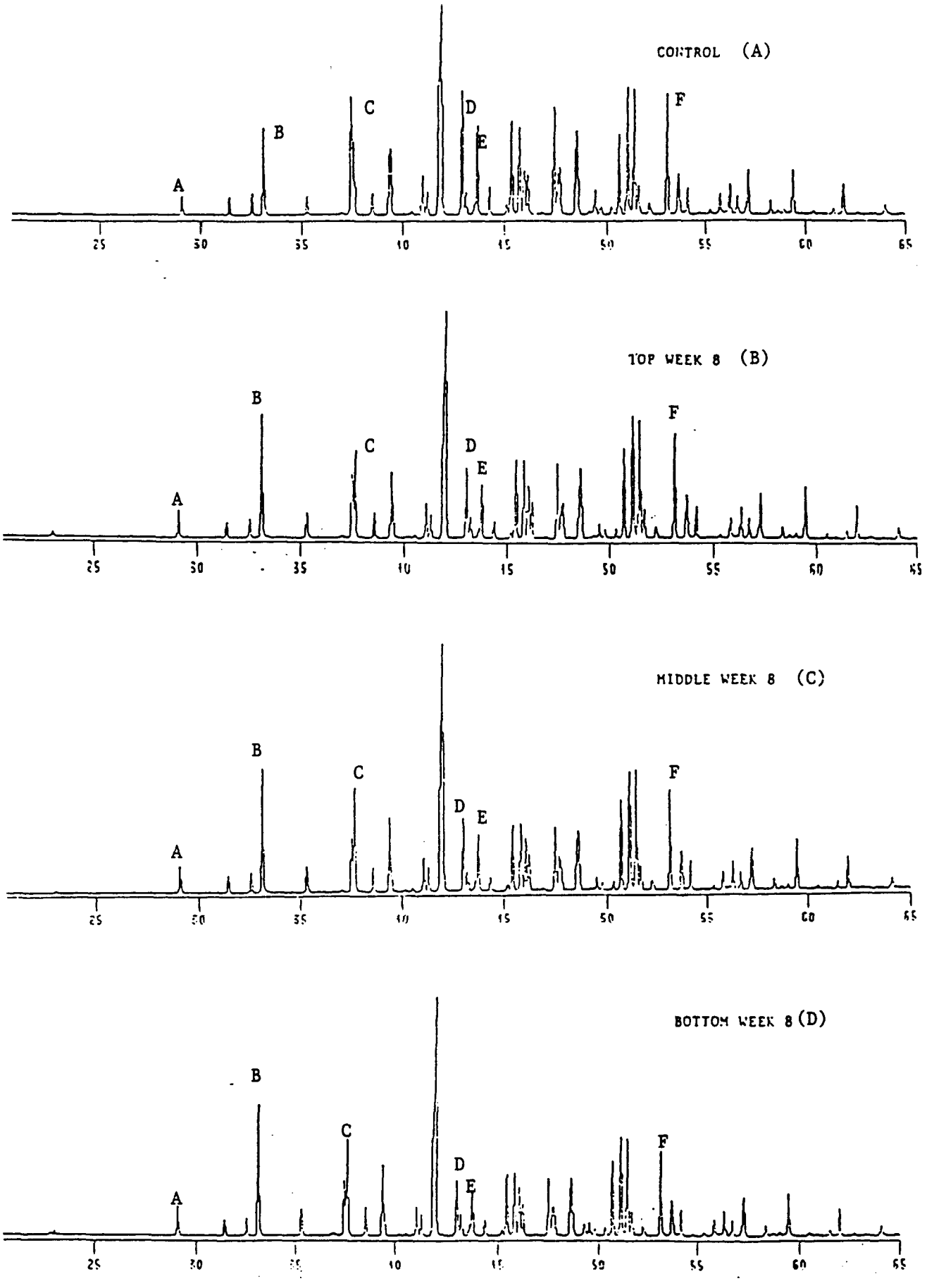

Figure 8-2. Capillary gas chromatograms showing the anaerobic dechlorination of Aroclor 1242 after 8 weeks of operation in the Hudson River model sediments. A) control; B) surface of sediment; C) middle of sediment; D) bottom of sediment.

reactor after 4 and 8 weeks of operation. An increase in the mono- and dichlorobiphenyls was observed relative to levels of week zero. Those levels were unchanged in autoclaved controls. In addition, a decrease in tri-, tetra-, penta- and hexachlorobiphenyl relative to levels in controls was also observed. In general, dechlorination was highest in the middle and bottom of the reactor. The individual congeners that underwent dechlorination are shown in Figure 8-2. In general, congeners 2,3-3,4 and 2,3,4-4-CB (peak F), 2,3-4-CB (peak E), 2,3-3- and 2,3,4-CB (peak D), 4-4- and 2,5-2-CB (peak C) were first dechlorinated as indicated by a reduction in their peak height relative to the control. Congeners 2-4- and 2,3-CB (peak B) as well as 2-2- and 2,6-CB (peak A), were the major dechlorination products after 8 weeks of operation.

No dechlorination was observed in batches incubated at 12°C, whereas incubations at 30°C and at room temperature were similar to the Hudson River model sediment in the extent and pattern of dechlorination. Gas production was monitored in the batch incubations. In general, the volume of gas measured was lowest in the 12°C incubations and highest in batches kept at room temperature and at 30°C.

SUMMARY AND CONCLUSIONS

So far, anaerobic reductive dechlorination has been demonstrated in laboratory batch experiments. In PCB-contaminated sediments, anaerobic microbial communities seem to mediate the dechlorination reaction. However, in nature dechlorination is a slow process. This study is the first attempt to initiate dechlorination through substrate addition while simulating at the same time *in situ* conditions. Methanol injection was able to initiate anaerobic conditions and dechlorination without using a reducing agent. The pattern of the shift in congener distribution is indicative of active dechlorinating activity taking place spatially and temporally within the reactor. In addition, the similarity of the results between the batch incubations and the river model sediment suggests that it might be possible to translate small-scale work to *in situ* conditions.

FUTURE WORK

The focus will be on the aerobic phase of the study. Once an appreciable amount of dechlorination products have been obtained, the addition of methanol will be stopped and the anaerobic layer will be oxygenated by adding hydrogen peroxide through the injection wells. Eventually, most of the sediment should be aerobic. At this point, the upper aerobic layer of the sediment that contains aerobic organisms adapted to PCB degradation will be pumped into the reactor, introducing PCB degraders into the bulk of the sediment. It is hoped this will mediate the oxidation of the less chlorinated congeners.

Chapter 9

DIFFERENTIATION OF ANAEROBIC MICROBIAL DECHLORINATION PROCESSES

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INTRODUCTION

It has long been recognized that the gas chromatographic patterns of the PCB mixtures recovered from environmental samples often differed from those of the commercially used compositions (e.g., Aroclors) originally released, indicating that changes in PCB congener distribution had occurred. These changes were originally attributed to "weathering" of the environmental PCBs. Several years ago we began to recognize that "weathering" encompassed a wide variety of physico-chemical and (usually) biochemical alteration processes, each of which was associated with a characteristic pattern of selection among the various PCB congeners present in the commercial PCB product originally released. As a result, each environmental alteration process leaves a distinctive distribution of residual PCBs, and hence a characteristic pattern of alteration in the gas chromatogram. Since then, we have been collecting "weathered" PCB samples and chromatograms from all accessible sources, and studying them to determine the nature of the transformation processes that had been underway. These surveys have shown that the environmental processes capable of altering PCB congener distributions included inter-phase partitioning, aerobic microbial biodegradation, and aerobic microsomal metabolism, all of which were known prior to our studies, and anaerobic microbial dechlorination, which was not.

The reductive dechlorination of PCBs that is effected by anaerobic microbial action was originally recognized in the gas chromatograms of aquatic sediments from a variety of sources [GE Reports, 1984-1989; Brown et al., 1984; 1987a; 1987b; 1990], but has more recently been duplicated in several laboratories [GE Reports, 1987-1990; Quensen et al., 1988]. From the very beginning it was recognized that anaerobic microbial reductive dechlorination is not a single process, as if carried out by just one dechlorinase enzyme system in just one strain of bacteria, but instead a group of related processes, each characterized by its own pattern of selection among PCB congeners as substrates, and hence yielding a mixture of residual and partially dechlorinated PCB congeners which exhibits a characteristic gas chromatographic profile. These profiles were given letter designations which reflected either the order of their discovery or their source. Thus far, 11 different GC profiles (B, B′, B*, C, E, F, G, H′, H*, N, and W) have been seen repeatedly in environmental samples, and at least two others (K and L) seem indicated by limited data from the St. Lawrence. Six of these environmental profiles (B, C, E, H, H′, and N) have now been duplicated in laboratory cultures. In addition, the laboratory cultures have often yielded alteration patterns (e.g., H, H′, J, M, N, or Q) which were different from those exhibited by the sediments used as

inocula. The reasons for this appear to be two-fold. First, the particular strain of PCB-dechlorinating microbe that becomes predominant under conditions of laboratory culture may not be the one that was predominant when the bulk of the environmental dechlorination occurred. Second, some of the patterns observed in the environmental samples result from the action of two or more individual dechlorination systems, which may be segregated under the appropriate conditions of laboratory culture. Thus, the environmental Pattern C of upper Hudson River sediments has been found to result from the combined action of the dechlorination systems responsible for Patterns M and Q [GE Report, 1989] and Pattern H′ probably results from the action of System H with a small contribution from B, M, or J, depending on the site.

In order to differentiate the observed dechlorination processes, it appeared important to develop objective criteria for characterizing the congener selection patterns seen. Accordingly, a detailed analysis of the congener selection patterns in the 15 best-documented dechlorination systems was undertaken.

RESULTS AND DISCUSSION

The resulting Table 9-1 shows that the 15 dechlorination systems examined differ in four general characteristics. First, in their relative preferences for attacking chlorines located in the *ortho*, *meta*, and *para* positions on the ring. Systems M and N have a strong preference for removing *meta* chlorines; Q and probably E for removing *para* chlorines; while the remainder attack chlorines in either position with comparable ease. However, only the systems from the Housatonic River drainage (F, G, and certain new cultures described elsewhere in this report) have thus far been found to remove *ortho* chlorines.

Second, the observed systems differ in their preferences for attacking lightly, moderately, or heavily chlorinated PCB congeners. Most of them appear to have mild preferences for attacking the more lightly chlorinated congeners, in line with the mildly inhibitory effects of opposite ring substitution which have been previously detailed [Brown et al., 1987b; 1990]. However, Systems E and F seem to prefer to attack the higher congeners, and G and N show an intermediate behavior. These sort of preferences are most marked for System F, which attacks most heavily chlorinated PCBs both preferentially and indiscriminately, and for System M, which attacks only a few congeners carrying more than four chlorines, but can be very active on the lower congeners.

Third, the systems differ in their overall selectivity pattern in attacking higher PCB congeners, both in terms of substitution pattern on the ring being attacked and the sensitivity of the process to *ortho* substitution on the opposite ring. These patterns have been worked out in detail for Systems B, H, and H′ [Brown et al., 1987b; 1990], but can be summarized in more general terms for the other systems as in Table 9-1.

Table 9-1. Congener selectivity patterns shown by environmental PCB dechlorination systems‡

System	Source	Position and Range of Attack	Higher PCB Removal Pattern	Range of Reactivities for PCBs with Indicated Mono- or Dichlorophenyl Group					
				3,4-	2,3-	2,5-	2,4-	4-	3-
B	Upper + mid-Hudson R (NY) + cultures	*m,p*-L	III-4	4	4	2b	1	0?	1?
B′	Upper Hudson R (NY)	*m,p*-L	III-4	4	4	2-3b	2	0?	2
B*	Sheboygan R (WI)	*m,p*-L	III-4	4	4	3b	1	0?	2
C	Upper Hudson R (NY) + cultures	*m,p*-L	III-4	4	4	4	3-4	4	2
E	Upper Hudson R (NY) + cultures	*m,p*-H	II-4	?	?	?	?	?	?
F	Silver Lake (Pittsfield, MA)	*o,m,p*-H	III-4	4	<3	0	0	0	0
G	Silver Lake (Pittsfield, MA)	*o,m,p*-M	III-4	4	4	2-3b	0?	?	2
H	(Several sites† + cultures)	*m,p*-L	II-3	3	2	0	0	0	0
H′	(Several sites† + cultures)	*m,p*-L	II-3	3	3	0	0	0	0
H*	Sheboygan R + Harbor (WI)	*m,p*-L	II^+-3	3	2	0	0	0	0
J	New Bedford (MA) cultures	*m,p*-L?	III-4	4a	3	0	0	0	0
M	Upper Hudson R (NY) cultures	*m,p*-LL	I-4	4a	4	4	0	0	2
N	Woods Pond (MA) + cultures (on 1260)	*m,p*-M	III-4	?	?	?	0	?	?
Q	Upper Hudson R (NY) cultures	*m,p*-L	III-4	4	4	0?	3-4	4	0?
W	Waukegan Harbor (IL)	*m,p*-L?	II^+-3	3-4	3-4	2b	0	0?	2

‡KEY. Chlorine positions attacked: *m,p* = *meta, para*; *o,m,p* = *ortho, meta, para*; underline indicates preference. Chlorination range attacked: L, M, H = mild preference for lower, medium, or higher congeners, respectively; LL = strong preference for lower congeners. Pattern of attack on higher congeners: I = 236-XY > 234-XY >> 245-XY, etc. II = 234-XY > 245-XY > 235-XY >> 236-XY; 2345-XY, 2346-XY >> 2356-XY. II^+ = ditto except 234-XY ≃ 245-XY. III = 234-XY ≥ 245-XY > 236-XY > 235-XY but range of relative reactivities small; 2345-XY, 2346-XY, 2356-XY all reactive. Effect of opposite ring substitution pattern on reactivity of indicated chlorophenyl group (or 245-chlorophenyl group in a higher congener): 0 = indicated group never dechlorinated; 1 = only congeners lacking 2′ substitution attacked; 2 = congeners substituted 2′ but not 2′X′ attacked; 3 = congeners substituted 2′X′ but not 2′6′ attacked; 4 = all congeners carrying indicated group attacked. Modification of usual 2′6′ substitution effects on reactivity: a = 2′6′ substituted congeners attacked at least as easily as less *ortho* substituted types. b = indicated *ortho* substituted type may be more reactive than non-*ortho* substituted species (e.g., 25-3, 25-4 CB).

†New Bedford (MA); Pensacola (FL); Bridgeport (CT); Woods Pond (MA); mid-Hudson R. (NY); upper Hudson cultures.

Finally, and most characteristically, the various systems differ in their selectivity patterns for attacking the lower PCB congeners, again in terms of both the substitution pattern on the ring being attacked and the sensitivity to various types of substitution on the opposite ring (Table 9-1, last six columns). Basically, most systems can attack 3,4- or 2,3-dichlorophenyl groups in most of the lower congeners, but encounter increasing limitations with 2,5-, 2,4-, 3-, or 4-chlorophenyl groups. The limitations are manifest mainly in the range of PCB congeners containing such groups which can undergo attack; increasing substitution on the opposite ring, especially *ortho* substitution, limits the range of reactivity.

Incidentally, on Table 9-1 no data is given in the last six columns for System E because so far it has only been observed in environmental samples which also exhibited dominant System B or C dechlorination in the lower congener range, or in early cultures on Aroclor 1260, which contains almost no lower congeners to start with. Data has also been omitted for the action of System N or lower congeners, in this case because we are not absolutely sure whether the system responsible for transforming Aroclor 1260 in culture (and observed in Woods Pond) is identical to that responsible for transforming Aroclor 1242 in parallel cultures examined by Quensen. The latter system is very similar to System M in its lower congener reactivity preferences, but also (unlike M) attacks the higher congeners present (at low levels) in 1242 as well.

SUMMARY AND CONCLUSIONS

In summary, as a result of the differences in range of PCB congeners attacked it is possible to distinguish among, and thereby characterize, at least 13 different anaerobic microbial dechlorination systems (allowing Systems C and H′ as composites) and to recognize that several others may be already hinted at from fragmentary chromatographic data. Thus far, only Patterns H and H′ have appeared in either sediments or cultures from more than one drainage basin. Otherwise, it would appear that each of the more heavily studied drainage basins (e.g., the Hudson, Housatonic, Acushnet, Sheboygan, and Waukegan) has produced its own distinctive assemblage of dechlorination systems in response to the availability of PCBs as terminal electron acceptors for anaerobic microbial metabolism. Whether these assemblages arise from local mutations to produce the requisite dechlorinases, or from natural selection guided by local environmental features, is still unknown.

Chapter 10

GENETIC STUDIES OF BACTERIAL PCB DEGRADATION: 1. BACTERIAL SURVIVAL ON PCB-CONTAINING SOIL 2. ANALYSIS OF *bph* GENES

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INTRODUCTION

The ability to develop a practical process for aerobic PCB biodegradation depends largely upon obtaining organisms with suitable characteristics. Two of the most important attributes are (1) high levels of degradative activity against a wide variety of PCB congeners, and (2) the ability to survive and retain activity on PCB-containing soils long enough for significant degradation to occur. The survivability of naturally occurring and genetically engineered PCB-degrading bacteria on contaminated soil is being examined in the laboratory. In addition, other properties which may influence the utility of these strains are being investigated.

We are also continuing our efforts to develop recombinant bacteria with superior PCB-degrading abilities. Biphenyl dioxygenase (encoded by the *bphA* gene) is the enzyme primarily responsible for PCB degradation. Our approach is to construct a wide-host-range plasmid in which *bphA* activity is controlled from a high expression promoter. This should lead to the increased production of biphenyl dioxygenase and thereby an increase in PCB-degrading activity. In order to accomplish this goal the nucleotide sequence of the *bphA* gene is being determined and the influence of adjoining DNA regions on PCB degradation is being examined.

RESULTS AND DISCUSSION

Survival of Genetically Engineered Bacteria on PCB-Containing Soil

The need to repeatedly add organisms to contaminated soil severely reduces the utility of either *in situ* or reactor-based bioremediation processes. The use of soil isolates in such processes does not guarantee that these organisms will survive on PCB-laden soil. For example, in the field study conducted by Unterman et al., [1988a], a soil isolate (LB400) with good activity against many PCB congeners, exhibited poor soil survivability and therefore had to be repeatedly applied to the site [GE Report, 1988]. Previous studies have demonstrated that the genetically engineered *Escherichia coli* stain FM4560 (containing the LB400 *bph A, B* and *C* genes), degrades PCBs nearly as well as LB400 while displaying characteristics which may be advantageous in a bioremediation process [Mondello, 1989; GE Report, 1988]. Among the potential advantages was greater survivability in laboratory media containing PCBs.

Recently, the survivability of FM4560 and LB400 were compared on PCB-contaminated soil from the Glens Falls dragstrip site. This material contains ~ 550 ppm of highly evaporated Aroclor 1242 and thus is similar in composition to Aroclor 1248. A series of 2 dram vials containing 0.2 g of dragstrip soil were inoculated with 0.1 mL of either FM4560 or LB400 (~10^8 cells/vial), sealed, and incubated at 23°C without shaking. Separate vials were sampled at each timepoint by the addition of 2.0 mL of phosphate buffer (50 mM, pH 7.5), and vortex mixing at maximum speed for 2.0 minutes. Samples were serially diluted and spread onto minimal medium plates containing biphenyl, 0.02% succinate and 5 μg/mL tetracycline (for LB400 samples). FM4560 cells were plated onto both MaConkey and Luria agar plates containing 200 μg/mL ampicillin. Colony formation was used to measure cell survival. The presence of active *bph* genes in LB400 was determined by growth on biphenyl, while *bph* gene activity in FM4560 colonies was demonstrated by their ability to produce yellow *meta* cleavage product when sprayed with an ether solution of biphenyl or 2,3-dihydroxybiphenyl.

Similar survival curves were obtained for FM4560 and LB400 (Figure 10-1). Early timepoints show a significant increase in cell numbers resulting from growth on stored intracellular nutrients. After 72 hours the cell numbers returned to their original levels and continued to decrease such that by 8 days ~ 20% of the cells remained viable. Of the initial inoculum, 2% of the cells were found to survive after incubation for 28 days.

The presence of active *bph* genes in the surviving cells was examined at each timepoint. For LB400 the percentage of viable cells unable to grow using biphenyl remained relatively stable for the first 8 days at ~ 2.5% and then increased to 4.2% for the remainder of the experiment. Virtually all (99.8%) of the FM4560 colonies contained active *bph* genes. Those that did not were restreaked onto selective media and tested again. In each instance the cells were found to contain active *bph* genes. An examination of the plasmids in these cells revealed no alterations in their *Eco*RI restriction patterns indicating that no substantial DNA rearrangements or deletions had occurred.

The survival of LB400 for long periods on PCB-contaminated soil was unexpected as previous investigators have reported that LB400 cells rapidly lost viability upon exposure to soil from the dragstrip site. The different results obtained in the previous experiment may be due to the use of a different experimental protocol. In those experiments bacterial cultures were added to the soil and allowed to air dry. The more recent studies were conducted in sealed vials where the moisture level was constant. Experiments are in progress to determine the effect of drying on cell viability. Somewhat less surprising was FM4560's ability to survive on PCB-containing soil. Several studies had demonstrated long-term survivability of recombinant *E. coli* strains on soils; however, none of this work involved PCB-contaminated material [Devanas and Stotsky, 1986; Trevors, 1987].

Instability of LB400 *bph* Genes

In 1989, we reported that cultures of LB400 grown without biphenyl contained an unusually large number of cells which had lost the ability to degrade PCBs [GE Report,

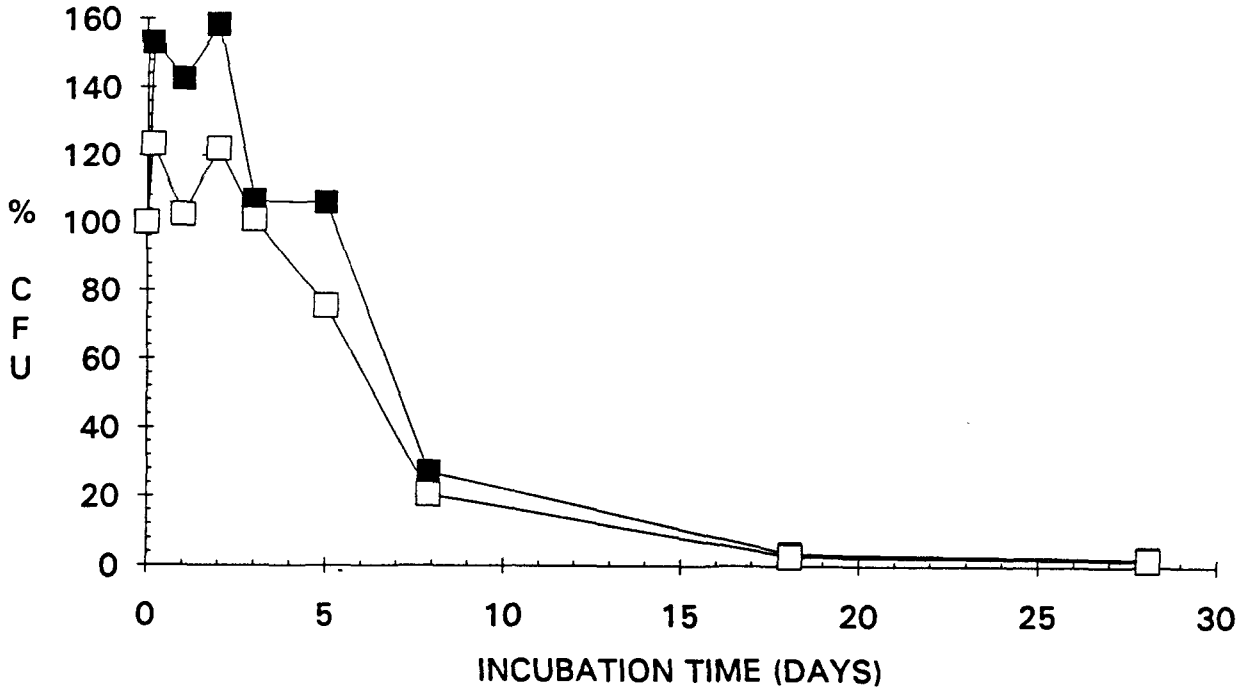

Figure 10-1. Survival of FM4560 and LB400 on PCB-contaminated soil. FM4560, Closed Boxes; LB400, Open Boxes.

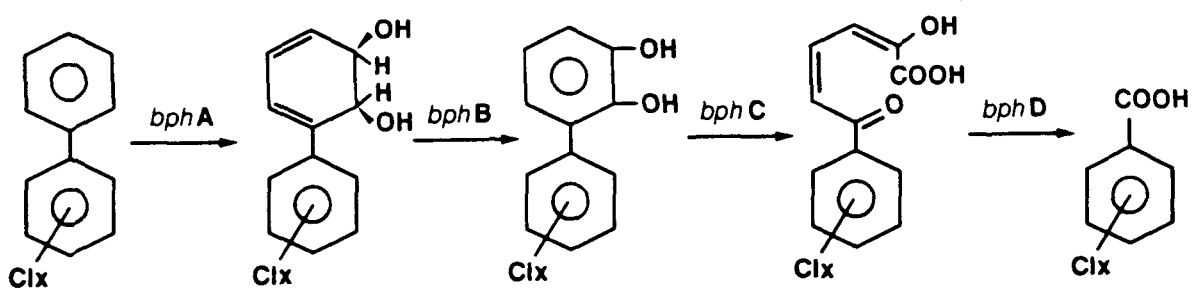

Figure 10-2. Degradation of biphenyl and chlorobiphenyls by the 2,3-dioxygenase pathway in *Pseudomonas* strain LB400. Gene designations: *bphA*, biphenyl 2,3-dioxygenase; *bphB*, dihydrodiol dehydrogenase; *bphC*, 2,3-dihydroxybiphenyl dioxygenase; *bphD*, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (*meta*-cleavage compound product) hydrase.

1989]. Analysis of these spontaneous mutants revealed that many had lost all of the enzymatic activities encoded by *bph A, B, C,* and *D* (Figure 10-2). Southern hybridization experiments revealed that these strains had undergone a deletion of their *bph* structural genes. The potentially serious effects of this phenomenon on LB400's utility in a PCB bioremediation process has led us to investigate this phenomenon in more detail.

1. Bph Mutation Frequency

Biphenyl grown colonies of LB400 were inoculated into *Pseudomonas* minimal medium (PMM) containing 0.5% sodium succinate as the carbon source. The cells were maintained in logarithmic growth phase for 100 generations by serial passage into fresh media. The ratio of Bph^-/Bph^+ cells was determined by colony counts on minimal medium containing biphenyl and 0.05% succinate. The results of six independent experiments are shown in Table 10-1. The percentage of mutant strains in the cultures ranged from 0.6 to 3.2 and with an average of 1.5%. Of the 64 mutant strains isolated, 62 (96.9%), were stable (reversion frequency $<1/10^{10}$). Southern hybridization analysis of of these mutant strains confirmed that they had lost the *bph* structural genes.

TABLE 10-1. Spontaneous mutagenesis of the LB400 *bph* genes

Experiment	# of Colonies	# Bph^-	# Stable[a]	% Loss[b]
1	452	9	8	1.8
2	310	10	10	3.2
3	591	6	6	1.0
4	1067	6	6	0.6
5	1515	22	21	1.4
6	1287	11	11	0.9

Average = 1.5%

[a] these mutants reverted to the Bph^+ phenotype at a frequency of less than 1 in 10^{10}

[b] % loss determined by number of stable mutants/number of colonies examined

2. Deletion Specificity in the LB400 Genome

Wild-type cultures of LB400 can be grown on minimal salts medium and succinate without the addition of growth factors such as amino acids, nucleotides, or vitamins. A mutation in any of the >100 genes involved in the biosynthetic pathways for these compounds will result in an auxotrophic strain unable to grow in a minimal medium. To determine whether

the high spontaneous mutation frequency is specific for the *bph* genes, the number of auxotrophic mutants appearing after growth of LB400 on a complete medium was examined. A culture of LB400 was maintained in logarithmic growth phase on Luria-Bertani medium for 100 generations by serial passage into fresh medium. The percentage of auxotrophic mutants in this population was determined by replica plating colonies of these cells grown on Luria agar plates onto minimal agar plates supplemented with 0.2 % succinate. Of the 987 colonies tested, none were found to be auxotrophic, indicating that the deletion phenomenon does not occur throughout the LB400 genome and may be *bph* specific.

3. Growth Rate Comparison

If the Bph^- mutant strains grow faster than the wild-type they will eventually dominate the culture and PCB degrading activity will be lost. In order to determine whether the Bph^- strains had a selective advantage in growth rate over the Bph^+ cells we compared the growth curves of the two organisms in PMM + 0.5 % succinate. As shown in Figure 10-3, both LB400 (Bph^+) and LS2 (Bph^- mutant of LB400) have nearly identical growth curves and therefore the ratio of Bph^-/Bph^+ cells in a mixed culture will tend to remain constant.

4. *Bph* Gene Stability in Recombinant Bacteria

Escherichia coli strain FM4560 contains the recombinant plasmid pGEM456 (ampicillin resistance [Ap^r], *bphA, B,* and *C*) and has PCB-degrading ability similar to that of LB400 [Mondello, 1989]. In order to evaluate the ability of this strain to stably retain its PCB-degrading ability, FM4560 was maintained in logarithmic growth phase for 72 hours with and without antibiotic selection in Luria-Bertani broth. The presence of active *bph* genes was determined by the ability to accumulate *meta* cleavage product when treated with either solutions of biphenyl or 2,3-dihydroxybiphenyl. When grown in the presence of ampicillin (to select for plasmid-containing cells), all colonies tested throughout the experiment contained active *bph* genes. In the absence of antibiotic selection, an average of 8.3% of the cells had lost both antibiotic resistance and *bph* activity by 21 hours (Figure 10-4). The loss of Bph^+/Ap^r cells in the population decreased exponentially and by 72 hours was reduced to an average of 31.7%. The loss of *bph* gene activity was always accompanied by the loss of antibiotic resistance suggesting that the entire pGEM456 plasmid had been lost from these cells. An examination of 24 Bph^-/Ap^s strains for plasmid content confirmed the absence of plasmid DNA in these cells. These data indicate that the pGEM456 plasmid is lost or maintained as a single unit and is not subject to the deletion phenomenon associated with the *bph* genes in LB400. Similar results were also observed in the soil survival experiment described above. The loss of plasmid DNA from cells growing in the absence of antibiotic selection is common and results from the faster growth rate of plasmid-free cells. These data suggest that in the recombinant strain, PCB-degrading activity can be stably maintained through simple antibiotic selection.

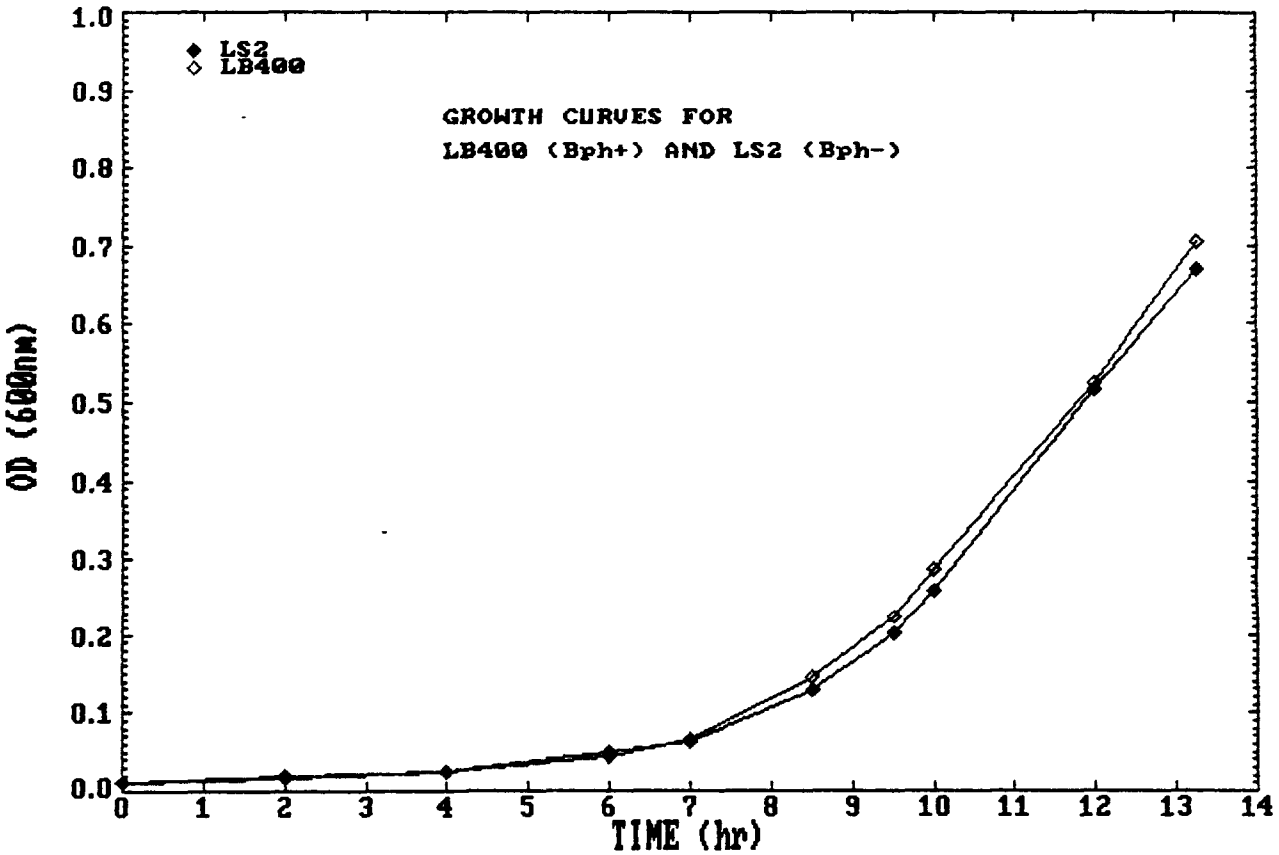

Figure 10-3. Comparison of the growth curves for strains LB400 and LS2.

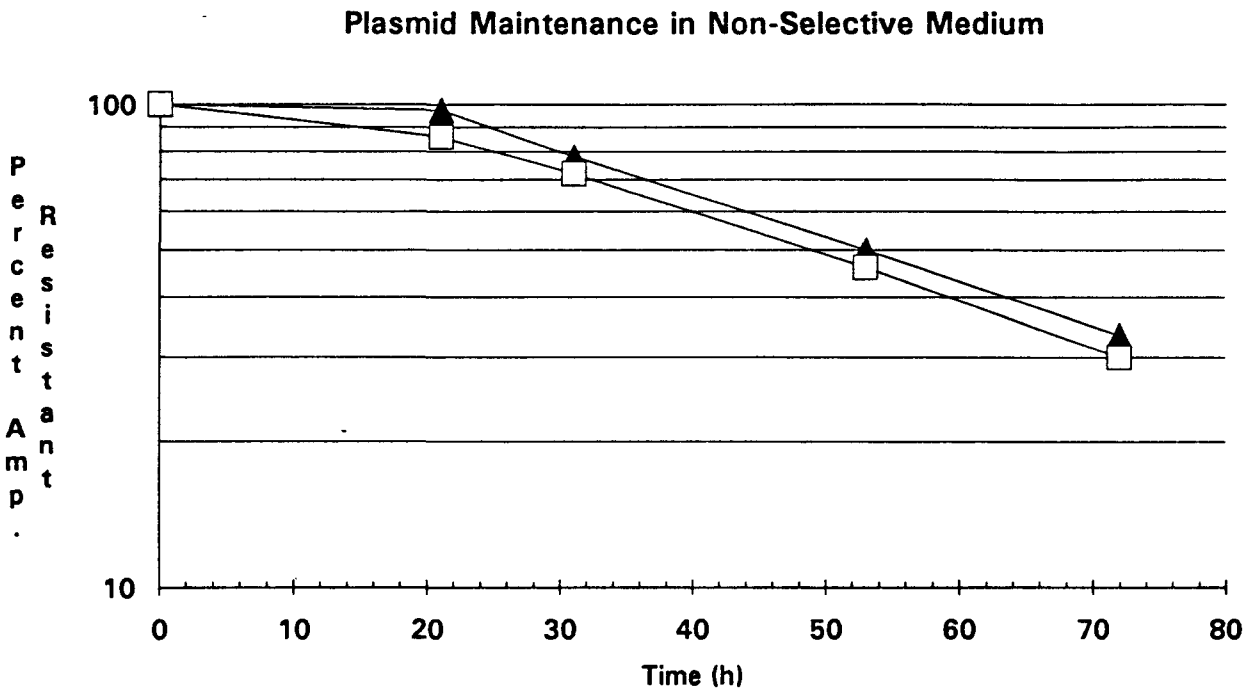

Figure 10-4. Plasmid stability in *Escherichia coli* FM4560 grown in nonselective medium. Data represent results of two independent experiments.

Analysis of the LB400 *bphA* Gene

1. Sequencing the *bphA* gene

To date, ~ 50% of the LB400 *bphA* gene has been sequenced. The procedure involved the use of Exonuclease III and S1 nuclease to construct a series of nested deletion clones from a plasmid containing the 2.9 kilobase *Eco*RI fragment from pGEM456. Sequencing was performed using T7 DNA polymerase and dideoxy nucleotide termination reactions with [^{35}S]dATP as the label. The reactions were analyzed on 6% polyacrilamide wedge gels containing 8 M urea. Two contiguous sections of DNA sequence have been obtained and work is currently underway to fill the gap between the two segments.

2. Evidence for Multiple Bph Promoters

Several lines of evidence suggest that the LB400 *bph* genes are not arranged as an operon. Previously reported transposon mutagenesis studies have found none of the polar effects commonly observed in operons (i.e., the inactivation of downstream genes). The absence of these effects usually indicates that the downstream genes contain their own promoter regions. The existence of multiple Bph promoters is also suggested by recent cloning experiments in which a 3.7 kilobase DNA fragment (encoding the *bphA* gene) and a 2.0 kb fragment (containing *bphB* and *C*) were inserted into pUC-13. In both cases, the *bph* genes were expressed regardless of fragment orientation, consistent with the presence of a promoter on each fragment.

3. Wide-Host-Range Plasmids Encoding the *bphA* Gene

The cloning of the *bphA* gene onto broad-host-range plasmids allows this gene to be transferred to a variety of bacterial strains from diverse environments. Once this is accomplished the strain-specific expression of *bphA* can be studied. A 3.7 kb DNA fragment encoding the LB400 *bphA* gene was inserted into cloning vectors pMMB66EH and pKT240. These plasmids are capable of being transferred to and maintained in many different genera of bacteria. The recombinant plasmids (designated pGEM438 and pGEM240 for the pMMB66EH and pKT240 derivatives, respectively) have thus far been mobilized into strains of *E. coli*, *Alcaligenes eutrophus*, and *Pseudomonas*. In all cases, PCB-degrading activity in these strains (as measured by resting-cell assays on PCB mixes 1B and 2B), were much lower than that of FM4560. Among the possible reasons for this are (1) the lower copy number of the vector plasmids, (2) the absence of a structural or regulatory region of DNA required for high activity, or (3) the accumulation of an inhibitory intermediate from PCB degradation.

SUMMARY AND CONCLUSIONS

Both the naturally occurring and recombinant PCB-degrading bacteria showed better than expected survivability on PCB-containing soil, and were easily detectable even after 28 days. This may mean that given the proper conditions, multiple additions of cells to the soil

would not be necessary. Instability of the *bph* genes in LB400 has been further characterized and found to occur in greater than 1.5% of the population. The phenomenon appears to be *bph* specific and does not produce cells with a growth rate advantage over the wild-type strain. The *bph* genes in the recombinant strain FM4560 were found to be stable if antibiotic selection for the plasmid was maintained.

Evidence for at least two separate promoters in the *bph* region suggest that these genes are not organized as an operon. This may influence our strategy for constructing bacteria with enhanced PCB-degrading ability.

FUTURE PLANS

Once the complete *bphA* gene sequence is available, a computer analysis will be performed to determine nucleotide and amino acid homology with sequences contained in the Genbank database. In addition, transcriptional analysis of the *bph* region will be performed to identify and confirm the existence of potential regulatory sites (i.e., promoters, ribosome binding sites and terminators, etc.). This information will be used to construct a recombinant plasmid in which the *tac* promoter is used to control the expression of *bphA*.

Laboratory studies to evaluate the ability of genetically engineered bacteria to degrade PCBs on soil have been initiated. These investigations will help determine whether currently available recombinant strains have significant advantages over LB400 for PCB degradation.

Chapter 11

PCB BIODEGRADATION AND NITRATE REDUCTION

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INTRODUCTION

It has become increasingly evident that future bioremediation processes for PCBs will employ consortia of microorganisms, either naturally constituted, reconstituted, or genetically engineered. Such cooperative interactions may prove especially useful for contaminated sites which contain pools of PCBs under aqueous deposition. Towards this end, we have initiated a program designed to obtain assemblages of bacteria which may facultatively utilize nitrate as well as oxygen as electron acceptors for PCB dissimilation. Thus, enrichment cultures were made from municipal sewage and PCB-contaminated sites, including Hudson River sediments. Samples from these sources were inoculated into mineral salts media amended either with biphenyl, hexachlorobenzene, Aroclor 1242, or mixtures, and were incubated in air or with nitrate under reduced oxygen tensions. At this point, we have isolated a number of organisms which are capable of biphenyl or selected Aroclor congener metabolism, and which also demonstrate nitrate reduction. Current endeavors are directed toward the linkage of these two major metabolic functions.

RESULTS AND DISCUSSION

A compilation of results which relate to the salient gross physiological properties of these microorganisms thus far obtained is encompassed in Table 11-1. As can be seen from these data, a number of consortia and single isolates would appear to utilize biphenyl or PCBs and concomitantly reduce nitrate or produce gas. While it is generally recognized that oxygen at μMolar concentrations represses the synthesis and activity of nitrate and nitrite reductase [Coyne and Tiedje, 1990], there has been at least one report [Krul, 1976] of a constitutive nitrate reductase in denitrifying *Alcaligenes* sp. From among this array of isolates, we have chosen initially to investigate two consortia, "TS-1" and "95-SW", in more detail.

Properties of Consortium "TS-1"

The identity of the individual microbial components present within TS-1 (Table 11-1) are as follows: *Citrobacter freundii, Pseudomonas fluorenscens, Pseudomonas putida,* and *Comanonas testosteroni*. Other reports [Pettigrew et al., 1990] and earlier references therein have also indicated that the latter organism possesses aromatic degradative capabilities. We will concentrate our efforts on the biochemistry of this isolate.

TABLE 11-1. Qualitative screen for nitrate reduction in the presence of biphenyl or Aroclor 1242

Microbial Isolate	Nitrate[3] Reduction Plates (3 days)	Nitrate[3] Reduction Tubes[1] (stab) (3 days)	Biphenyl[1] NO_3 Tubes	Aroclor1242-NO_3[2] NO_3 tubes
Hexachlorobenzene isolate	+	gas/-	+	-
P. putida isolate	+	gas/±	+	-
Hudson R. isolate #1	+	+ +	+ + (strong)	+
P. fluorescens isolate	+	gas/-	+	-
TS-1 consortium	+	gas/-	+ +	+
Comonoas testosteroni	+	+ + (strong) + +	-	
Sludge aroclor consortium	+	gas/-	(gas)/+ +	+ +
Isolate E-4y	-	-		
Hudson R. isolate #5			+	+
Hudson R. isolate #16		+	+	+
Sludge isolate #15			+	+ +
Gull Creek isolate #2			±	-
Hudson R. isolate #17[4]			+	-
Hudson R. isolate #18		+	-	
Sludge isolate #16			+	+ + + (very strong)
Sludge isolate #2			+	-

[1] PAS salts with 0.5% biphenyl, 4 mM KNO_3

[2] PAS salts with 200 ppm Aroclor 1242, 4 mM KNO_3

[3] Yeast extract-peptone medium

[4] Intense yellow-orange colony

Comamonas testosteroni reached cell densities between 10^7 to 10^8 per mL when grown on biphenyl, Aroclor, or both after 24 hours (Figure 11-1). Even in the absence of yeast extract, the culture demonstrated comparable cell densities when grown aerobically on a mixture of Aroclor and biphenyl (data not shown). Capillary GC analyses of *C. testosteroni* activity on Aroclor 1242 (Figures 11-2A and 11-2B) indicate changes in concentration of several congeners (indicated by arrows). Five dichlorobiphenyls (25-, 24-, 2-3-, 2-4-, and 23-CB), three trichlorobiphenyls (25-3, 35-2-, and 23-4-CB), and one tetrachlorobiphenyl congener (24-26-CB) underwent degradation in this experiment.

Growth of *C. testosteroni* in the presence of 2-4- and 4-4-CB indicated that the metabolic transformations of these compounds during 48 hours of growth resulted in the formation and accumulation of chlorobenzoic acids (Figure 11-3). A recent report by Pettigrew et al. [1990], who investigated an isolate identified as *Comamonas testosteroni* (LPS10A), also determined that these compounds are an endpoint of PCB metabolism.

The aerobic metabolism of biphenyl carried out by this microorganism, as established by GC-MS of intermediary metabolites, provided proof that the earlier proposed *meta* cleavage pathway [Smith and Ratledge, 1989] is operational (Figure 11-4). The following intermediates were unequivocally identified (data not shown): 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate, 2-oxo-penta-4-enoate, γ-benzoic acid, γ-benzoyl-butyraldehyde, and γ-benzoyl-butyric acid.

Consortium 95-SW

This consortium is a mixture of two microorganisms isolated from aquatic sediments, tentatively identified as a *Pseudomonas* sp. and a *Bacillus* sp. (Table 11-2). The individually resolved isolates do not show nitrate reductase activity with biphenyl. Using resting-cell assays [Bedard et al., 1986] in screw cap vials, complete metabolism of biphenyl was observed after 6 hours of incubation. Assays with individual congeners demonstrated significant degradation (95% loss of 2-4-CB and 20% loss of 4-4-CB in 48 hours; 50% loss of 23-234-CB in 96 hours). Preliminary experiments with Aroclor 1242 indicate a decrease in several congeners, especially 24- and 25-CB and 245-CB (data not shown).

TABLE 11-2. Nitrate reductions by 95-SW consortium

Microorganism	Nitrate medium, Plates, [Beef Ext. & Peptone]	Nitrate medium, Slants [Biphenyl]
95-S *Pseudomonas*	(-)	(-)
95-W *Bacillus*	(+)	(-)
95-S & W Mix of both	(+)	(+)

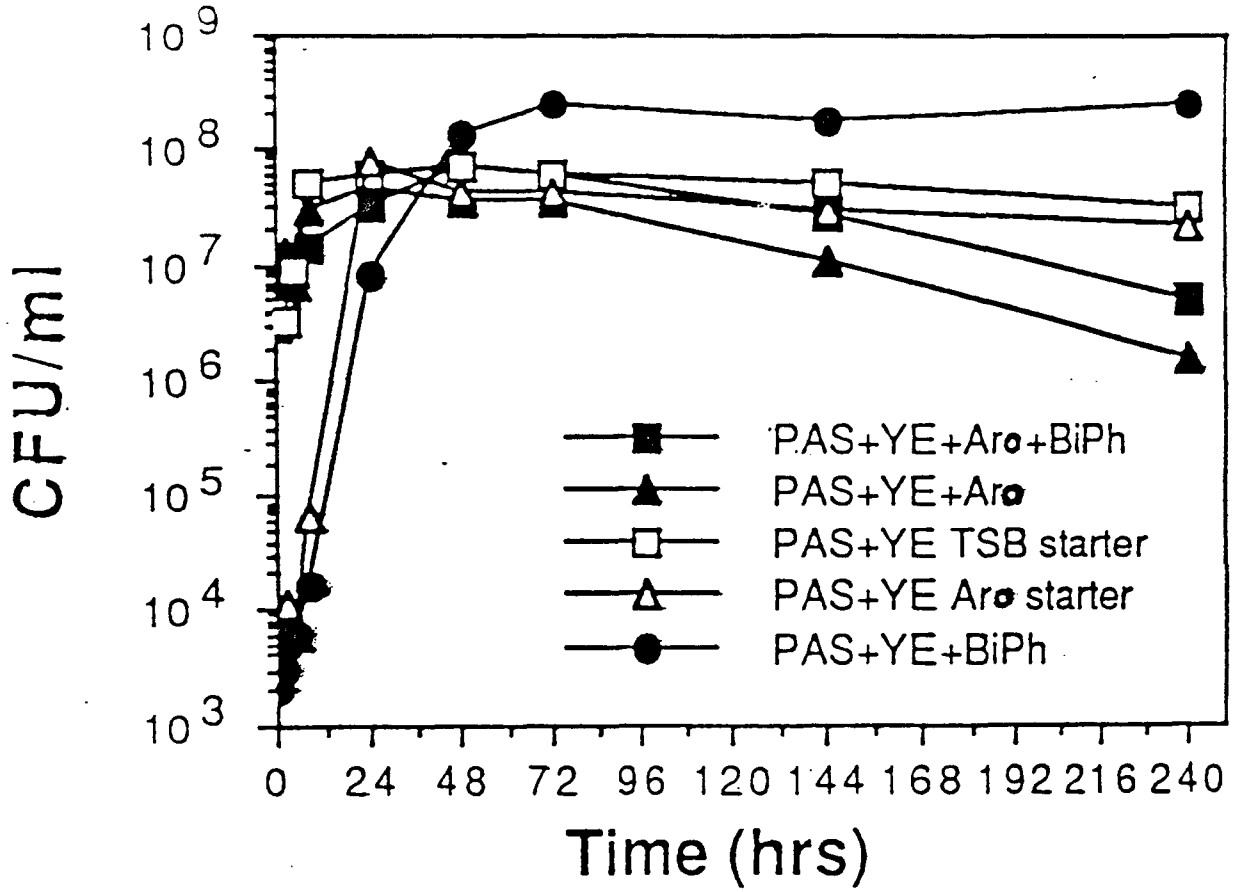

Figure 11-1. *C. testosteroni* grown on PAS and yeast extract. (BiPh = biphenyl, Aro = Aroclor 1242)

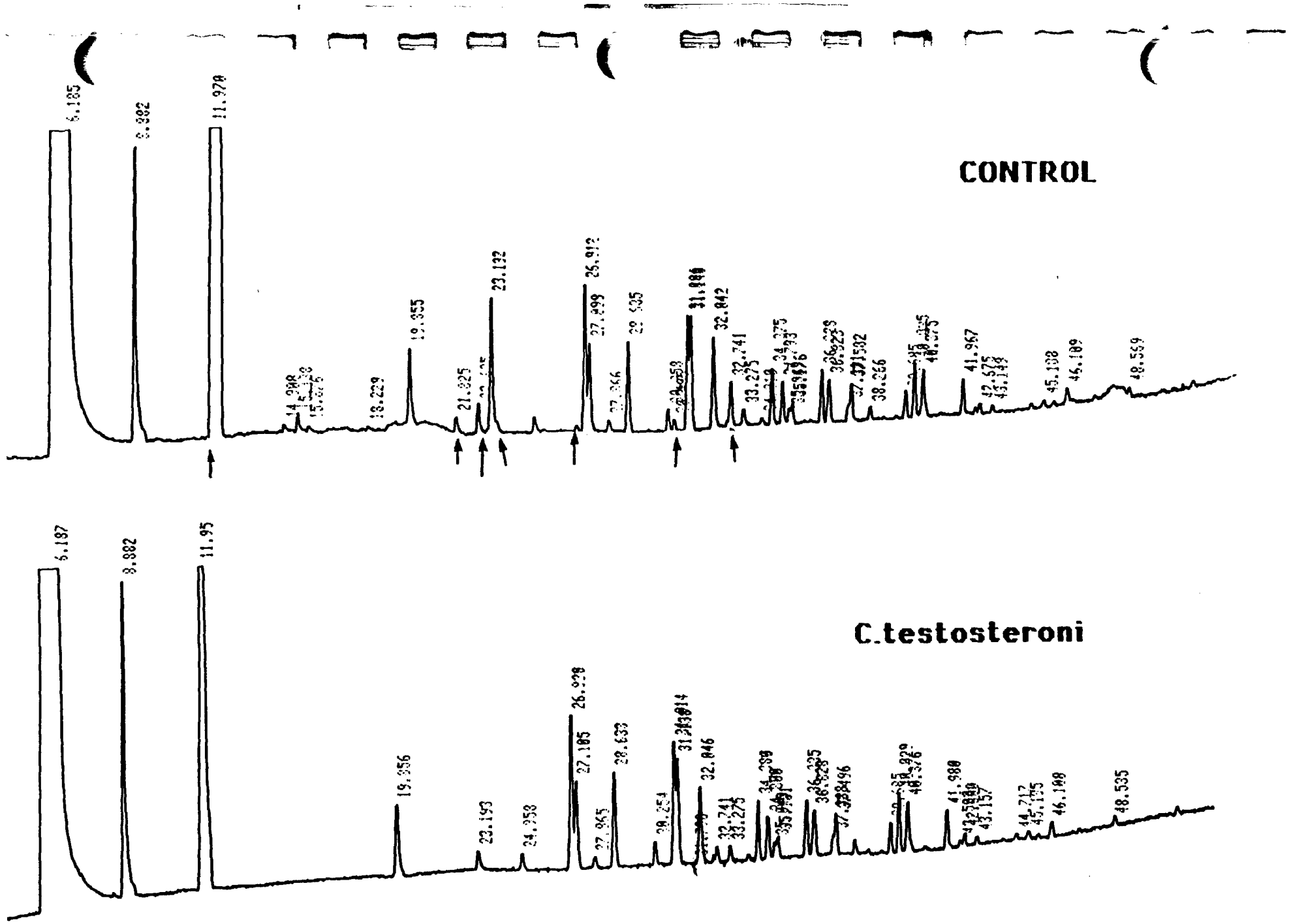

Figure 11-2. Capillary GC analyses of 1242 after incubation with *C. testosteroni*. Upper curve indicates control. Lower curve indicates post-metabolism. Arrows indicate individual congeners which appear to have been transformed.

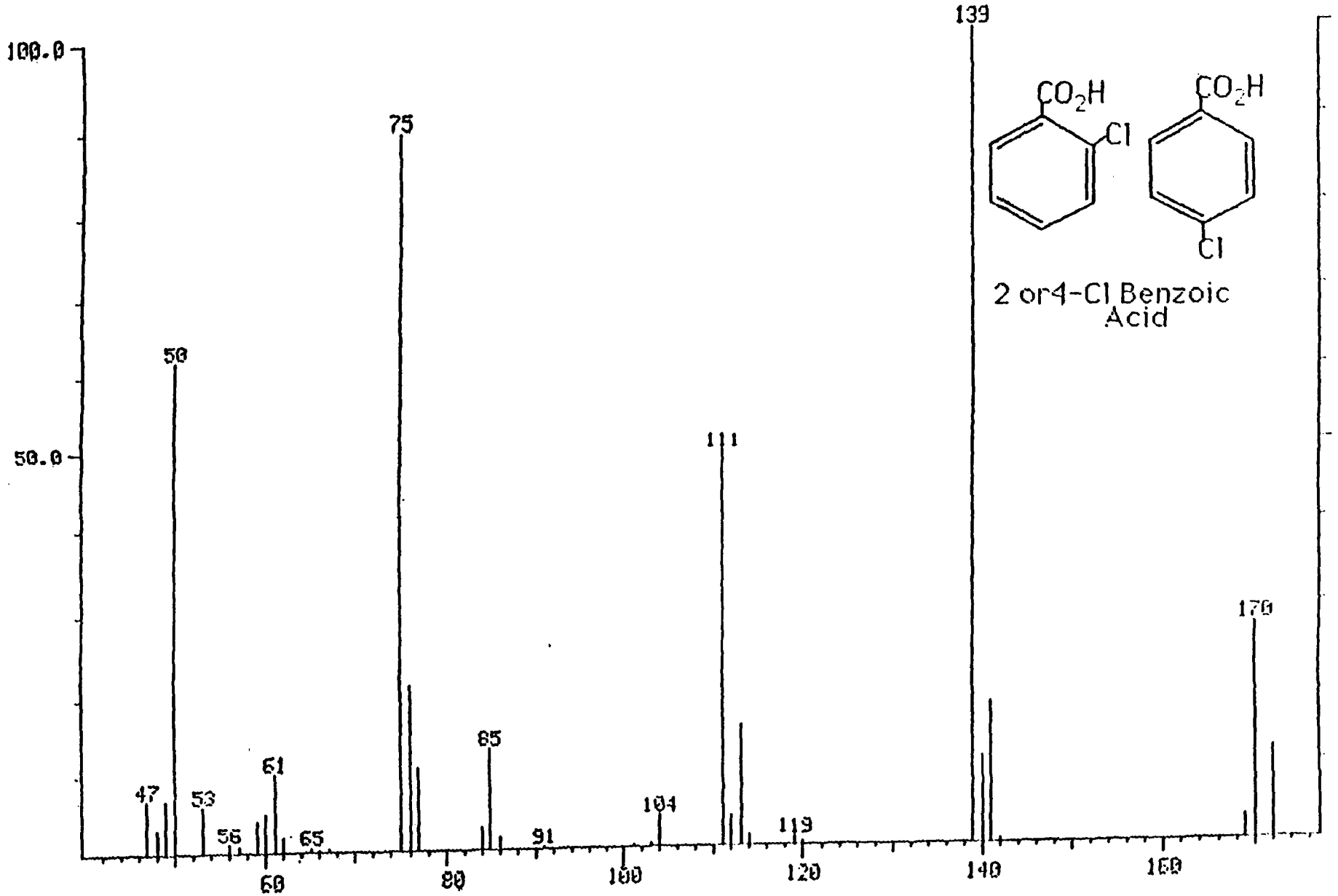

Figure 11-3. Mass spectra of the metabolic products of *C. testosteroni* grown in the presence of 2-4- and 4-4-CB.

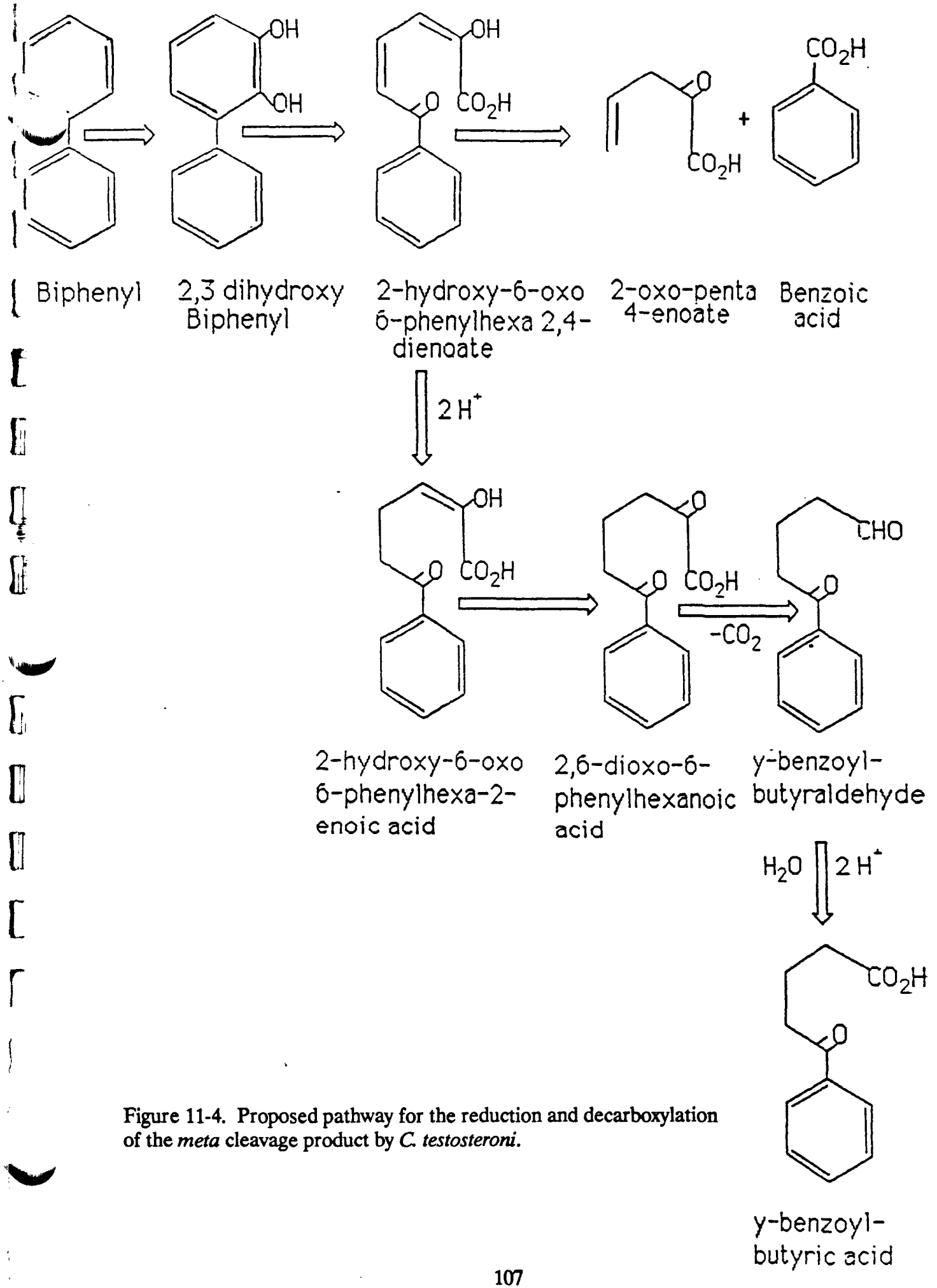

Figure 11-4. Proposed pathway for the reduction and decarboxylation of the *meta* cleavage product by *C. testosteroni*.

SUMMARY AND CONCLUSIONS

Enrichment cultures were made from municipal sewage as well as from a number of PCB-contaminated sites, including Hudson River sediments. Samples from these sources were inoculated into mineral salts amended either with biphenyl, Aroclor 1242, or both, and were grown in air or with nitrate as electron acceptor. Pure isolates include a *Comamonas testosteroni*, a Pseudomonad, and a *Bacillus* sp. Cell densities of the former approaching 3 x 10^8/mL can be achieved when this organism is grown on a biphenyl-Aroclor mixture. High performance liquid chromatography (HPLC) analysis following extraction and workup of growth supernatants from either substrate revealed at least five major metabolites. From biphenyl, further GC/MS analysis identified these compounds: 2-oxo-penta-4-enoic acid, 2-hydroxy-6-oxo-6-phenylhexa-2-enoic acid, γ-benzoylbutyric acid, γ-benzoylbutyraldehyde, and benzoic acid. The most likely biochemical explanation for the appearance of these intermediates involves the initial activity of oxygenase and *meta* cleavage enzymes, followed by a series of oxido-reduction states. *C. testosteroni* also accumulates a melange of yellow-colored compounds when grown on Aroclor 1242. The exact nature of these remains to be established.

Several of the above enrichments reduce nitrate in the presence of biphenyl and/or Aroclor 1242 when grown anaerobically. Such putative anaerobic dissimilatory routes must initially involve different activation and ring-cleavage steps. However, in facultative strains, these intermediates may be convergent with later steps found in the aerobic metabolic route.

FUTURE PLANS

Our proposed objectives for the coming year are:

1. To continue to probe the enzymology of catabolizing enzymes involved in ring fission of chlorinated biphenyls and to link these studies with possible biological dechlorination.
2. To continue to resolve and study the physiology of biphenyl, haloaromatic, and Aroclor-transforming bacterial isolates with special regard to those which possess N-oxide-reducing activities.
3. To select from among the former strains or combinations thereof, those which are either constitutive for nitrate reductase or which can express such enzyme activity in the presence of reasonably low oxygen tensions.
4. To attempt to link Aroclor biotransformation under anoxic conditions with nitrate reduction by introducing such reconstituted assemblages into a laboratory aquatic setup. For such studies, the experimental procedures developed by Kuhn et al. [1988] will be followed.

Chapter 12

AVAILABILITY OF PCBs IN SOILS AND SEDIMENTS TO SURFACTANT EXTRACTION AND AEROBIC BIODEGRADATION

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INTRODUCTION

Surfactant extraction and aerobic biodegradation of aged PCB contamination in soils and sediments can be viewed analogously. In both processes the hydrophobic PCB molecules must move from the soil/sediment matrix and transfer into the aqueous phase before uptake can occur. In surfactant systems this uptake occurs by adsorption into surfactant micelles. In biological systems it occurs by partitioning into microorganisms [GE Report, 1988]. For either process, phases which exist in the soil/sediment matrix and compete for the PCB molecules can substantially hinder the rate and extent of transport observed.

This chapter presents research examining the effect of two such phases, naturally occurring organic matter (NOM) and oil, on surfactant extraction and aerobic biodegradation of PCBs in soils and sediments. A soil pretreatment is presented which enhances both processes by modifying the NOM present in the soil or sediment matrix.

RESULTS AND DISCUSSION

Surfactant Extraction

Sodium dodecylbenzene sulfonate (SDBS) has been used successfully to extract PCBs from Oakland soil [GE Report, 1989]. This soil is primarily montmorillite clay with little organic carbon content. Montmorillite is considered difficult to extract due to its high internal surface area. The soil is contaminated with Aroclor 1260 to a level of 3500 mg/kg, a high PCB loading. Despite these facts, better than 95% extraction of PCBs was achieved in less than 45 minutes with a 1% aqueous solution of SDBS at 80°C. These extractions were run at a 4:1 solution to soil ratio with agitation. Solution PCB loadings approached 1000 mg/L.

Glens Falls dragstrip soil has been characterized as a Windsor Loamy Fine sand. The soil is predominantly a fine sand with some silt, clay, and organic matter present. The organic carbon content is 2-3% by weight as measured by combustion of the organic carbon. The soil is contaminated with partially evaporated Aroclor 1242 at a level of 100 mg/kg. The expectation was that this low level of PCBs would be removed easily from the sandy loam soil by a SDBS treatment comparable to that given the Oakland soil.

This was not the case. Only about 40% of the PCBs present in the dragstrip soil were removed after 2 hours of extraction with the surfactant. The kinetic results are compared with those from Oakland soil in Figure 12-1. The numbers plotted represent the percentage of total PCBs present in the supernatant at any time. These results indicate little difference in the percentage of PCBs removed from the two soils at early timepoints, but clearly demonstrate a difference in final extent of extraction. The absolute amount of PCB solubilized was also different. Final solution PCB loadings in the dragstrip soil extraction only reached 25 mg/L at a 2:1 solution to soil ratio, far below that achieved with the Oakland soil.

Several experiments were run to examine the mechanism causing the poor extraction performance of the dragstrip soil. The data presented in Figure 12-2 show the effects of three levels of surfactant concentration on the kinetics of extraction from this soil. The extractions were run at 80°C, again with a 2:1 solution to soil ratio and agitation. Increasing the SDBS concentration in aqueous solution from 1 to 3% increased the amount of total PCB solubilized from 40% to 85% after 2 hours. The initial rates of extraction were not significantly altered by the increased surfactant concentration.

The results of three consecutive extractions on a single soil sample are given in Table 12-1. One percent surfactant solutions were used with the conditions just described. After each extraction the slurry was centrifuged, the supernatant withdrawn, and fresh surfactant solution added to make up the volume removed. About 75% of the PCBs were removed by the three extractions combined. The ratio of solution PCB concentration to soil PCB concentration stayed relatively constant for all three extractions. The results of these two experiments make it clear that the extraction is not being limited by surfactant loading or by kinetic rate. Instead, the process appears to be equilibrium limited.

TABLE 12-1. Consecutive extractions from dragstrip soil using a 1% SDBS surfactant solution

	Sol'n [PCB]	Soil [PCB]	Soil/Sol'n [PCB]
Start	---	99 mg/kg	---
Extraction 1	20 mg/L	66 mg/kg	3.3
Extraction 2	15 mg/L	40 mg/kg	2.7
Extraction 3	9 mg/L	25 mg/kg	2.8

There is literature [Means et al., 1980; Chiou et al., 1983; Gschwend and Wu, 1985] indicating that nonpolar molecules such as PCBs undergo equilibrium partitioning into NOM when both are present in an aqueous medium. This is much like the distribution of these molecules between the two phases which form when octanol and water are mixed. NOM exists in the form of large macromolecules in many soils and sediments, the result of a partial breakdown of organic material, primarily lignin, deposited over decades. These macromolecules are largely nonpolar, although they are heterogenous and may contain polar

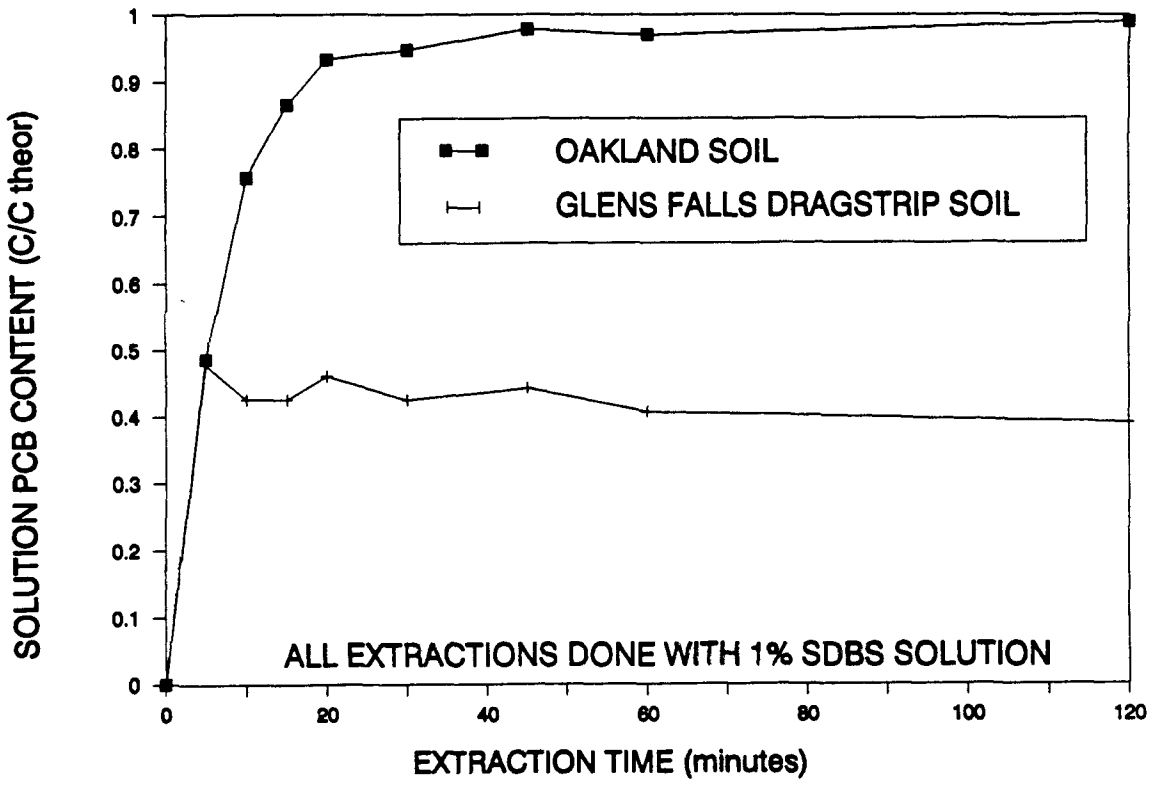

Figure 12-1. Kinetic data for surfactant extraction of PCBs from two soils

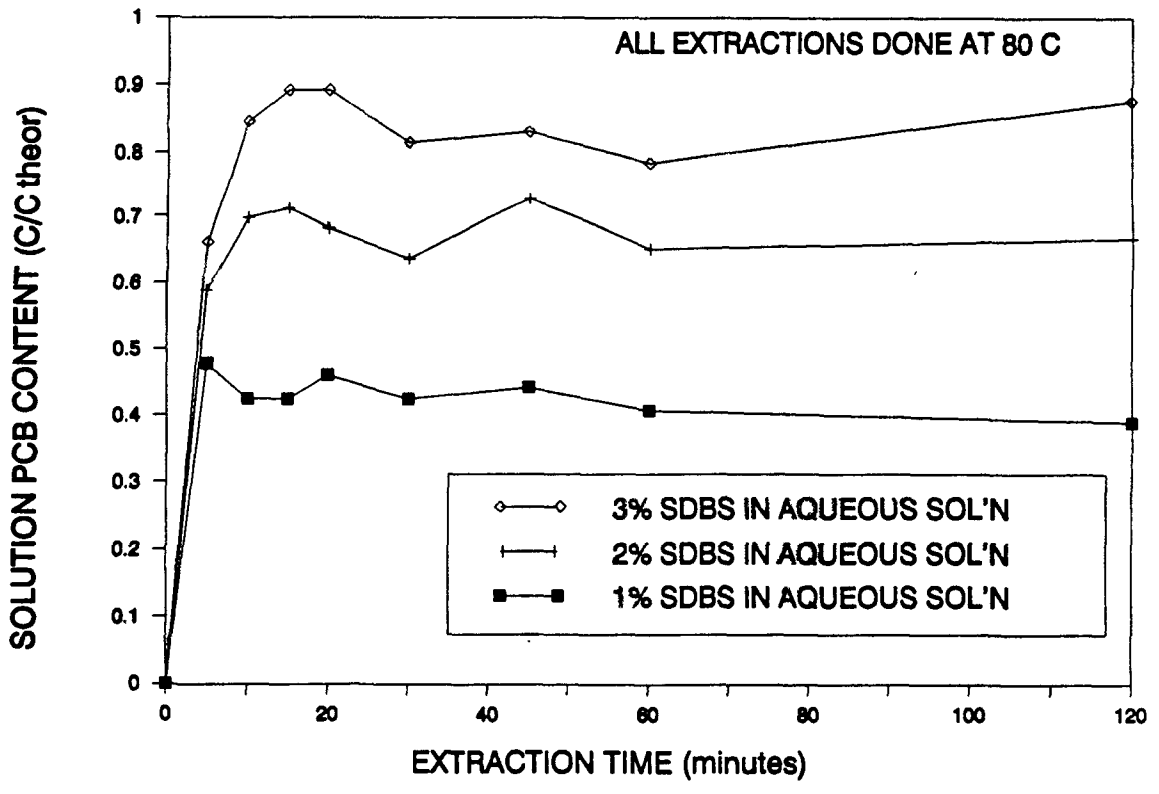

Figure 12-2. The effect of surfactant concentration on the extraction kinetics of PCBs from Glens Falls soil

constitutive groups. When present with PCBs in aqueous media, they act as a sorptive phase for the hydrophobic PCB molecules, shielding them from the polar aqueous environment.

Partitioning into NOM is generally described by an equilibrium partition coefficient, K_{oc}, defined as the concentration of solute present in the natural organic carbon phase in mg/kg divided by the concentration of solute present in solution in mg/L. Typical K_{oc} values for PCBs in water range from 10^5 to 10^8 L/kg [Karickhoff et al., 1979], depending on the average number of chlorine atoms attached to the biphenyl molecule. In natural systems the organic carbon will make up only a fraction of the total soil or sediment mass. In this case, a general partitioning constant, K_p, is defined as

$$K_p = K_{oc} f_{oc} \qquad [1]$$

where f_{oc} is the mass fraction organic carbon in the soil or sediment.

The organic carbon partition coefficient of a hydrophobic organic molecule is strongly correlated with its solubility [Chiou et al., 1979]. The addition of surfactant above the critical micelle concentration creates a second organic phase in a water/soil or water/sediment system. Surfactant micelles and soil/sediment NOM now compete for hydrophobic molecules. Increasing the surfactant concentration increases the number and volume of micelles in the system, increasing its capacity to take up these molecules. There is a shift from molecules partitioned into the soil NOM to molecules partitioned into the surfactant micelles. This phenomenon explains why varying the SDBS concentration had such a significant effect on the extent of extraction of PCBs from dragstrip soil.

A series of experiments using dragstrip soil and soil extract were run to further confirm that equilibrium partitioning is controlling the extraction process. Five gram samples of dragstrip soil were spiked with from 0 to 2 mL of concentrated (1000 mg/L) PCB extract obtained from a hexane/acetone soxhlet extraction of the soil. The hexane and acetone were allowed to evaporate and 15 mL volumes of 1% SDBS solution were added to the soil. The samples were placed in an ultrasonic bath at 50°C for 1 hour, then allowed to stand and approach equilibrium for 24 hours. The samples were centrifuged and the supernatant assayed for PCB content. The soil was dried, extracted, and also analyzed for PCB content. The final soil PCB concentration was adjusted for the contribution of residual surfactant solution dried onto the soil.

In addition to these trials, another series of experiments were run to extend the range of equilibrium measurements to low PCB concentrations. In this case, sample soil loadings were varied from 1-5 g and then combined with 15 mL of 1% SDBS solution. No PCB spiking was done. The samples were processed as described above.

The results of both series of experiments are combined in the curve in Figure 12-3. In an ideal equilibrium partitioning situation a plot of soil PCB concentration versus solution PCB concentration at equilibrium should appear as a straight line through the origin with K_p as the slope. This is generally the case here. The fact that the line does not appear to go through

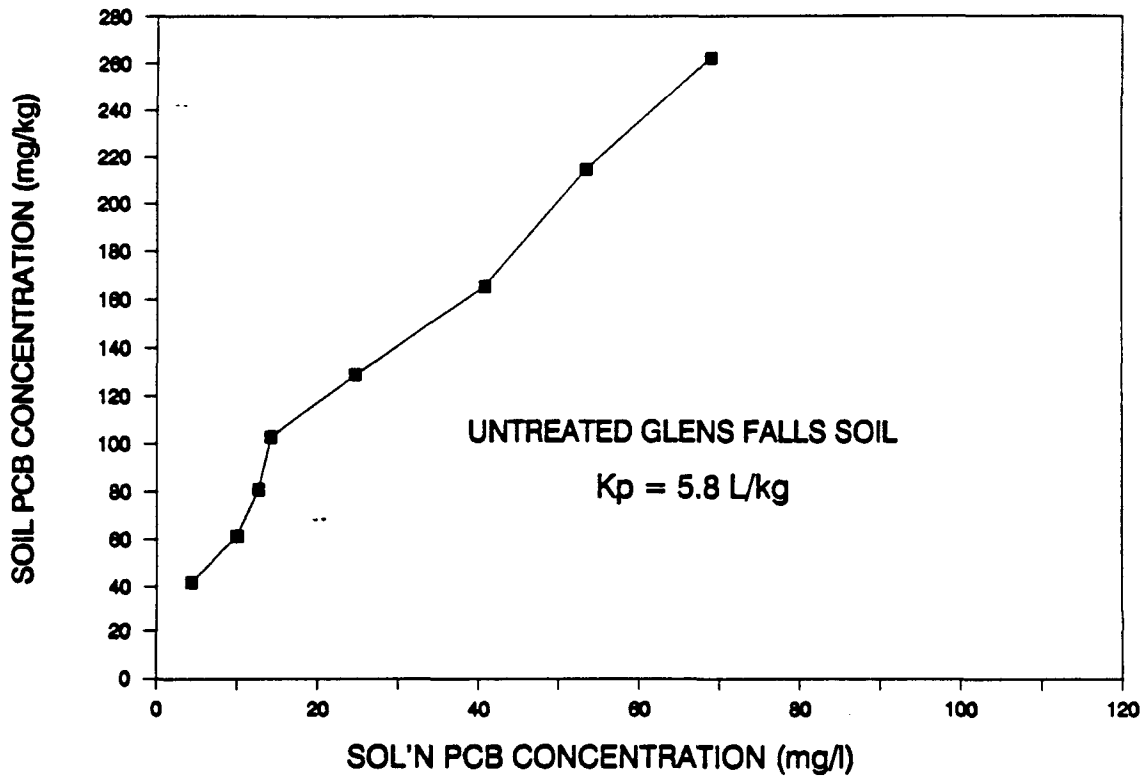

Figure 12-3. Equilibrium partitioning of PCBs between Glens Falls soil and surfactant solution

the origin may reflect diffusional resistance in the soil matrix. True equilibrium may not have been reached in the 24-hour equilibration periods. However, the general equilibrium hypothesis is supported by these data.

Aerobic Biodegradation

It is generally assumed that aerobic microorganisms require substrates in soluble form in order for biotransformation to occur. The transport of slightly soluble substate into the aqueous phase can be rate limiting in many biological processes. Substrates sequestered in NOM macromolecular matrices are also presumably not available to the organisms. As in surfactant systems, the transport of hydrophobic molecules from this phase, through an aqueous medium, and into a second lipophilic phase, this time in a microorganism, is of significant importance to biodegradative processes.

The bioavailability of aged PCBs in soils and sediments can be assessed by removing the PCBs from the soil/sediment matrix via soxhlet extraction, followed by biodegradation of the extract. The extent of biodegradation observed can then be compared to that in the original matrix under similar conditions. These experiments have been done for PCBs in Glens Falls dragstrip soil.

Soxhlet extractions were performed using hexane/acetone as the extraction solvent. The biodegradation studies shown were done with *Pseudomonas* sp. JB1P3C organisms [GE Report, 1989] in 72-hour resting-cell assays. The results appear in Table 12-2. The presence of a large difference in starting PCB concentration in the assays makes it difficult to compare the degradation numbers directly. Yet given that aerobic biodegradation generally proceeds more efficiently (i.e., degrades a higher percentage of the material present) at lower concentrations, these results indicate significant limitations in PCB bioavailability do exist in the soil, consistent with the surfactant extraction results.

TABLE 12-2. PCB bioavailability in Glens Falls dragstrip soil

Soil Sample	Starting [PCB] (ppm)	Cell OD	Mass Sediment (g)	% Degraded
GF dragstrip	20.0	5.0	0.2 (wet)	35%
GF (soxhlet)	130.0	5.0	---	54%

A simple trial was performed to show that this bioavailability limitation could be due to the NOM present in the dragstrip soil. One hundred microgram portions of Aroclor 1242 dissolved in hexane were deposited into a series of vials, with the hexane allowed to evaporate. To these vials were added neutralized Aldrich humic acid dissolved in 0.5 mL of water in ratios of 10:1, 50:1, 100:1, and 200:1 by weight to the Aroclor. The vials were shaken for 24 hours to allow the PCBs to come into equilibrium with the humic acid, then JB1P3C cells were added and standard resting-cell assays performed.

The results are shown in Figure 12-4. Inhibition of both the rate and extent of biological activity was observed at humic acid ratios of 100:1 and above. This equates to 1% humic acid in the assay. Duplicate vials were checked for pH shifts that might have occurred over the course of the experiment, but none were found. It has been noted in the literature that hydrophobic compounds tend to partition more strongly into commercial humic acid than they do into NOM [Chiou et al., 1987]. Therefore, this experiment qualitatively demonstrates the potential of NOM to account for the limited biodegradation results observed in dragstrip soil.

Natural organic matter is not alone in acting as a sorptive phase for PCBs in the environment. In a recent communication, Boyd and Sun [1990] showed experimentally that oil and grease can be up to 10 times more effective than NOM as a sorptive phase for hydrophobic compounds. They reformulated the expression for the generalized partition constant in equation [1] to include the contribution of this additional phase

$$K_p = K_{oc} f_{oc} + K_{oil} f_{oil} \qquad [2]$$

where f_{oil} is now the oil and grease fraction in the soil or sediment. Thus the extent of PCB partitioning can be expressed as a combined function of both the organic carbon and oil/grease content of the soil/sediment matrix.

Both NOM and oil/grease are present in many environmental samples [GE Report, 1988]. Table 12-3 shows a compositional analysis of two sediment samples, one from the upper Hudson River and one representing a composite of several samples from Woods Pond in Massachusetts. Both samples are high in organic carbon, as measured by combustion of the dry sediment. This is especially true of the Woods Pond sample. The fraction of oil and grease present in each was determined by performing a soxhlet extraction on the sediment and measuring the loss in mass. Both straight hexane and hexane/acetone were used as extraction solvents, yielding similar results. This indicates oil and grease make up a substantial part of the extract. Samples of the extract were then submitted for infrared (IR) analysis.

TABLE 12-3. Compositional analysis of selected sediments

	Upper Hudson	Woods Pond
Water Content (%)	41 - 46	73 - 77
Extractable Organic (%) *	0.55	2.7
Combustible Fraction (%) *	7.8	23.1
PCB Content (mg/kg)	57	98

* indicates analysis done on a dry sediment weight basis

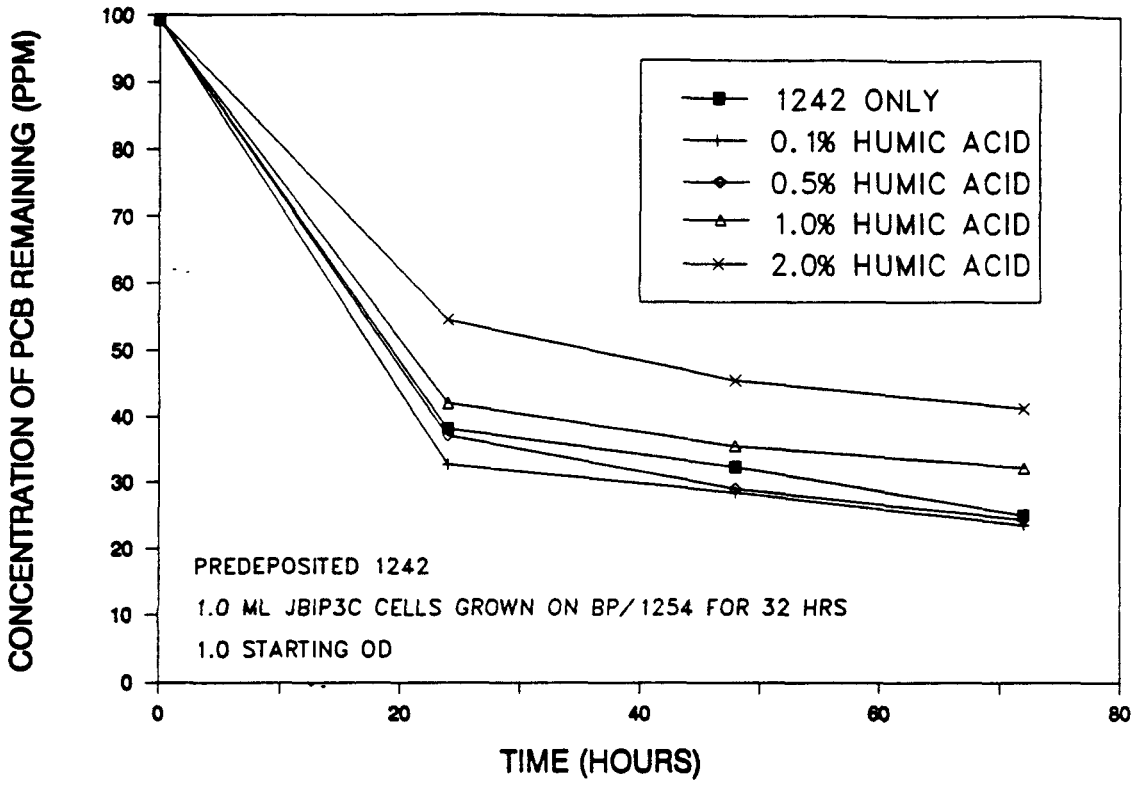

Figure 12-4. The influence of Aldrich humic acid on PCB biodegradation by JB1P3C

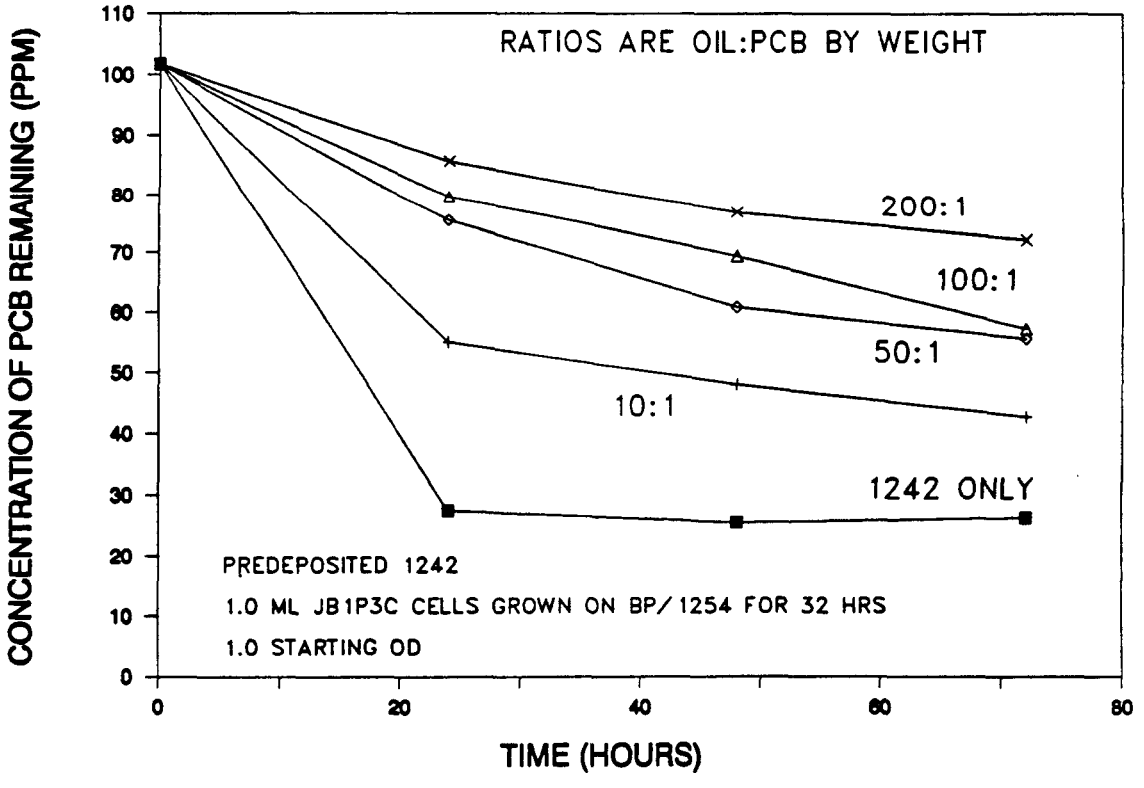

Figure 12-5. The influence of mineral oil on PCB biodegradation by JB1P3C

The IR analyses indicate the presence of an aliphatic hydrocarbon oil in both samples. The Woods Pond sample probably contains mostly mineral oil, based upon the large amount of this material historically used at nearby manufacturing sites. There is no single likely candidate for the type of oil found in the Hudson River sediment.

General Electric has demonstrated in the past that the presence of oils can make PCBs unavailable for aerobic biodegradation [GE Report, 1988; Bedard, 1990]. To further confirm those results, an experiment was carried out where light mineral oil was added with Aroclor 1242 to small vials in ratios of 10:1, 50:1, 100:1 and 200:1 by weight. Water was added and the vials were shaken for 24 hours to allow the oil and PCB to approach equilibrium. JB1P3C cells were then added to the vials and standard resting-cell assays performed.

The results appear in Figure 12-5. The inhibition of biodegradation is much more severe here than observed with the Aldrich humic acid. Inhibition begins at the lowest concentration of oil and increases progressively as additional oil is added to the system. Partitioning of the PCBs into the oil phase is the logical explanation for these observed results. The magnitude of these effects indicate strong partitioning and support Boyd's findings that the partitioning coefficients for oil phases may be much greater than those for NOM.

These results have been obtained with aerobes, but there is no biological or physical basis for expecting this phenomenon to depend upon type of organism. Anaerobic dechlorination may also be affected by partitioning-related bioavailability issues. However, several factors should be taken into consideration.

Partitioning is ideally a reversible, dynamic equilibrium process. If a system at equilibrium is perturbed, such as by removing PCBs from the aqueous phase, it will return to equilibrium given sufficient time. This time to equilibrium is determined by the rate of transport of the PCB out of the partitioning phase and may be limited by adsorption or diffusion phenomena in that phase. In aerobic biodegradation trials in the laboratory, organisms are usually present at high concentrations and degradation is fast. The organisms act as sinks for PCBs, with depletion of some PCB congeners occurring on a time-scale of hours. If the transport of PCBs out of the partitioning phase cannot keep up, the system operates under nonequilibrium conditions, with PCBs depleted in the aqueous phase. Bioavailability limitations result. If cells run out of energy or die over time, as commonly occurs in resting-cell assays, biodegradation may cease altogether.

In anaerobic dechlorination, both in the laboratory and in nature, organisms are generally present at much lower cell concentrations. Anaerobes transform PCB molecules into lower chlorinated congeners, rather than oxidizing them as in the aerobic case. They act over time-scales of months, rather than hours [GE Reports, 1988; 1989]. These observations suggest that anaerobic systems should operate at conditions much nearer to equilibrium concentrations of PCB in the aqueous phase than do the aerobic systems we have studied. However, in cases such as Woods Pond, whose sediment contains high levels of both NOM and oil, it is conceivable that the equilibrium concentration of PCBs is so low that it cannot even maintain the active organism population necessary to carry out anaerobic dechlorination. This may

explain the modest natural dechlorination seen to date on that site [GE Report, 1990].

Enhanced Partitioning

The partitioning of PCBs into soil humic materials adds significant difficulty and cost to a surfactant extraction process and will impair attempts at biological remediation as well. One method of addressing this problem is to seek either to remove the NOM from the soil or sediment matrix or to change the character of that NOM so that it no longer is a strong sorptive phase for hydrophobic compounds.

Certain organic components of soils can be solubilized with dilute caustic solutions [Mortensen, 1979]. Humic and fulvic acids, which make up most of the dragstrip natural organic fraction, are in this category. A third component of NOM, humin, is not solubilized by this treatment. Sodium hydroxide in concentrations of 0.1 N to 0.5 N is commonly used to remove these solubilized fractions in soil analysis. If heat is added with the caustic, the acids undergo partial hydrolysis. Both solubilization and hydrolysis of the humic material can be expected to have a positive effect on the partitioning of PCBs with regard to extraction.

This thesis was tested in a pair of experiments involving caustic-treated dragstrip soil. In the first experiment 0.5 N NaOH caustic solution was added to dragstrip soil in a 4:1 ratio and shaken for 1 hour. The contents were then centrifuged and the dark brown supernatant containing the dissolved humic material drawn off. A second volume of clean caustic was then added to the soil and this procedure repeated until the supernatant was no longer deeply colored. Approximately 10% of the PCBs originally present in the soil were removed in the supernatant. The caustic-treated soil was then neutralized, dried, and 5 g samples were spiked with concentrated dragstrip extract (0-2 mL). Fifteen mL volumes of 1% SDBS solution were added to each sample and equilibrium partitioning experiments were carried out as previously described.

In the second experiment, the 0.5 N caustic solution was added to the soil in a 1:1 ratio and the slurry was refluxed for 3 hours using a heating mantle and condenser. This time the supernatant was not removed. Instead, the slurry was neutralized and air dried. The dissolved organic fraction remained with the soil. Five gram samples were then spiked with concentrated soil extract as before and the partitioning experiments performed.

The results of these experiments are given in Figure 12-6. Soil from both caustic treatments show a significant decrease in PCB partitioning compared to the untreated standard (K_p = 1.3 and 0.9 L/kg versus 5.8 L/kg for the standard). Soil subjected to both caustic and heat treatment showed the least partitioning despite the fact that its solubilized organic component was not removed. The organic phase must be altered in order for this to occur, making the PCBs more available for extraction.

A kinetic study was carried out using the caustic and heat-treated dragstrip soil to verify this result. The soil was slurried with surfactant and the PCBs extracted (1% SDBS, 80°C, 2:1 solution to soil ratio) as the supernatant was monitored for PCB concentration. The results

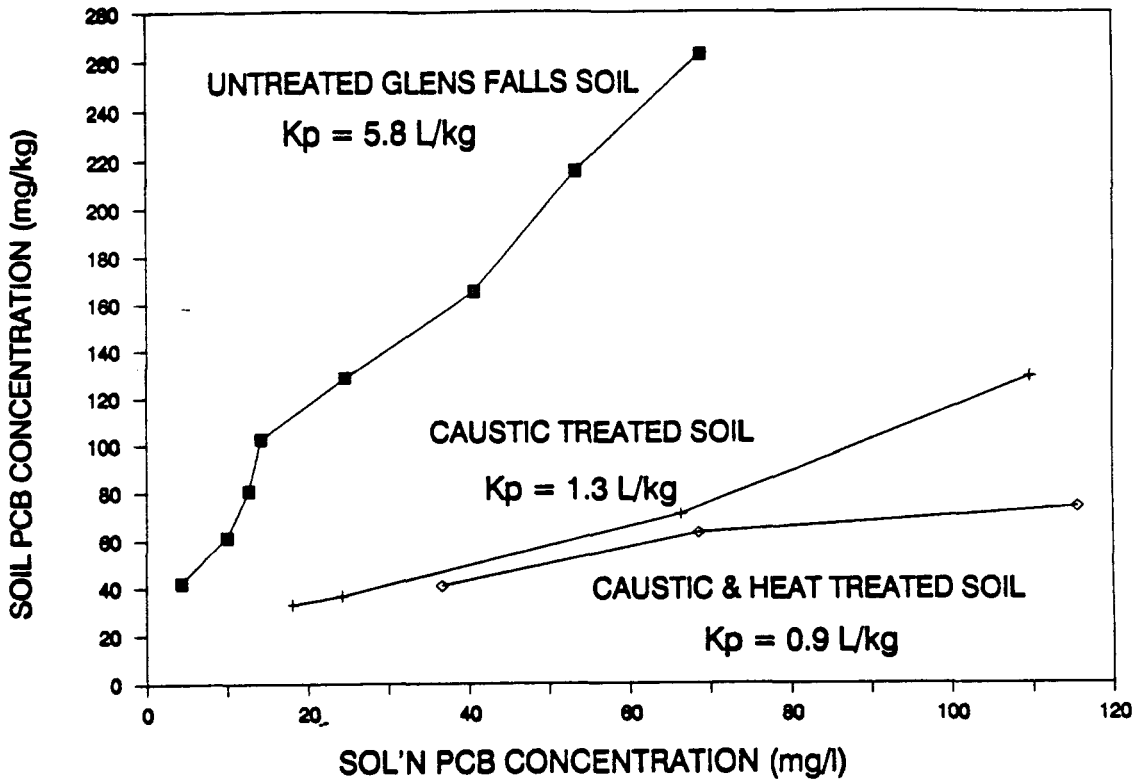

Figure 12-6. The effect of soil pretreatment on the partitioning of PCBs between Glens Falls soil and surfactant solution

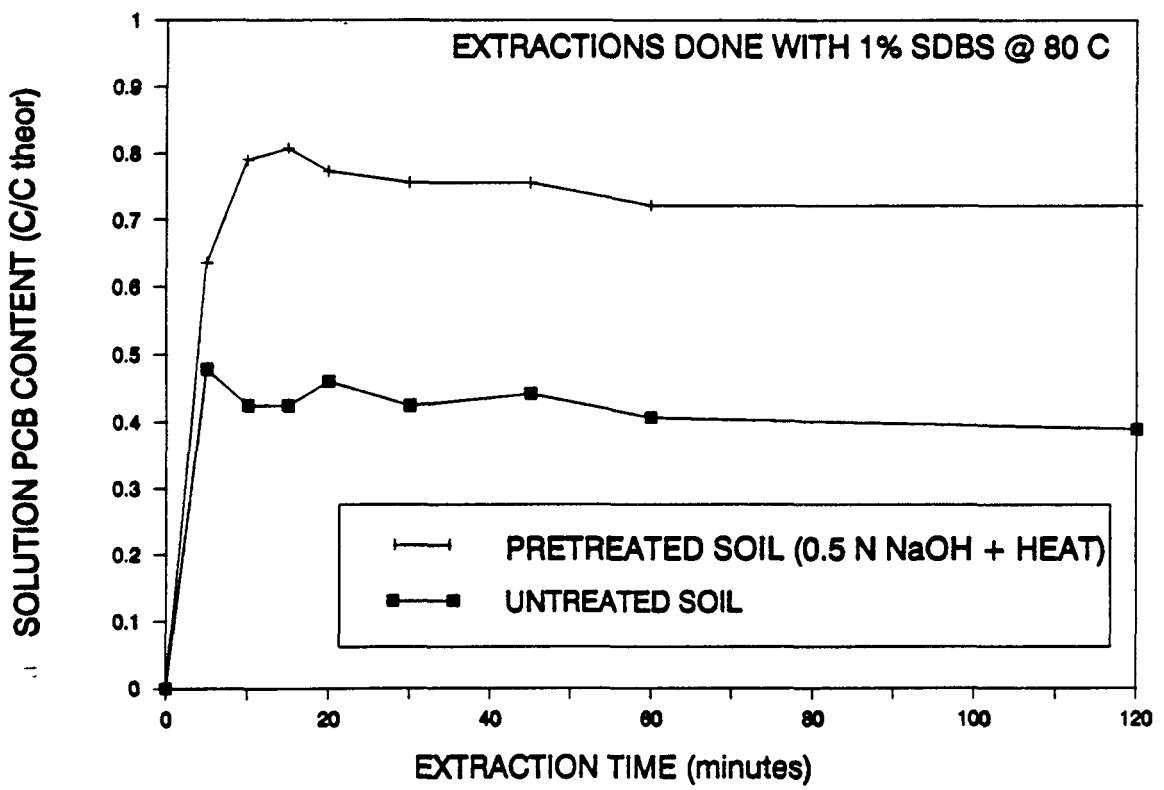

Figure 12-7. The effect of soil pretreatment on the kinetics of surfactant extraction from Glens Falls soil

are shown in Figure 12-7. About 75% of the PCBs in the soil are solubilized when the pretreatment is used, as opposed to <40% for untreated soil. It appears the ability of the soil humic matter to sorb PCBs is decreased by the pretreatment, but not eliminated.

Biodegradation experiments were also performed using the treated soils. Reliable data are available only on the soil with the soluble organic material removed. Fifty-five percent of the PCBs in the NOM-depleted soil were biodegraded by JB1P3C in resting-cells assays. This contrasts with 35% PCB biodegradation by JB1P3C observed using untreated dragstrip soil and is comparable to the soil-free degradation value given in Table 12-2.

SUMMARY AND CONCLUSIONS

The analogy between surfactant extraction and aerobic biodegradative processes is used to elucidate the effect of natural organic matter on PCB availability in soil and sediment matrices. PCB equilibrium partitioning into the NOM phase is demonstrated to have a detrimental effect on the extent and efficiency of PCB removal for both of these processes. Oil and grease also form a secondary phase in soils and sediments, and are shown to have a similar detrimental effect on biodegradation. The presence of one or both of these phases in most soils and sediments requires that their impact be better understood and factored into any extractive or biodegradation studies undertaken.

Partitioning into NOM can be reduced by pretreating the soil with a mild caustic (0.5 N NaOH) solution and heat. The treatment solubilizes and hydrolyzes the humic and fulvic fractions in the soil, limiting the extent to which PCBs can sorb into these fractions. The resulting enhanced PCB availability is beneficial to both extractive and biodegradative processes.

FUTURE WORK

We plan to continue to explore the PCB bioavailability issue as it impacts the aerobic bioremediation of river sediments. The caustic soil treatment may be beneficial as a pretreatment for a bioreactor system, but is of limited value if an *in situ* approach is desired. Greater understanding of the interaction between equilibrium partitioning and biological uptake will be required to achieve more effective bioremediation strategies.

Chapter 13

SYNTHESIS OF PROPOSED INTERMEDIATES IN THE AEROBIC DEGRADATION OF PCBs; RING-FISSION PRODUCTS

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INTRODUCTION

A major pathway for the destruction of PCBs by aerobic microorganisms [Furukawa et al., 1979; GE Reports, 1984-1989] closely resembles the pathway elucidated earlier for oxidation of biphenyl itself [Catelani et al., 1973; Gibson et al., 1973]. Oxidative cleavage of biphenyl-2,3-diol yields the ring-fission product (I, Ar = phenyl) which is further degraded in at least two different ways (Figure 13-1). Isomerization to the di-keto tautomer (Ik) and cleavage by hydrase leads to benzoic acid via path A. Path B has been proposed [Omori et al., 1988] to explain the isolation of keto-acid II as a metabolite of I.

Both paths A and B (Figure 13-1) have also been observed in PCB metabolism. Chlorobenzoic acids (Cbas) are major metabolites of PCBs in numerous organisms. Keto-acid II (Ar = 2-chlorophenyl) has been found as a metabolite of both 2-CB and 2,2′-CB in a recombinant strain of *E. coli* that harbors a plasmid (from *Pseudomonas* sp. LB400) encoding the first three enzymes of the biphenyl/PCB pathway but not the hydrase needed for cleavage of I to benzoic acid [Bedard, GE Report, 1989].

There is some indication of a third way (path C) by which the ring-fission product (I) can be degraded [Barton and Crawford, 1988]. Oxidation of 4-CB by a *Pseudomonas* sp. was reported to produce large amounts of 4-chloroacetophenone (4-CA). The corresponding ring-fission product (I, Ar = 4-chlorophenyl) was not shown to be an intermediate in this case, so we cannot consider that path C is firmly established.

Oxidation of 23-3-CB by several different strains led to attack on the 3-chlorophenyl ring and isolation of both 2,3-CA and 2,3-Cba [Bedard et al., 1987; Bedard and Haberl, 1990, in press]. Other congeners possessing a 3-chlorophenyl ring behaved similarly. It was proposed [GE Report, 1987] that the pathway in this case involved the tri-keto acid III (Figure 13-2). Attack at either C_4 or C_6 could lead to cleavage at the C_4C_5 bond or the C_5C_6 bond, respectively, to produce the observed products. Many instances are known in which unsymmetrical β-diketones undergo non-enzymic hydrolytic cleavage in both possible directions [Hauser et al., 1948].

The goals of the present research were to develop chemical syntheses for compounds I and III, and to explore their reactions under non-enzymic conditions, as well as their transformation by appropriate microorganisms. In this report we describe the preparation of I (Ar = phenyl) and of six compounds of type III, and preliminary results of a study of their non-enzymic cleavage reactions.

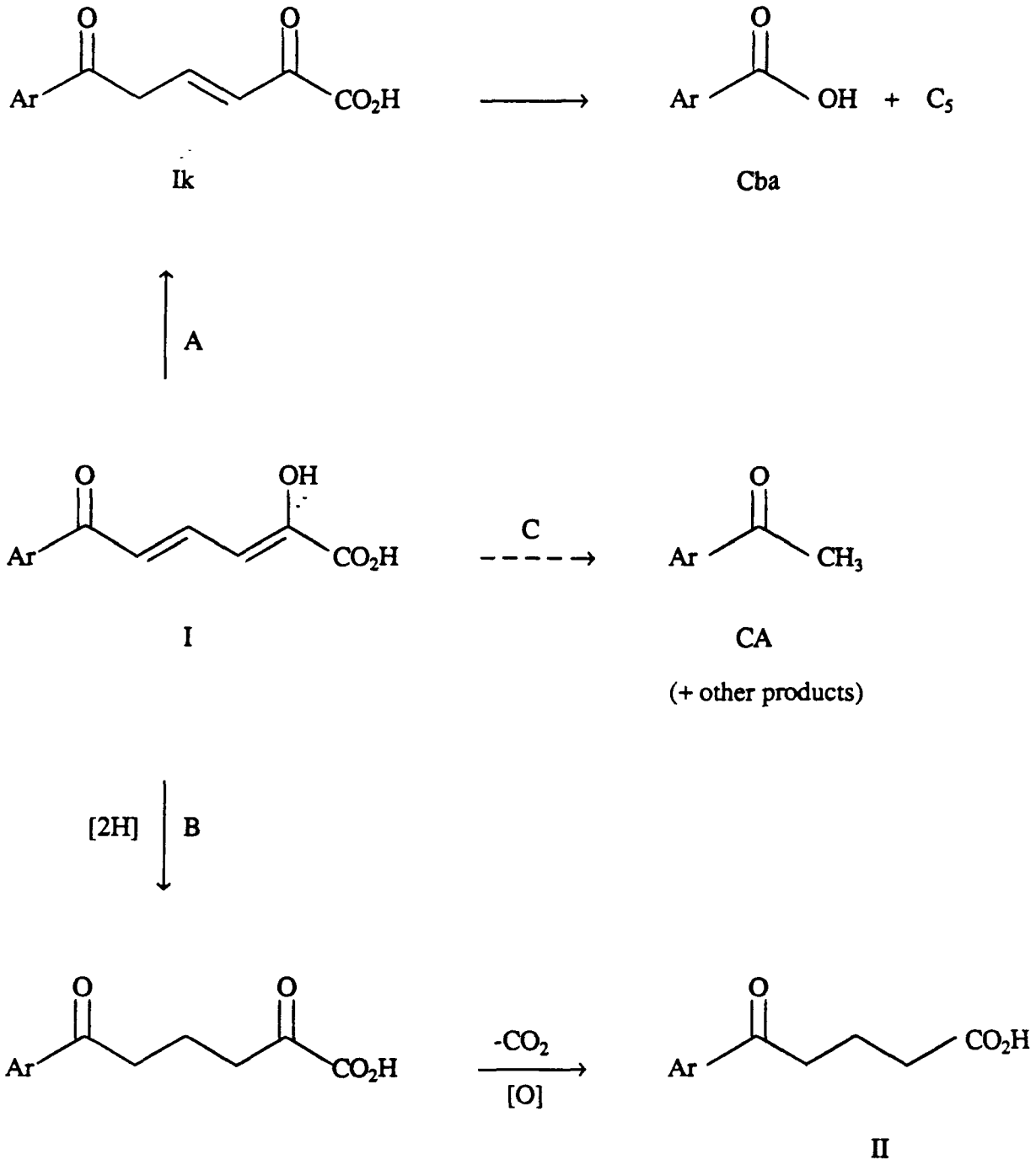


Figure 13-1. Paths for metabolism of the ring-fission product (I).

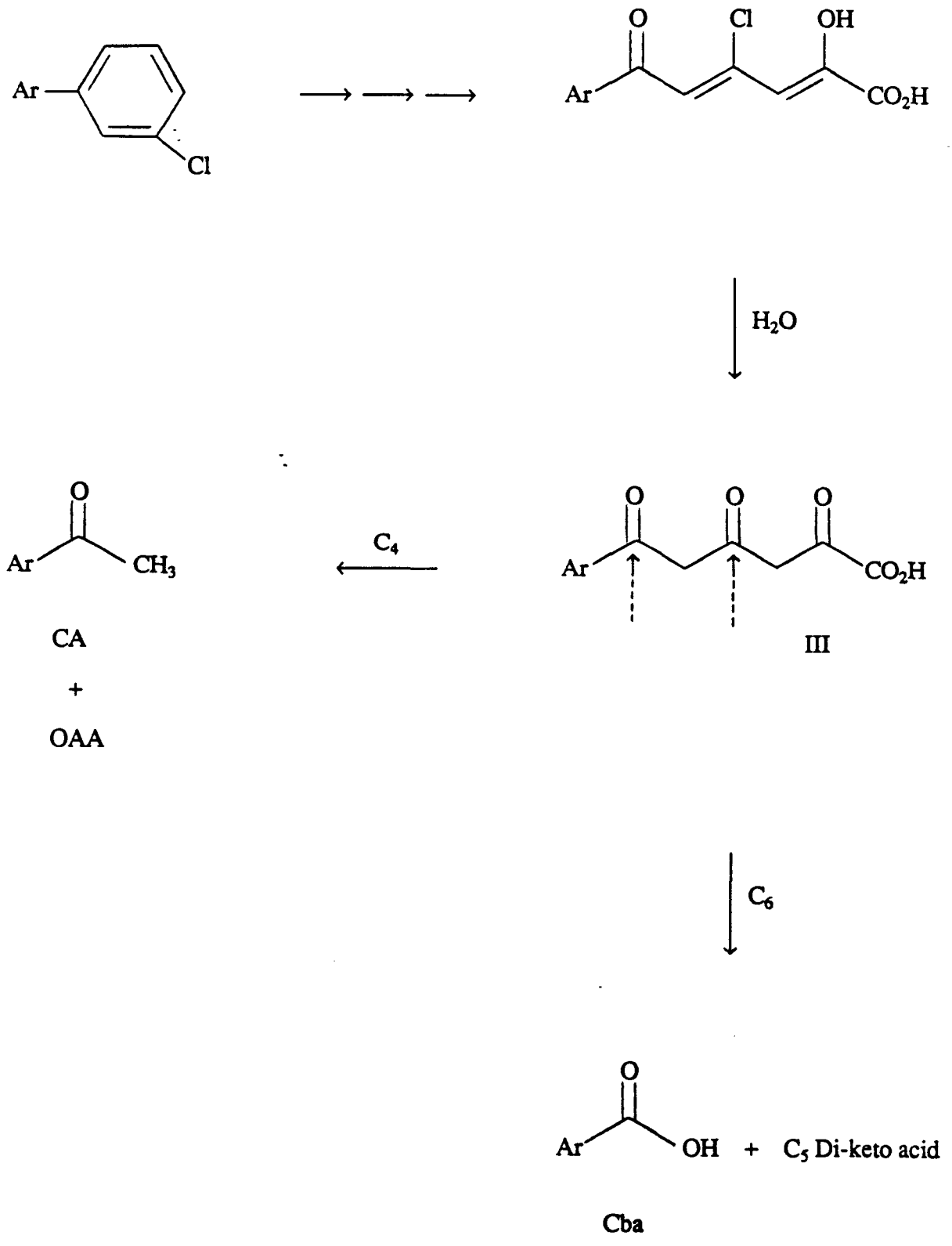


Figure 13-2. Proposed pathway for degradation of a 3-chlorophenyl ring. Compound III can exist as a mixture of tautomeric forms in which one or two of the three keto groups is in the enol form. The numeral III is used throughout the text to refer to any one or all of these species.

RESULTS AND DISCUSSION

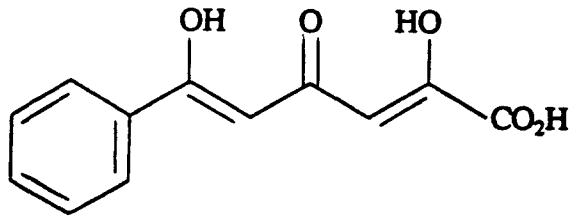
Synthesis of the Ring-Fission Product (I)

The ring-fission product from biphenyl (I, Ar = phenyl) has been isolated from microbial cultures and characterized [Catelani et al., 1973] but no chemical synthesis of the compound has been reported. We have found that the methyl ester is readily prepared from crotonophenone (1-phenyl-2-buten-1-one) and dimethyl oxalate (DMO). Hydrolysis in aqueous LiOH, followed by acidification, affords the free acid as yellow crystals which darken on storage.

Comparison of the physical properties of our product and that described by Catelani reveals satisfactory agreement (Table 13-1). Samples of our product decomposed upon heating at temperatures somewhat higher than he reported, but decomposition points are notoriously dependent on particle size and heating rates, and the observed difference may not be of much significance.

Synthesis of Tri-Keto Acids (III)

The prototype tri-keto acid III (Ar = phenyl) was recently synthesized for the first time in this laboratory [Stiles, unpublished]. Condensation of benzoylacetone with dimethyl oxalate (DMO) in the presence of magnesium methyl carbonate (MMC) provided yellow crystalline product in yields above 50%. X-ray crystallographic analysis [Selegue, unpublished] indicated



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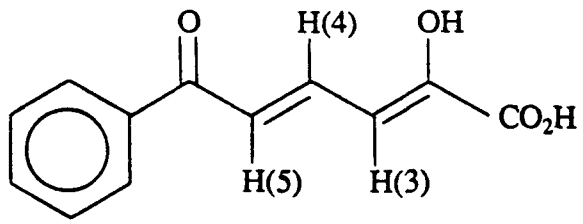
that the crystalline substance exists as the tautomer IIIA. NMR spectra of solutions in acetone-d_6 indicated the presence of small quantities of the two possible monoenols in equilibrium with the dienol illustrated. No evidence was seen of the completely ketonized tautomer.

We have now succeeded in preparing five chloro substituted derivatives of III, starting in each case with the appropriate chloro substituted benzoylacetone. Table 13-2 summarizes these synthetic results.

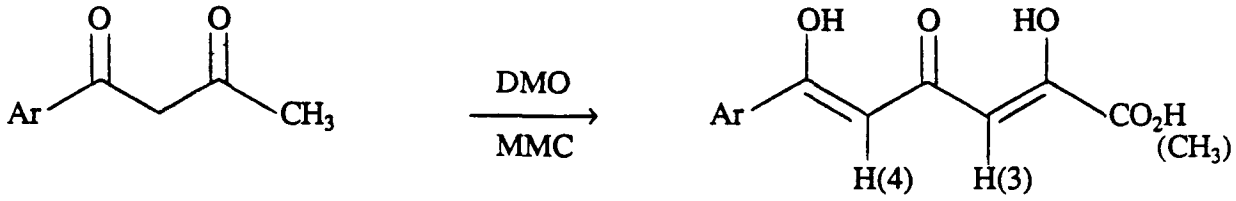
TABLE 13-1. Physical properties of the ring-fission product



		Present Work	Catelani et al., 1973
NMR: (δ)	H(3)	6.53 d (J = 12)	6.52 (J = 12, 0.5)
	H(4)	7.91 dd	7.91
	H(5)	7.39 d (J = 15)	7.34 (J = 16, 0.5)
M.S.:		218 202 201 173	218 202 173
		157 128 105 77	157 105
U.V.:[a]	pH 2	337 nm (4.2)	336 nm (4.3)
	pH 12	435 nm (4.5)	435 nm (4.5)
M.P.:		ca 134° dec.	112° dec.

[a]All of our samples have exhibited a weak absorption of variable intensity near 250 nm. We consider it probable that this absorption is due to an impurity.

TABLE 13-2. Yield and NMR data for the tri-keto acids III-VIII



	Ar	NMR δ (acetone-d_6)		Yield (%)[a]
		H_3	H_4	
III	C_6H_5	6.44	6.62	82
IV	2-ClC_6H_4-	6.43	6.34	79
V	4-ClC_6H_4-	6.44	6.66	91
VI	2,3-$Cl_2C_6H_3$-	6.41	6.31	81
VII	3,4-$Cl_2C_6H_3$-	6.41	6.75	71
VIII	2,4-$Cl_2C_6H_3$-	b	b	high

[a]Free acid plus methyl ester.

[b]Compound VIII was badly contaminated with a product containing one methoxyl group in place of one of the chlorines. It was not possible to assign the NMR signals in the 6-7 δ region with certainty.

Non-Enzymic Cleavage of the Tri-Keto Acids

Treatment of tri-keto acid III (Ar = phenyl) with 1 M NaOH at room temperature resulted in slow (half-time ca. 8 hours) hydrolytic cleavage to form benzoic acid, acetophenone, oxalic acid, and acetic acid. The 4-chloro derivative (V) behaved similarly. Yields are summarized in Table 13-3. A reaction scheme is presented in Figure 13-3. The products can be accounted for on the basis of attack at either C_2 or C_6 of the tri-keto acid. Products that would result from attack at C_4 (benzoylacetic acid, oxaloacetic acid) were not found.

In separate experiments we confirmed previous reports [Hauser et al., 1948; Lehninger and Witzemann, 1942] that the diketones IX and X undergo the indicated reactions at rates that are rapid compared to that which we observe for the tri-keto acids. Measurement of the ratio CA/Cba formed from the diketones IX and IXa (Ar = 4-chlorophenyl) gave values of 2.5 and 1.2, respectively. From these data and the yields given in Table 13-3, the ratio C_6/C_2 for the two modes of attack on the tri-keto acids III and V can be calculated to be 1.0 and 1.1, respectively. The effect of *p*-chloro substituent thus has only a small effect on the point of attack. Studies of the more highly chlorinated derivatives VI-VIII will be instructive on this point.

Non-Enzymic Cleavage of the Ring-Fission Product (I)

The ring-fission product (I) proved more resistant to alkali than the tri-keto acid (III). At 50-55° in either K_2CO_3 or NaOH (1 M) a slow reaction could be readily observed by following the decay in absorbance at 435 nm. However, after complete disappearance of the 435 peak (several days were required) analysis of products did not give a satisfactory material balance. Oxalic acid (>50% yield) benzoic acid, and acetophenone were readily identified, but the ratio of the latter two varied widely in different experiments. Efforts to improve the analysis of this reaction mixture are continuing.

Although oxalic acid is a major product of the non-enzymic hydrolysis of both I and III, this substance has not been reported to be a metabolite of biphenyl or PCBs. Evidently, the enzyme directs attack on the ring-fission products at points more remote from the ionized carboxyl group than C_2. An analogous situation exists with acetylpyruvic acid (X). Non-enzymic hydrolysis leads quantitatively to oxalic acid and acetone, as shown in Figure 13-3, but the compound is metabolized to acetate and pyruvate [Lehninger and Witzemann, 1942; Meister and Greenstein, 1948].

SUMMARY AND CONCLUSIONS

The ring-fission product (I) has been prepared for the first time by chemical synthesis. Studies of PCB metabolism may be facilitated by the availability of this compound in gram quantities.

TABLE 13-3. % Yields of products from alkaline hydrolysis of tri-keto acids III and V

		$ArCO_2H$	$ArCOCH_3$	Oxalate[a]	Acetate[b]
III		69[c]	37[d]	80	n.d.
V	{	67[bc]	21[c]	82	+
		70[d]	25[d]		

[a]By permanganate titration following isolation of Ca salt.

[b]Identified by ^{13}C-NMR.

[c]Determined from weight of isolated product.

[d]Determined by ^{1}H-NMR.

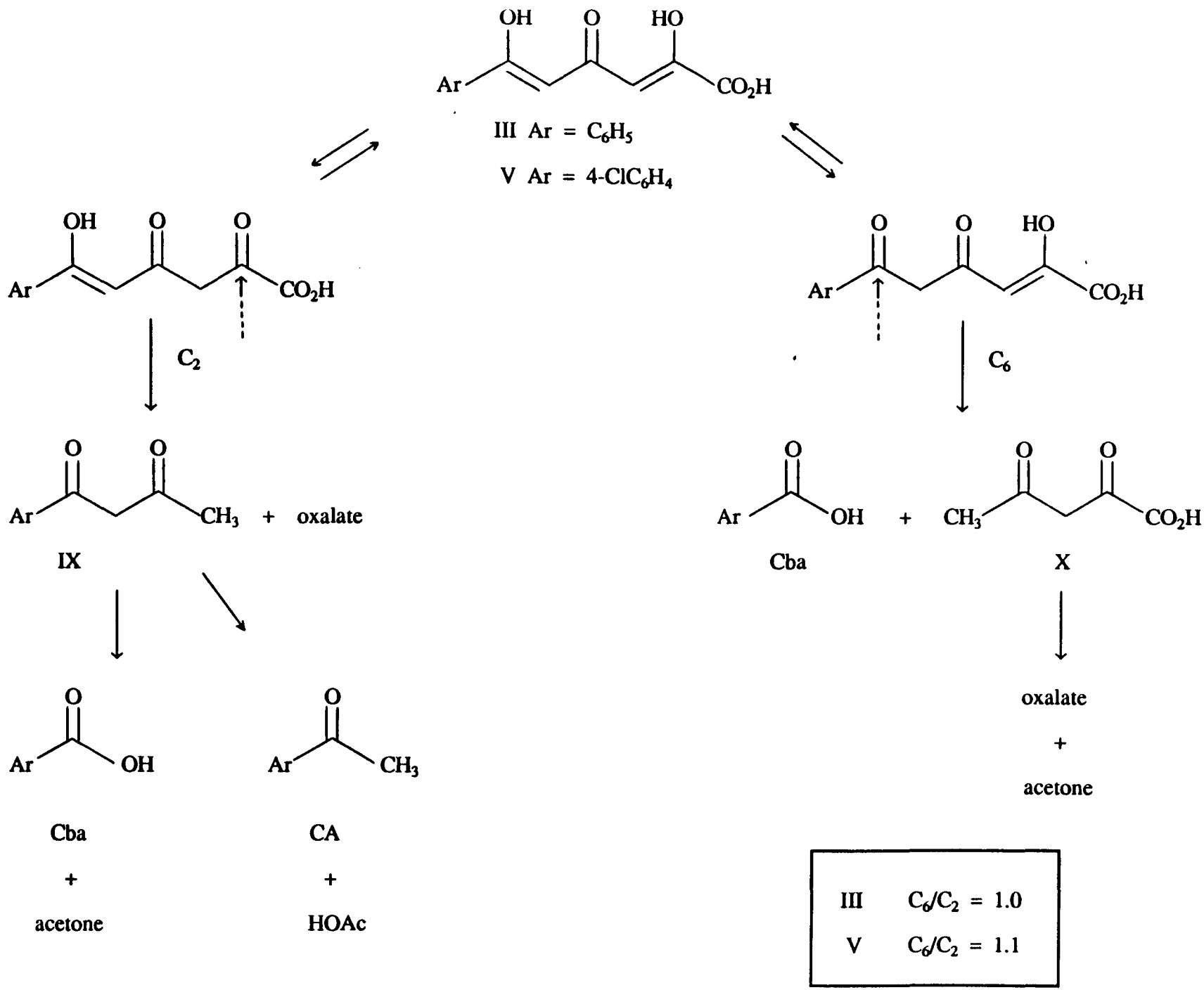


Figure 13-3. Scheme for the alkaline hydrolysis of III and V. Acetone was not determined; yields of other products are given in Table [illegible].

The tri-keto acid III (Ar = phenyl) and five chloro substituted derivatives (IV-VIII) have been synthesized.

Non-enzymic hydrolysis of I, III, and V yielded both benzoic acid and acetophenone (4-Cba and 4-CA in the case of V). These compounds are the major aromatic products formed in the aerobic degradation of many PCBs, and this result may thus lend support to the proposed hydrolytic pathways involving I and III. However, alkaline hydrolysis may not be a good model for the hydrase reaction, and experiments with I and III in microbial cultures should be completed before firm conclusions can be drawn.

Oxalic acid is a major product of the non-enzymic hydrolytic cleavage of compounds I, III, and V. This compound has not been found as a metabolite of biphenyl or PCBs. We suggest that oxalate may be a key substance in delineating the differences between enzymic and non-enzymic mechanisms of attack on this class of compound.

FUTURE PLANS

Chloro derivatives of I, and additional derivatives of III, will be prepared as need arises. Examples will be chosen which correspond to those PCB congeners that are of interest as substrates for aerobic degradation.

Studies of the alkaline cleavage of I and III, including the chloro derivatives, will be continued. We propose to study the effects of pH and of ring substitution on the rate of this reaction, which may be viewed as a crude model for the hydrase reaction.

Efforts will be made to observe the hydrolysis of synthetically produced I and III by cultures of LB400, H850, and/or such other strains as may be available for the purpose.

Chapter 14

THE QUANTITATION OF POLYCHLORINATED BIPHENYLS BY GAS CHROMATOGRAPHY AND TANDEM MASS SPECTROMETRY

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INTRODUCTION

The focus of this research has been to develop methods of analysis for PCB congeners involved in anaerobic dechlorination studies [Brown et al., 1987a; Chen et al., 1988; Quensen et al., 1988]. A major problem with detecting this dechlorination is that with routine gas chromatography (GC) and mass spectrometric (MS) methods, the PCB congeners of interest are often not resolved. Electron capture detection of chromatographically separated PCB isomers is a highly sensitive technique, but coeluting and overlapping components are difficult, if not impossible to identify and quantitate [Duinker et al., 1988; Schulz et al., 1989]. Although MS detection offers enhanced specificity of analysis, detection limits are often not adequate for the samples of interest. Thus, it is desirable to develop methods which will allow the accurate quantitation of individual congeners which may or may not be chromatographically resolvable from other congeners.

It has long been known that chlorinated aromatic molecular anions will undergo an exchange of a chlorine atom for an oxygen atom in the presence of an oxygen diradical in the source of a mass spectrometer [Hunt et al., 1975; Hass et al., 1979; Mitchum et al., 1980]. By using a triple-stage quadrupole mass spectrometer [Yost and Enke, 1983], this reaction can also be observed when oxygen is introduced into the second quadrupole (collision cell) in an MS/MS experiment [Kostiainen and Auriola, 1988 and 1990]. In addition, the oxygen-chlorine exchange reaction is specific for odd-electron molecular anions which allows for the elimination of many of the interferences (especially from more highly chlorinated congeners) present in other methods. As such, the monitoring of this reaction may be used to quantitate PCB congeners which coelute with congeners having a different degree of chlorination.

By using this GC/MS/MS method of analysis, river sediment samples containing Aroclor 1242 which have been subjected to anaerobic activity have been quantitated. Also, river sediment samples which have undergone apparent *in situ* dechlorination have been analyzed. This method offers excellent detection limits, comparable to electron capture detection, while simultaneously providing the high specificity of MS/MS techniques.

EXPERIMENTAL

Sample Preparation

River sediment samples were spiked with Aroclor 1242 and then autoclaved. Three samples were then inoculated and allowed to incubate for 16 weeks. After incubation, three live and three autoclaved aliquots were each extracted with 50/50 acetone/hexane. Each extract was subjected to Florisil cleanup and then shaken with mercury for sulfur removal and finally concentrated to 10 mL. Prior to analysis, the three live samples were combined into a single sample, as were the three autoclaved samples. Sediment samples from the H7 Hudson River site and from Silver Lake were each extracted and prepared in a similar fashion. The Aroclor 1242 standard was provided by John Quensen, and all other individual congeners and standards were purchased from Ultra Scientific Company, Rhode Island.

Instrumental

All GC/MS and GC/MS/MS methods were performed with a Finnigan TSQ-70B mass spectrometer using a Varian 3400 gas chromatograph equipped with a 30 meter x 0.250 μ column of 0.25 μ DB-5 phase (J & W Scientific). The GC was temperature programmed at 140°C for 2 minutes, 140°C to 320°C at 6°C per minute and 320°C for 2 minutes. Helium was used as the GC carrier gas, ammonia was used as the chemical ionization reagent gas, and pure oxygen was introduced into the collision cell. The instrument utilizes a 20 kV dynode which was set at 5 kV. All reactions were generated using electron energies of 70 eV, collision offset energies of 2-3 eV (laboratory), and collision pressures from 0.5 mTorr to 1.5 mTorr. The instrument was tuned for unit resolution in both Q1 and Q3 modes of operation.

RESULTS AND DISCUSSION

Selectivity of MS/MS Detection

The goal of this work is to develop methods of analysis for quantitating PCB congeners which chromatographically coelute with more highly chlorinated congeners. Of particular interest are the six congeners which have been shown to exhibit dioxin-like toxicity; 34-34-CB, 234-34-CB, 245-34-CB, 345-34-CB, 2345-34-CB, and 345-345-CB [Kannan et al., 1988]. It is desirable to be able to quantitate these congeners, in particular 34-34-CB and 234-34-CB, in river sediment samples containing Aroclors. While electron capture detection offers excellent sensitivity, this method is not of use in distinguishing coeluting congeners. Though MS methods offer better selectivity with reduced sensitivity, coeluting structural isomers are still indistinguishable. In addition, quantitation of congeners in the presence of more highly chlorinated isomers by MS is difficult, if not impossible due to the lack of uniqueness of fragment ions. This is illustrated in Figure 14-1 which shows the negative chemical ionization (NCI) mass spectra of both a tetrachloro- and pentachlorobiphenyl. With NCI only a limited amount of fragmentation is observed as opposed to other modes of ionization such as

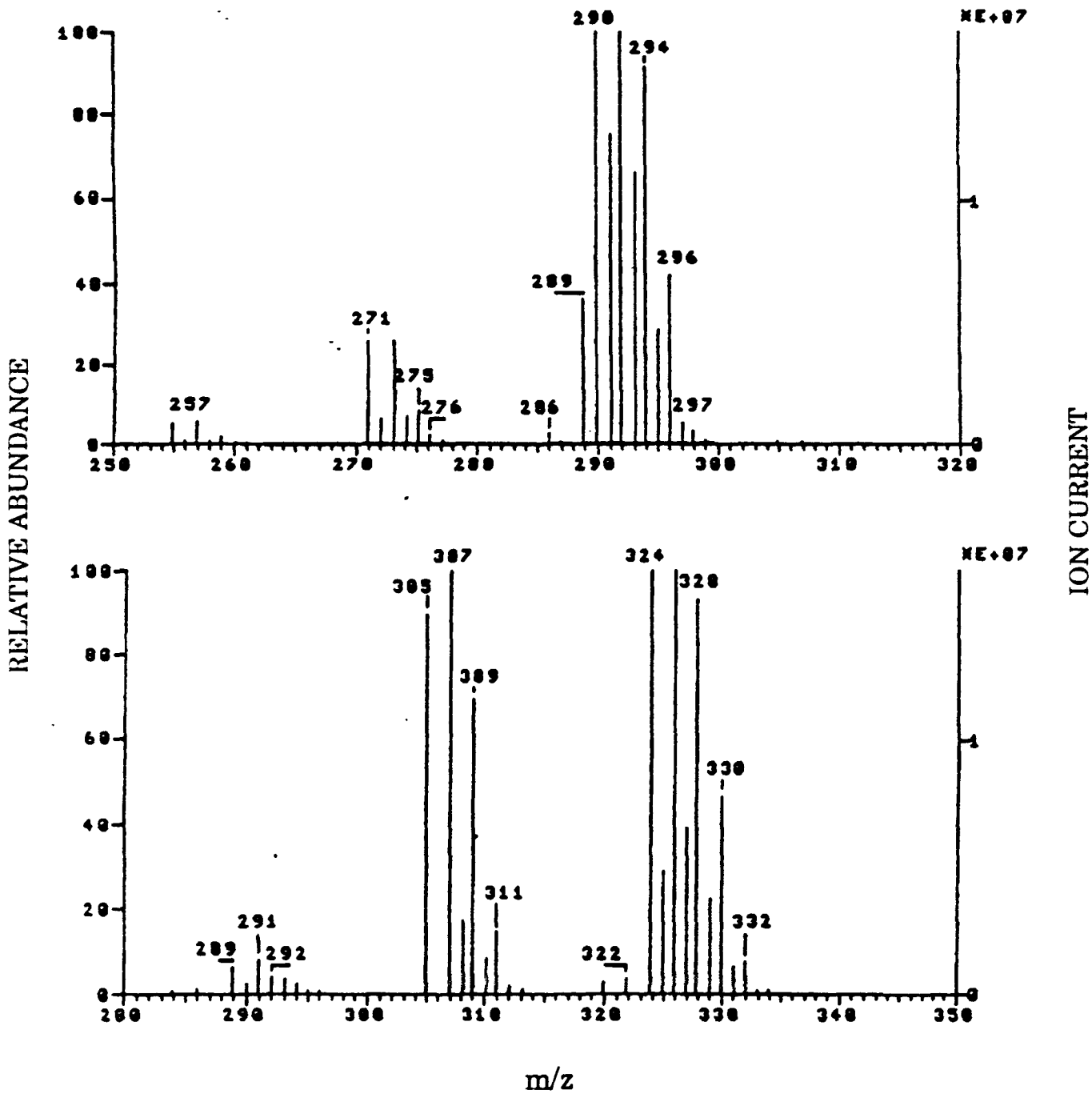

Figure 14-1. The primary (parent) mass spectra of a tetrachlorobiphenyl (top) and a pentachlorobiphenyl generated with negative chemical ionization.

electron impact (EI) ionization [Dougherty et al., 1972; Field, 1980; Daishima et al., 1989]. Also, the most abundant ions in the spectrum of the tetrachlorobiphenyl (the molecular ion region) also appear in the spectrum of the pentachloroisomer. It is for this reason then, that MS/MS methodology was explored for the purpose of enhanced specificity of analysis.

By forming the parent ion [M^-] under conditions of electron capture negative chemical ionization and selecting it in the first quadrupole of a triple-stage quadrupole mass spectrometer and introducing oxygen into the second quadrupole (collision cell), the exchange reaction of oxygen for chlorine may be observed. The m/z 290 ion which is the ^{35}Cl molecular anion of tetrachlorobiphenyls is also present in the primary mass spectrum of pentachlorocongeners as a result of the loss of chlorine from the ^{13}C molecular anion (Figure 14-1). The oxygen-chlorine exchange reaction, however, is specific for the odd-electron molecular anion. Therefore, the MS/MS detection of m/z 290 forming m/z 271 will be completely free of interference from congeners with other degrees of chlorination.

Since the degree of electron affinity of the [M^-] ion relative to that of the [$M\text{-}H^-$] ion is dependent upon chlorination of the aromatic ring and to some extent the positions of this chlorination, not all PCBs form [M^-] ions in the source and are thus not amenable to this reaction. With lower degrees of chlorination, the [$M\text{-}H^-$] ion is an even electron moiety and will not undergo chlorine exchange with oxygen. As a result, none of the mono or di substituted congeners and only a few of the tri substituted congeners are able to be analyzed in this fashion. Thus, shown in Figure 14-2 are the total ion current chromatograms for an Aroclor 1242 sample analyzed by monitoring the reaction products of the molecular anions of the tri- through nonachlorobiphenyls with oxygen. Since the congeners of particular interest are the aforementioned six toxic congeners, and these all consist of at least tetrachloro substitution, this method is of great utility in their analyses.

Choice of Chromatographic Internal Standard

Many PCB analyses are performed chromatographically using octachloronaphthlene as an internal standard. For ECD or MS routines this is a reasonable choice since it is chromatographically resolvable from PCB congeners and exhibits a response factor of the same order of magnitude as that of the PCB congeners. The response factor of an analyte is defined as the ratio of its detector response to its concentration. The response factors for several congeners with respect to octachloronaphthalene are listed in Table 14-1. This internal standard may not be the best choice, however, for the oxygen-chlorine exchange reaction. While octachloronaphthalene will undergo chlorine exchange with oxygen, it is not nearly as responsive as the PCB congeners. Response factors for PCB congeners vary up to three orders of magnitude with respect to this internal standard. The apparent reason for this low response of octachloronaphthalene is that fully-chlorinated aromatics exhibit a lesser tendency to undergo this reaction. This low response may also be one reason for not being able to observe a decachlorobiphenyl congener in the Aroclor 1242 sample. Since the response factors vary dramatically with the degree and position of chlorine substitution, it may be of interest to

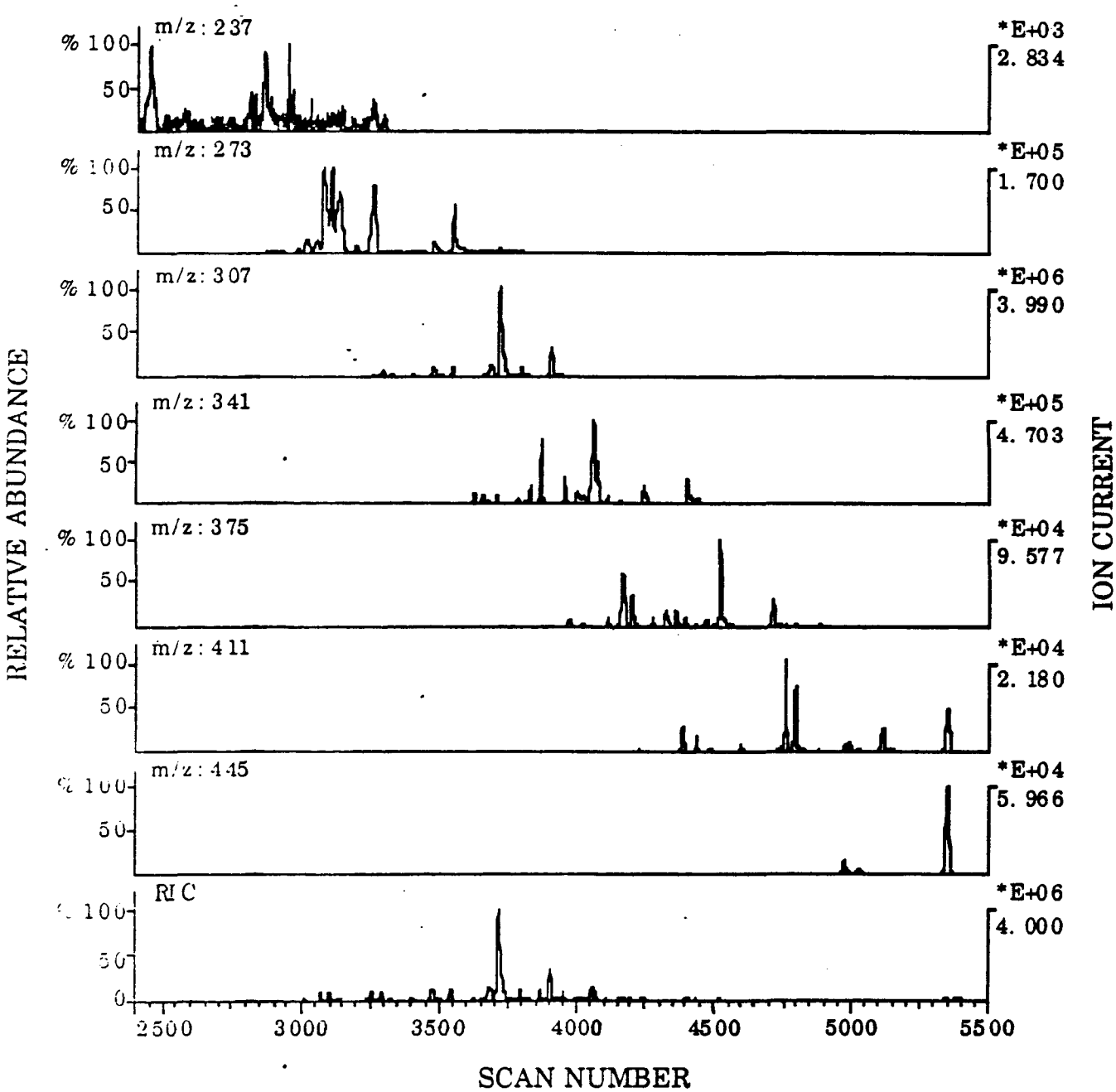

Figure 14-2. The total ion current chromatograms for the tri- through nonachlorobiphenyls of an Aroclor 1242 standard.

TABLE 14-1. Relative response factors (RRF) with respect to octachloronaphthalene and minimum detectable quantities (MDQ - in femtograms) for selected PCB congeners.

CB	RRF	MDQ
34-34	1.6	1200
234-24	290	4
234-34	18	110
236-34	88	12
245-34	11	180
345-34	14	140
234-236	94	11
236-245	194	5
245-245	134	8
2345-34	14	140
345-345	6.0	320
2346-236	48	22
2346-245	499	2
2346-2346	133	8
2345-2356	577	2
23456-345	164	6
23456-2345	234	5
23456-2346	222	5
23456-2356	122	9

explore using other or multiple internal standards for these analyses.

Detection Limits

A primary concern with the development of this method is the achievement of detection limits and sensitivities adequate for the samples of interest. Typical sediment samples contain anywhere from a few parts per million total PCBs to several hundred ppm. This means that many of the individual congeners of interest are on the order of parts per billion or less. While negative chemical ionization offers much better sensitivity and detection limits than EI/MS, it approaches the performance of an ECD only in selected-ion monitoring (SIM) modes of operation. For PCBs this involves the monitoring of chloride in particular. Using SIM, however, offers very little advantage in selectivity over the use of an ECD. By monitoring constant neutral losses of m/z 19 in an GC/MS/MS experiment, a sample may be specifically analyzed for chlorinated aromatics using the oxygen-chlorine exchange reaction. This technique is a scanning technique and as such has detection limits two to three orders of magnitude higher than that of an ECD or SIM. By monitoring only one reaction (for instance, m/z 324 to m/z 305), the high specificity of MS/MS is coupled with the low detection limits of SIM. A comparison of detection limits for these modes of operation for several congeners are also given in Table 14-1.

As is shown, detection limits for the more highly chlorinated are exceptionally low. Of particular interest are the detection limits for the nonachlorobiphenyls. These congeners are generally not observed in a quantifiable manner in an Aroclor 1242 sample; and yet, by monitoring the selected reaction m/z 466 ($M+4^-$) to m/z 447 in a chromatographic run, three (and only three) significant peaks are observed in quantities well above the detection limits. The quantities of the three nonachlorobiphenyls in an Aroclor 1242 standard were calculated to be 0.059% for 23456-2345-CB, 0.0066% for 23456-2346-CB, and 0.022% for 23456-2356-CB.

In Table 14-2 are listed the quantitative results of the six selected toxic congeners for the autoclaved and inoculated river sediment samples and for sediment samples taken from contaminated sites. As is shown, for all but one congener, ~ 80% dechlorination was observed. The advantage of this method is apparent from the fact that for two of these congeners (34-34-CB and 234-34-CB), quantitation is difficult by GC/MS due to the presence of co-eluting congeners (236-34-CB and 234-236-CB, respectively) in amounts one to two orders of magnitude greater. By using this selected reaction method, however, the co-elutates do not present an interference (see Figure 14-3).

SUMMARY AND CONCLUSIONS

Using a selected reaction monitoring mode of operation in a GC/MS/MS experiment and monitoring the exchange reaction of oxygen for chlorine, PCB congeners are able to be quantitated even when not chromatographically resolved. This method couples the high specificity of MS/MS with the excellent detection limits of selected-ion monitoring coupled with

TABLE 14-2. Results of analysis of PCB samples for the toxic congeners. See text for explanation of samples. Values listed are amounts in mg/L and percents of total PCBs.

CB	34-34	234-34	245-34	345-34	2345-34	345-345	
H7:AUTO	0.29	0.86	0.67	0.006	0.17	0.003	mg / L
H7:LIVE	0.00	0.12	0.14	0.001	0.009	0.00	mg / L
HUDSON RIVER	0.17	0.14	0.72	0.001	0.18	0.00	mg / L
	0.10	0.08	0.42	0.001	0.11	0.00	%
SILVER LAKE	0.19	0.25	2.20	0.048	7.00	0.069	mg / L
	0.04	0.02	0.16	0.003	0.50	0.005	%
H7:JUNE	1.5	0.65	1.7	0.037	0.35	0.010	mg / L
	0.6	0.26	0.68	0.015	0.14	0.004	%

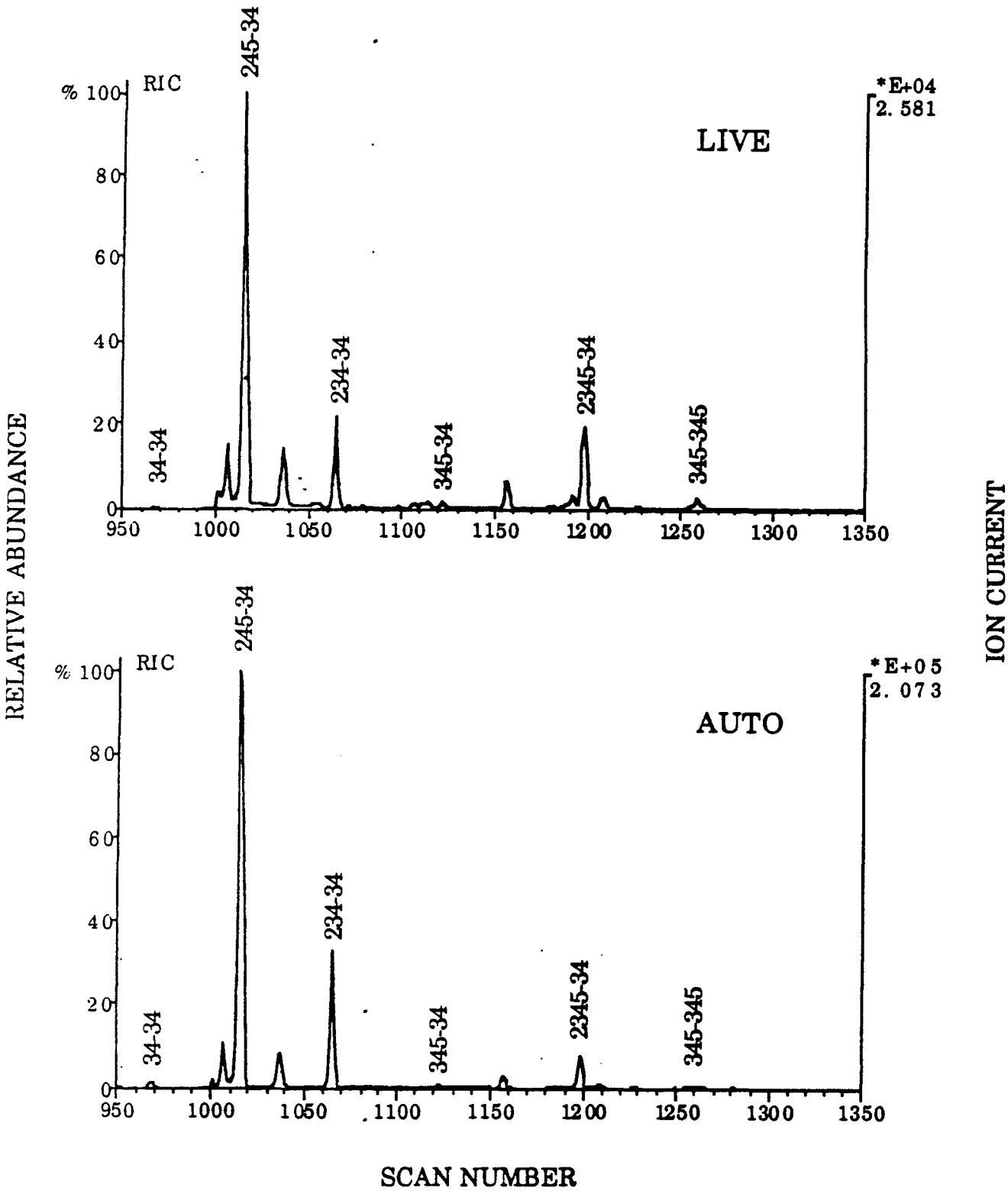

Figure 14-3. The total ion current chromatograms generated by monitoring the selected reactions for the toxic congeners (see text) in the analysis of two river sediment samples spiked with Aroclor 1242 with one inoculated (Live) with bacteria from the H7 site under anaerobic conditions and the other autoclaved (Auto). Note the difference in intensities for the two chromatograms.

negative chemical ionization. For the more highly chlorinated congeners, detection limits are even lower than that of an ECD. Due to the highly selective nature of this technique, individual PCB congeners may be quantitated in chromatographic analysis times of 30 minutes or less.

FUTURE WORK

1. Quantitation. The concentrations of individual congeners within a standard Aroclor mixture need to be determined. In addition, the precision and accuracy of the method need to be defined.
2. Internal Standards. The use of internal standards in conjunction with, or in addition to, octachloronaphthalene needs to be explored.
3. Instrumental Modifications. The instrument (TSQ-70B) needs to be modified in order to more accurately control the gas flow into the source and collision cell.
4. Other Oxidants. Reactants other than oxygen may prove to be useful for this method.
5. Other Aroclors. This method will be used for other Aroclors such as 1016, 1254, and 1260.
6. Other PCB Reactions. It would be desirable to explore other reactions of PCB ions in MS/MS experiments which could be used to distinguish isomers of the same mass.
7. Other Compounds in PCB Samples. This method will be used to detect and quantitate other chlorinated species such as PCDDs and PCDFs in PCB containing samples.

Chapter 15

RESEARCH PLANS FOR 1990-1991

A sequential biological process combining anaerobic reductive dechlorination and aerobic oxidation continues to show great promise for degrading even highly chlorinated Aroclor mixtures. Now that this process has been demonstrated in the laboratory, the challenge remains to apply it in the field under real environmental conditions. Future research plans are focused on gaining a greater understanding of these complementary modes of PCB biotransformation and the factors which influence their activity in the environment.

Anaerobic

The characterization and isolation of single strains or consortia of PCB-dechlorinating cultures remains as a significant goal in this research. Other areas of interest include further identification of consortia capable of *ortho* dechlorination and additional quantitation of the toxicity reduction in dechlorinated PCB mixtures. Research to examine the factors which influence the rate and extent of the dechlorination process on endogenous PCBs in different soils and sediments will continue, with an increased emphasis on bioavailability issues. Efforts to scale up the anaerobic process in anticipation of *in situ* application of this technology will increase.

Aerobic

The potential advantages of recombinant PCB-degrading strains over natural isolates will continue to be explored in the laboratory. These efforts include sequencing the *bph* genes and overexpression of these genes in the recombinants. Further characterization of the aerobic degradative pathway via both chemical and biological routes will also continue. Finally, laboratory aerobic degradation results using natural isolates will be extended and scaled up. The results of this work will be applied toward developing an aerobic process to degrade the endogenous PCBs found in extensively dechlorinated sediments.

Other

Efforts in the analytical area will include improving the precision and accuracy of the GC/MS/MS method and extending the method to detect other chlorinated species, such as PCDDs and PCDFs. Other nonbiological methods of removing PCBs from soils, such as radio frequency heating, will continue to be explored in the coming year.

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