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CHARACTERIZATION OF PCB (POLYCHLOROBIPHENYL) DECHLORINATION BY ANAEROBIC MICROORGANISMS FROM HUDSON RIVER SEDIMENTS

by

Dingyi Ye

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Crop and Soil Sciences

1994

ABSTRACT

CHARACTERIZATION OF PCB (POLYCHLOROBIPHENYL)DECHLORINATION BY ANAEROBIC MICROORGANISMS FROM HUDSON RIVER SEDIMENTS

By

Dingyi Ye

Investigation on the anaerobic microbial dechlorination of polychlorobiphenyls (PCBs) was conducted to identify the dechlorinating physiological groups and to characterize their dechlorination processes. The investigation was performed with microorganisms eluted from the Hudson River sediments. Addition of specific inhibitors of sulfate-reducers (molybdate) and methanogens (2-bromoethanesulfonic acid, BESA, henceforth), as well as sulfate, inhibited both PCB dechlorination and methane formation, suggesting that either sulfate-reducing bacteria or methanogenic bacteria, or both, were involved in the dechlorination. The observation that cultures obtained under highly selective pressure for methanogens dechlorinated PCBs, mainly at the *para* position, provided more evidence that methanogenic bacteria are probably among the physiological groups capable of anaerobic PCB dechlorination. *Ortho* dechlorination of 2-CB and 2-2-CB/2,6-CB by the transferred methanogenic cultures was observed, indicating that the Hudson River contains

microorganisms with the potential for complete dechlorination of PCBs to biphenyl. Inocula that were pasteurized or treated with ethanol or both, retained a *meta*-preferential dechlorination activity. This dechlorination activity was stable over time and through

and through serial transfers. Neither methanogens nor thermophiles were responsible for this retained dechlorination activity. The results indicated that microorganism responsible for the dechlorination by the pasteurized cultures were probably anaerobic sporeformers. Dechlorination by the pasteurized cultures was also inhibited by BESA, sulfate, and molybdate. It appeared that microorganisms capable of dechlorination in the pasteurized cultures were probably sulfidogens. Only sporeforming bacteria were expected to survive the harsh pasteurization, and among known sulfidogens the only genus forming spores is *Desulfotomaculum*. Therefore, the experimental results suggest that the dechlorinating microorganisms in our pasteurized enrichment are probably *Desulfotomaculum*-like sulfate-reducing bacteria. Incubation temperature was also used in combination with pasteurization to more effectively differentiate three different activities Aroclor 1242, one that removes *meta* chlorines and two that remove *para* chlorines.

To my wife

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I wish to express my deep thanks to Dr. Stephen A. Boyd and Dr. James M. Tiedje for the Research Assistantship, for their valuable guidance and the continuous encouragement throughout my years of graduate study.

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GENERAL INTRODUCTION

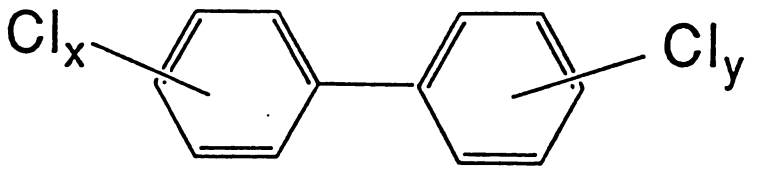
Polychlorinated biphenyls (PCBs) were widely used in industry for almost 50 years (1929-1978) (7), and during the 50-year period several hundred million pounds have been released into the environment (10). Despite the fact that the production and application of PCBs have been banned, their environmental fate is still a public concern because PCBs are recalcitrant and ubiquitous environmental contaminants and have possible toxic health effects (16).

In recent years microbially mediated anaerobic reductive dechlorination of PCBs was observed *in situ* (5, 6, 7) and was later confirmed in the laboratory (14). This process has also been reported by a number of laboratories with sediments from different aquatic systems (for reviews see 1). However, despite the environmental significance of anaerobic dechlorination of PCBs, research has, until now, failed to identify any dechlorinating physiological group and to characterize their dechlorination processes. Therefore, anaerobic dechlorination of PCBs by microorganisms from the Hudson River sediments was investigated to tentatively identify the microbial physiological groups capable of PCB dechlorination and to characterize the dechlorination processes performed by the dechlorinating physiological groups.

General Information of PCBs

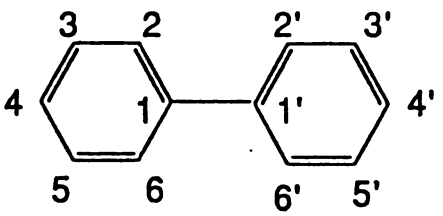
Structure and nomenclature. The polychlorobiphenyls (PCBs) are a family of chlorine substituted biphenyls. The general structure, numbering in the biphenyl ring

Polychlorobiphenyls (PCB's)

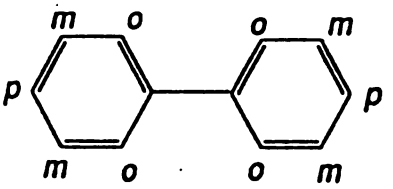


(A)

209 congeners are theoretically possible
Half are actually produced



Numbering in the Biphenyl Ring System



(B)

p - para *m - meta* *o - ortho*

Substituted Position in the Biphenyl Ring System

Fig. 1. (A) The general structure of PCBs. (B) The numbering and the chlorine substituted position in the biphenyl ring system.

system, and substituted position in the biphenyl ring system are shown in Fig. 1.

Chlorines can be attached to one ring or both, the number of the substituted chlorines may vary from 1 to 10. There are 209 theoretically possible congeners, however, only about half are actually produced (9).

Commercial PCBs were usually complex mixtures differing in the average chlorination levels under different trade names such as "Aroclor".

Physical and chemical properties of PCB. The major physical properties of PCBs are low water solubility, low vapor pressures, and excellent dielectric properties (9). The major chemical properties of PCBs are flame resistant and low reactivity, such as stable to oxidation and hydrolysis, and resistant to bases and acids (9).

Use of PCBs and the resulted environmental problems. Due to the relative inertness, flame resistant, and the excellent dielectric properties, PCBs were widely used in industry for almost 50 years. PCBs were mainly used as dielectric fluids (capacitors and transformer), industrial fluids (hydraulic system, gas turbines, and vacuum pumps), fire retardant, heat transfer applications, and plasticizers (adhesives, textiles, surface coatings, sealants, printing, copy paper) et al. (9).

it is estimated that during the 50-year period about several million pounds have been released into the environment, and this has caused environmental problems because PCBs have possible health effects, they are thermally and chemically stable, and the lipophilic nature of these compounds enables them to accumulate in food chain, to undergo bio-magnification, and to distribute worldwide.

Microbial Destruction of PCBs

Aerobic microbial degradation. Since PCBs are thermally and chemically stable, therefore, their environmental fate mainly depends on microbial destruction. Intensive studies on aerobic microbial degradation of PCBs have been reported (for review please see reference 1). These studies include soil communities, pure strains, and recombinant microorganisms. Although most soils contaminated with PCBs contain organisms with some level of PCB-degrading ability (1), the aerobic degradation is usually limited to the less chlorinated congeners, possibly because that higher chlorination levels may have steric hindrance effect on 2,3-dioxygenation which is a general pathway in aerobic degradation of PCBs (1).

Anaerobic reductive dechlorination. The anaerobic reductive dechlorination is a process in which chlorine is replaced by hydrogen and the aromatic rings remain intact. Unlike the aerobic degradation of PCBs which is generally limited to the less chlorinated congeners, the anaerobic dechlorination of PCBs converts a large array of highly chlorinated PCBs to lesser chlorinated congeners and the dechlorination products are known substrates for aerobic microorganisms (1). Anaerobic reductive dechlorination of PCBs removes chlorines from the biphenyl rings, hence reduces the toxicity of PCBs. Moreover, the reductive dechlorination of PCBs is especially noteworthy for the ability to transform highly chlorinated PCBs which are stable in the environment. The anaerobic reductive dechlorination is actually a major initial step to destruct most highly chlorinated PCBs. Without this initial destruction step, Those highly chlorinated congeners would, otherwise, likely persist in the environment.

However, despite the environmental significance of the reductive dechlorination of

PCBs, until now, no physiological group of the dechlorinating microorganisms has been identified.

The Objectives of This Study

The objectives of this study are: (1) to identify the physiological group(s) of the dechlorinating microorganisms from the Hudson River sediments, and (2) to characterize the dechlorination processes correlated to the defined physiological groups.

Overview of The Thesis

Effects of Molybdate, BESA, and Sulfate on Dechlorination of PCBs (Chapter 1). Both sulfate-reducing bacteria and methanogenic bacteria are major terminal electron sinks in the anaerobic food chain, and play important roles in the degradation of organic pollutants in anaerobic environments (for reviews, see references 3, 8, 9, 13). To clarify the roles these two physiological groups play in the anaerobic dechlorination of PCBs, specific inhibitors of sulfate reducers (molybdate) and methanogens (2-bromoethanesulfonic acid, BESA henceforth) (2, 17) were used to probe the functions of methanogens and sulfate-reducers in anaerobic dechlorination. Effects of sulfate on the dechlorination were also investigated. Sulfate is not a specific inhibitor of any microbial physiological group, however, addition of sulfate to sulfate-limited cultures usually stimulates growth of sulfate-reducing bacteria and concomitantly inhibits methanogenic bacteria (11, 12, 15). Therefore, sulfate may be used as both an inhibitor of methanogens and a stimulator of sulfidogens. The experimental results showed that addition of molybdate, BESA, and sulfate inhibited concomitantly PCB dechlorination

and methane formation, suggesting that either sulfate-reducing bacteria or methanogenic bacteria, or both, were involved in PCB dechlorination.

Methanogen experiment (Chapter 2). Results presented in Chapter 1 suggested that either sulfate-reducing bacteria or methanogenic bacteria, or both, were involved in PCB dechlorination. To further examine whether methanogens are capable of PCB dechlorination and to obtain an in-depth of physiological and ecological understanding about the microbial dechlorinating community, methanogen experiment was conducted. The experimental results showed that methanogenic bacteria are probably among the physiological groups capable of anaerobic dechlorination of PCB's. Furthermore, *ortho* dechlorination of 2-CB and 2-2-CB/2,6-CB by the transferred methanogenic cultures was observed, indicating that the Hudson River contains microorganisms with the potential for complete dechlorination of PCBs to biphenyl.

The heat- and ethanol-treatment experiments (Chapter 3). In one batch of the inhibition experiments described in Chapter 1, one of the triplicated autoclaved (121°C, 1 h) cultures retained the dechlorination activity (Appendix Section I). After the contamination was carefully examined and ruled out, this unexpected result suggested that the responsible microorganism(s) was(were) particularly heat resistant. Therefore, a PCB-dechlorinating inoculum was treated with either heat or ethanol, or both, to determine whether anaerobic sporeformers are capable of the PCB dechlorination. All treated cultures retained a *meta*-preferential dechlorination activity. Methanogens were not responsible for the retained dechlorination activity since no methane production was detected in any treated culture. Thermophiles were also not responsible for the dechlorination activity as evidenced by the fact that no dechlorination was observed at

37°C and above. These results indicate that anaerobic sporeformers are at least one of the physiological groups responsible for the reductive dechlorination of PCBs.

Effects of molybdate, BESA, and sulfate on the dechlorination by the pasteurized cultures (Chapter 4). Pasteurization eliminates methanogens which play an important role in anaerobic microbial processes (4, 20), but a partial dechlorination activity was still retained (19). Pasteurization also minimized the microbial diversity in the system since only sporeforming bacteria maybe expected to survive the treatment. Therefore, the effects of molybdate, BESA, and sulfate on PCB dechlorination by the pasteurized cultures were investigated to examine the relationship between the sulfate-reducer surviving the pasteurization and the retained dechlorination activity. BESA and sulfate completely inhibited dechlorination at their lowest screened concentrations, 1 mM and 2 mM, respectively. Molybdate partially inhibited the dechlorination at 2, 4, and 8 mM, and completely inhibited the dechlorination at 16 mM. It appeared that microorganisms capable of anaerobic dechlorination of PCBs in the pasteurized cultures were probably sulfidogens. Molybdate probably directly inhibited sulfidogens, while both sulfate and BESA probably inhibited the dechlorination by competing with PCB as electron acceptors. Only sporeformers were expected to survive the harsh pasteurization, and among sulfidogens the only known genus forming spores is *Desulfotomaculum*. Therefore, the experimental results suggest that microorganisms responsible for the dechlorination of PCBs in our pasteurized enrichment are probably *Desulfotomaculum*-like sulfate-reducing bacteria.

Differentiation of congener-specific dechlorination activity on Aroclor 1242 (Chapter 5). In the pasteurization experiment (Chapter 3), it was necessary to identify

if thermophiles were responsible for the dechlorination activity retained in the pasteurized culture. Therefore, effects of the incubation temperature were screened. Since temperature is also a pressure to select the dominant population, combination of both pasteurization and the incubation temperature will more effectively differentiate the microbial dechlorinating community. The experimental results confirmed that the dechlorination activity in the pasteurized cultures was not due to thermophiles. In addition to this conclusion, since both pasteurization and incubation temperature are selective pressures, combination of both pressures effectively differentiated the *para* and *meta* dechlorination activities of the system and hence demonstrated and partially characterized three classes of congener-specific *meta* and *para* dechlorination activity present in the Hudson River sediments.

The relationships among the Chapters. Despite that five chapters report different investigations on the anaerobic dechlorination of PCBs, these chapters are related each other.

Chapter 1 reports the preliminary investigations, and chapter 2 is a further investigation based on the conclusion of Chapter 1.

In one batch of the experiments described in Chapter 1, unexpected dechlorination by an pasteurized culture was observed, and it was this unexpected result that prompted the author to initiated the heat- and ethanol- treatment experiments summarized in Chapter 3.

Results of Chapter 3 indicate that anaerobic sporeformers are probably among the microorganisms capable of PCB dechlorination. However, it remained unclear which microorganism(s) the dechlorinating sporefomer(s) might be. Chapter 4 reports the effort

to tentatively identify and characterize the dechlorinating microorganisms in the pasteurized cultures.

As mentioned above, the original purpose of the experiment reported in Chapter 5 was to determine if thermophiles were responsible for the dechlorination by the pasteurized cultures. The results did clearly answer this question. In addition, this experiment successfully differentiated the *para* and *meta* dechlorination activities in the system. As shown in Chapter 5, three classes of dechlorination activity were demonstrated and partially characterized.

Besides the above relationships, the five chapters share common objectives: to identify the physiological groups of the dechlorinating microorganisms, and to characterize their dechlorination processes.

The appendix. The appendix consists of some experiments which are not included in the five chapters.

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Chapter 1

Effects of Molybdate, 2-Bromoethanesulfonic Acid (BESA), and Sulfate on Anaerobic Microbial Dechlorination of Plychlorobiphenyls (PCB's)

ABSTRACT

Addition of molybdate, 2-bromoethanesulfonic acid (BESA), and sulfate inhibited concomitantly PCB dechlorination and methane formation by microorganisms from the Hudson River sediments, suggesting that either sulfate-reducing bacteria or methanogenic bacteria, or both, were involved in the dechlorination. The inhibitory effects depended on experimental concentrations. BESA (50 mM) completely inhibited the on-set of dechlorination after 2 weeks of incubation, while 50 μM had no effect. When methanogensis was completely inhibited by 5 mM BESA, the cultures retained a partial dechlorination activity different from that of pasteurized cultures, indicating that the responsible microorganism(s) is(are) non-methanogenic and cannot survive pasteurization. In the presence of 3 ~ 5 mM sulfate, partial dechlorination was observed. This activity is noteworthy for its ability to attack 2,4-4-chlorobiphenyl (2,4-4-CB) and 2,4,6-2-CB. This specificity of dechlorination, together with different dechlorination specificities expressed by the BESA- and molybdate-amended cultures, as well as those observed with our different enrichments and other reported dechlorination patterns, indicate that diverse types of microorganisms in Hudson River sediments have the capacity to remove chlorines from certain positions of specific PCB congeners. The presence or absence of these active microorganisms may determine the actual PCB-dechlorination pattern observed *in-situ*.

INTRODUCTION

Both sulfate-reducing bacteria and methanogenic bacteria are major terminal electron sinks in the anaerobic food chain, and play important roles in the degradation of organic pollutants in anaerobic environments such as ground water and sediments, etc. (for reviews, see references 2, 5, 12, and 22). To investigate their activities in mixed communities, specific inhibitors of these two physiological groups are widely used. These inhibitors include molybdate which specifically inhibits sulfate-reducing bacteria, and 2-bromoethanesulfonic acid (BESA) which specifically inhibits methanogenic bacteria. The inhibitory properties of these reagents are based on unique physiological characteristics of the bacteria (1, 21), and are purported to have little effect on other microorganisms (14, 20). Thus, they may serve as powerful tools to probe the function of different physiological groups in the microbial community (14).

Unlike molybdate and BESA, sulfate is not a specific inhibitor of any microbial physiological group. However, addition of sulfate to sulfate-limited cultures usually stimulates growth of sulfate-reducing bacteria and concomitantly inhibits methanogenic bacteria, mainly because sulfate-reducing bacteria out compete methanogenic bacteria for hydrogen or acetate, and also due to bioenergetics reasons (7, 8, 17). Therefore, sulfate may be used as both an inhibitor of methanogens and a stimulator of sulfidogens.

In order to examine the role that methanogenic bacteria and sulfate-reducing bacteria play in PCB dechlorination, effects of molybdate, BESA, and sulfate on dechlorination were investigated. These studies are part of the efforts to characterize anaerobic microbial PCB dechlorination activity observed in mixed communities present in PCB contaminated sediments of the Hudson River (3, 15, 15a)

MATERIALS AND METHODS

Inoculum. The sediments for preparation of the inoculum were collected from the upper Hudson River near Hudson River Falls, N.Y. (site H7 in reference 3). Microorganisms were eluted from the sediments with the reduced anaerobic mineral medium (RAMM) (18) as described elsewhere (23). Sediment collection, shipment, and inoculum preparation were under strictly anaerobic conditions.

Preparation of the experimental batch. Both 160-ml or 60-ml serum bottles were used for different experiments. The 60-ml serum bottles received 10 g of PCB-free Hudson River sediments (collected from the Hudson River at River Miles 205, refer to reference 15) and 10 ml of RAMM containing 0.1% methanol and 10% inoculum, and was then incubated at 37°C for 1 week. After methane was detected, the bottles were autoclaved for 1 h on 3 consecutive days with incubation at 37°C between each autoclaving. After the third autoclaving, 10 ml of inoculum, 10 ml of RAMM, and 80 μl of 10% PCBs (Aroclor 1242, Monsanto Co., St. Louis, Mo.) in acetone were added. To evaluate the effects of inhibitors after on-set of dechlorination, similar experiments were set up using 50 μl of 10% PCB in acetone and 25 g dry sediment per 160-ml serum bottle. Stock solutions of molybdate, BESA, and sulfate were adjusted to pH 7 and autoclaved. The controls were autoclaved twice, 1 h each time with a 5 h incubation at 37°C before PCBs were added. The samples were shaken for 1 h and then incubated at 25°C in dark. Preparation of the experimental batches with 160-ml serum bottles followed the same procedures; the amounts of sediments, RAMM, PCB, and inoculum were proportionally increased.

Analysis of sulfate, molybdate, and determination of methane production. Sulfate and molybdate were analyzed with a high pressure ion chromatograph (Dionex) equipped with a HPIC AS4A column (20 cm) and a conductivity detector. The mobile phase was 1.7 mM $NaHCO_3$/1.8 mM Na_2CO_3, and the flow rate was 2.3 ml/min. Before being sampled, the culture bottles were shaken for 30 min, the headspace gas was analyzed for methane production, and 2 ml of the liquid portion of the samples were taken while being flushed with N_2, acidified with 1 N HCl, and bubbled with N_2 for 5 min to drive out H_2S. The samples were then centrifuged, filtered (0.22 μm, Millipore Co., Bedford, Mass.), and analyzed for sulfate and molybdate.

Methane production was measured by gas chromatography using a flame ionization detector.

PCB analysis and data summation. These procedures were as previously described (15).

RESULTS

Effects of molybdate, BESA, and sulfate after the on-set of dechlorination. After the on-set of dechlorination of Aroclor 1242 (200 μg/g dry sediment) was confirmed by GC analysis at 4 weeks, molybdate, BESA, and sulfate were introduced at a final concentration of 5, 25, and 20 mM, respectively, and then added again at these same concentrations 1, 2, and 3 weeks henceforth. PCB analysis showed that during 4 weeks of incubation no further dechlorination was observed in any culture including the non-amended cultures, suggesting that those congeners susceptible to microbial dechlorination probably had been dechlorinated to below a threshold concentration.

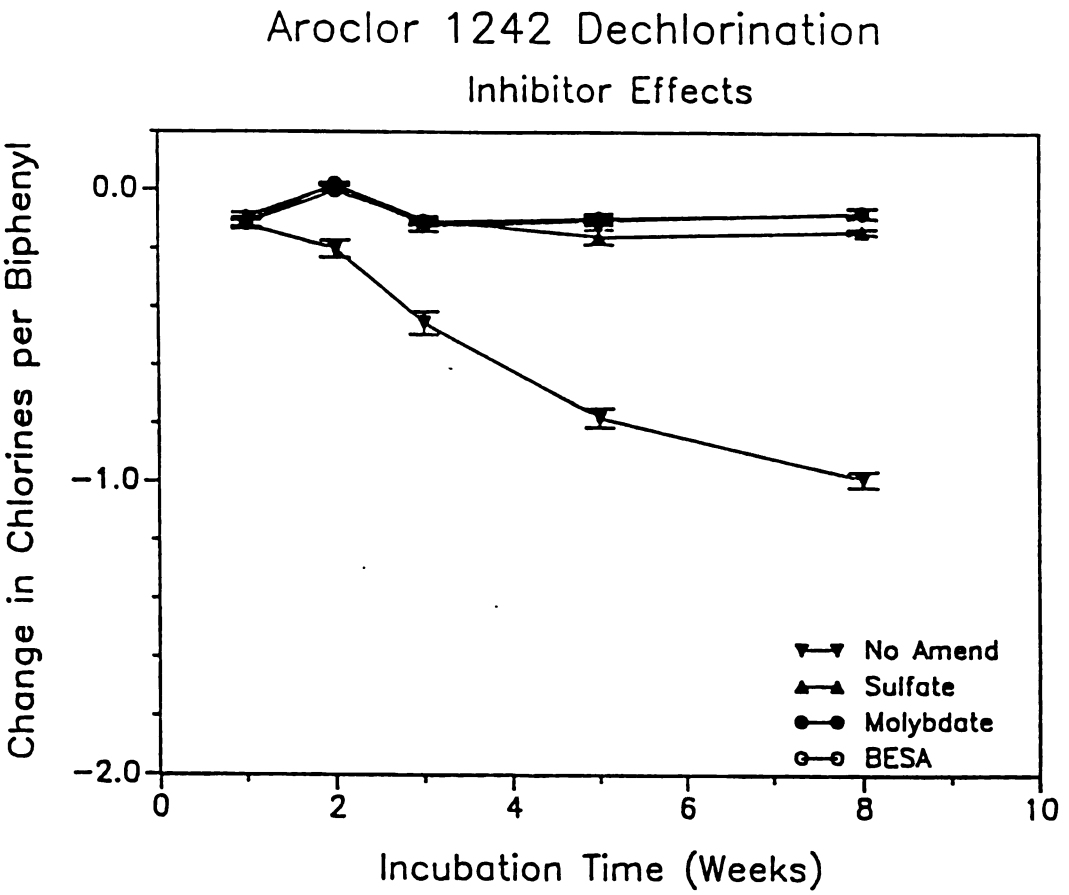

Fig. 1. Effects of molybdate, BESA, and sulfate on the on-set dechlorination of Aroclor 1242 by microorganisms eluted from Hudson River sediments. Error bars are the standard deviation (SD) of triplicate samples; where not shown, the error bars are smaller than the symbols.

Therefore, 400 μg/g sediment (dry weight) of Aroclor 1242 along with 5, 25, and 20 mM molybdate, BESA and sulfate were added, and that time was re-designated as the 0 time. The cultures were continuously incubated, and sampled at 1, 2, 3, 5, and 8 weeks. The cultures were re-amended with 5 mM of molybdate and 25 mM of BESA at 1 and 2 weeks; and 20 mM of sulfate at 1, 2, and 3 weeks. As shown by Fig. 1, dechlorination resumed in the non-amended cultures, whereas no dechlorination was observed in those cultures amended with either molybdate, BESA, or sulfate. The non-amended cultures produced methane, whereas no methane was detected in any amended culture.

Effects of BESA, molybdate, and sulfate on initial dechlorination. In this experiment BESA, molybdate and sulfate were added at the initiation of incubation. The sulfate experiment was performed using a different batch of inoculum from that in the molybdate and BESA experiments. The initial concentrations of molybdate and BESA were 5 and 50 mM, respectively. The same amounts of molybdate and half the amount of BESA added initially were re-fed at 2, 4, 6, and 8 weeks. The initial concentration of sulfate was 20 mM, and the same amount was added again at 4 and 8 weeks. The non-amended cultures produced methane, however, no methane production was detected in any amended cultures, except one of the triplicated molybdate-amended cultures which produced methane between 4 and 6 weeks (13.6% of that produced by the non-amendment group, data not shown). After supplement of molybdate at 6 weeks, no additional methane was detected in this culture.

BESA and sulfate completely inhibited dechlorination at the experimental concentration used. Molybdate delayed and partially inhibited the dechlorination.

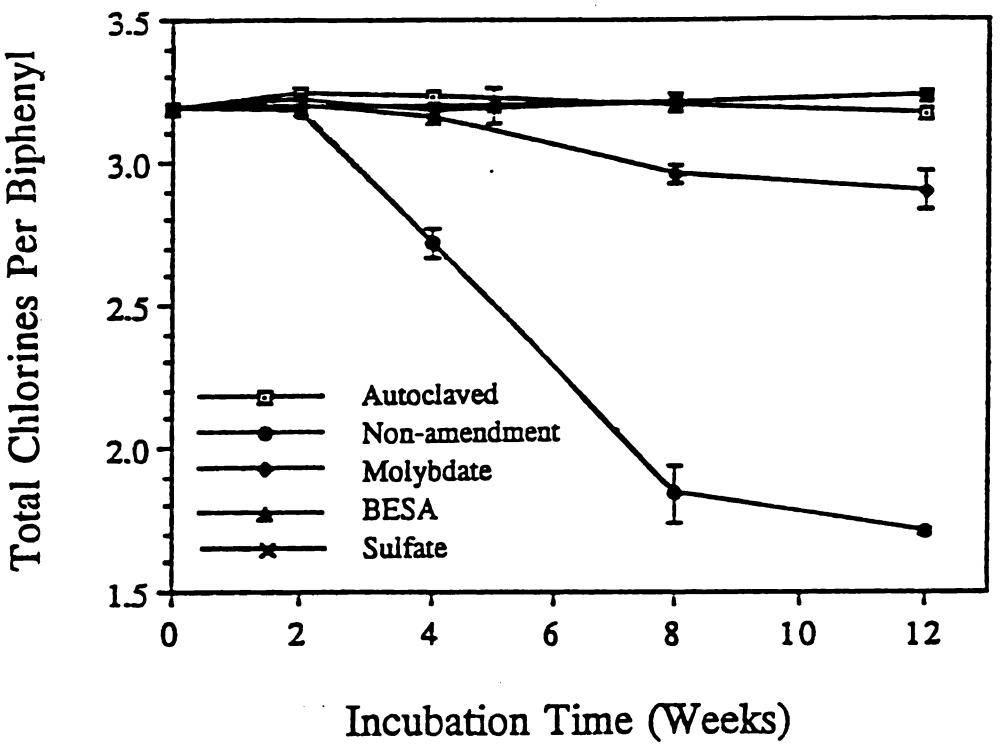

Fig. 2. The initial dechlorination of Aroclor 1242 by microorganisms eluted from Hudson River sediments incubated with either molybdate, sulfate, or BESA. Error bars are the standard deviation (SD) of triplicate samples; where not shown, the error bars are smaller than the symbols.

The color of the cultures amended with sulfate and with BESA turned dark black after 2 weeks of incubation, and these cultures gave off a strong sulfide-smell. In contrast to the dark black color of the sulfate- and BESA-amended cultures, the molybdate-amended cultures turned somewhat yellowish after 2 weeks of incubation and there was no sulfide-like odor.

Inhibition of dechlorination at low sulfate concentrations. In the experiments shown in both Fig. 1 and Fig. 2, dechlorination was completely inhibited by the 20 mM sulfate amendments. Therefore, an experiment with a lower concentration of sulfate was conducted. In this experiment, the initial concentration of sulfate was 5 mM, and we attempted to maintain this concentration during the entire incubation period. In another amendment group, 20 mM molybdate was added (at 0 time only) with 5 mM sulfate to ensure the presence of sulfate in the absence of sulfate reduction. As a control 20 mM molybdate was also added alone at 0 time. Because of the instrumental problems, both 3 and 4 week sulfate samples were not assayed until 5 weeks, and consequently, the concentration of sulfate was maintained between 3 ~ 5 mM.

As shown by Fig. 3A, dechlorination was significantly inhibited by all three amendments. After 8 weeks of incubation, the non-amended culture removed 0.96 chlorines from each biphenyl molecule; in contrast, only 0.31, 0.25, and 0.24 chlorines were removed from each biphenyl molecule by the cultures amended with molybdate, sulfate, and molybdate plus sulfate, respectively.

Inhibition by 5 mM sulfate was not reversed in the presence of molybdate since molybdate at the experimental concentration also inhibited the dechlorination. However, molybdate did inhibit sulfate consumption (Fig. 3B). After 2 weeks of incubation, the

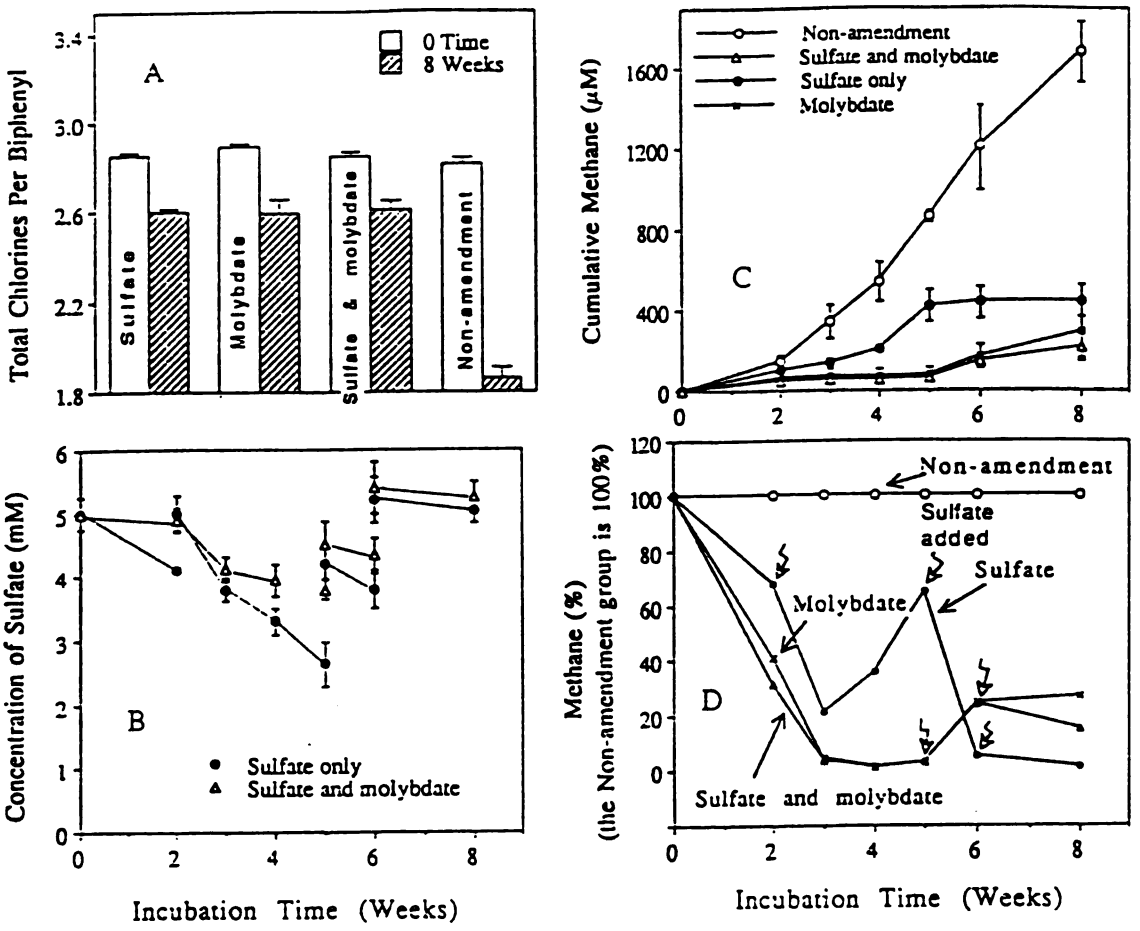

Fig. 3. Effects of molybdate (20 mM, added at 0 time only) and sulfate on dechlorination when the concentration of sulfate was maintained at 3~5 mM: (A) dechlorination of Aroclor 1242 by microorganisms eluted from Hudson River sediments with different amendments after 8 weeks of incubation; (B) concentrations of sulfate in the cultures amended with sulfate plus molybdate, or sulfate only; (C) methane produced by different amended groups; (D) methane produced by different amended groups as percentages of the non-amendment group. The hollow arrows indicate supplement of sulfate. Error bars are the standard deviation (SD) of triplicate samples; where not shown, the error bars are smaller than the symbols, except for Fig. (D).

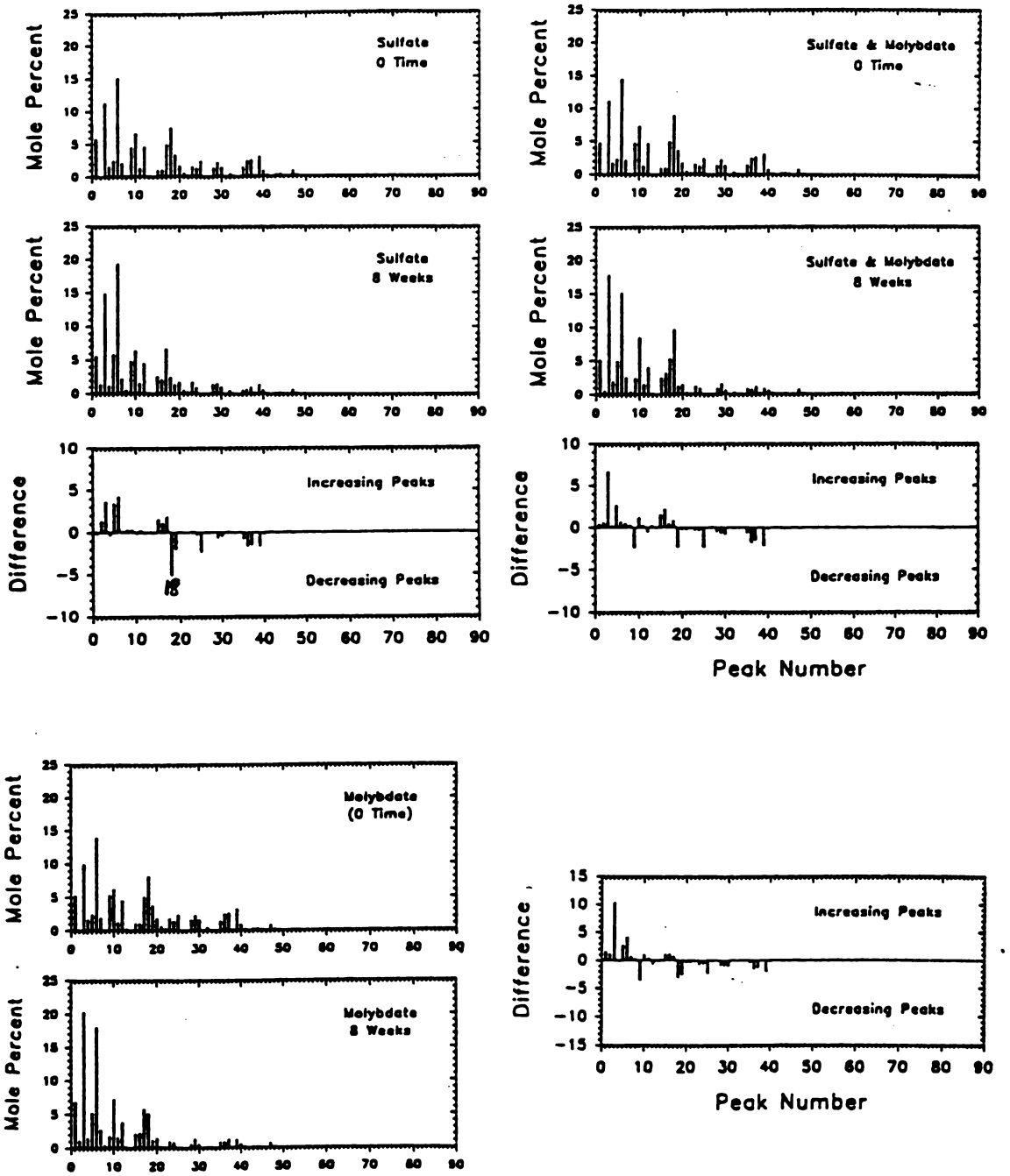

Fig. 4. Mole percentage of Aroclor 1242 peaks before and after 8 weeks of incubation with the following amendments: sulfate only (maintained at 3~5 mM); 20 mM molybdate (added at 0 time); and sulfate (maintained at 3~5 mM) plus molybdate (20 mM, added at 0 time). For peak assignments, see reference (15).

concentration of sulfate decreased 0.9 mM in the cultures amended only with sulfate, while in presence of 20 mM molybdate the concentration of sulfate only decreased 0.06 mM. HPIC analysis showed that after 2 weeks of incubation the concentration of molybdate decreased 1.0 and 1.8 mM in the with- and without- sulfate cultures, respectively.

Both sulfate and molybdate inhibited methane production. The inhibitory effect of 20 mM molybdate on methanogensis was stronger than that of 5 mM sulfate (Fig. 3C, 3D). As shown in Fig. 3D, sulfate resulted in suppression of methane production, except that supplement of sulfate to the cultures amended with sulfate plus molybdate at 5 weeks (3B) failed to inhibit methane production (3D). Methane production in this amendment group increased between 5 and 6 weeks until sulfate was added at 6 weeks (3D). Hydrogen in the headspace was also monitored with gas chromatography; no hydrogen cumulation was found in any culture (data not shown). Fig. 4 shows the dechlorination patterns by the three amended cultures. The sulfate-amended culture is noteworthy for its ability to attack peak 18, which represents 2,4-4-chlorobiphenyl (2,4-4-CB) and 2,4,6-2-CB (mainly 2,4-4-CB).

Effects of BESA at different concentrations. Cultures were amended with 50 μM, 5 mM, and 50 mM (25 mM at 0 time and 2 weeks) BESA added at 0 time only. A 2 week lag time was observed in the 5 and 50 mM amendments (Fig. 5A). The 50 μM amendment had no effect. The 50 mM BESA treatment continued to inhibit dechlorination during the 12 week incubation, and the 5 mM BESA delayed and partially inhibited the dechlorination (Fig. 5A). Methane formation was partially inhibited by 50 and 5 mM BESA during the first 3 weeks of incubation, and was completely shut off

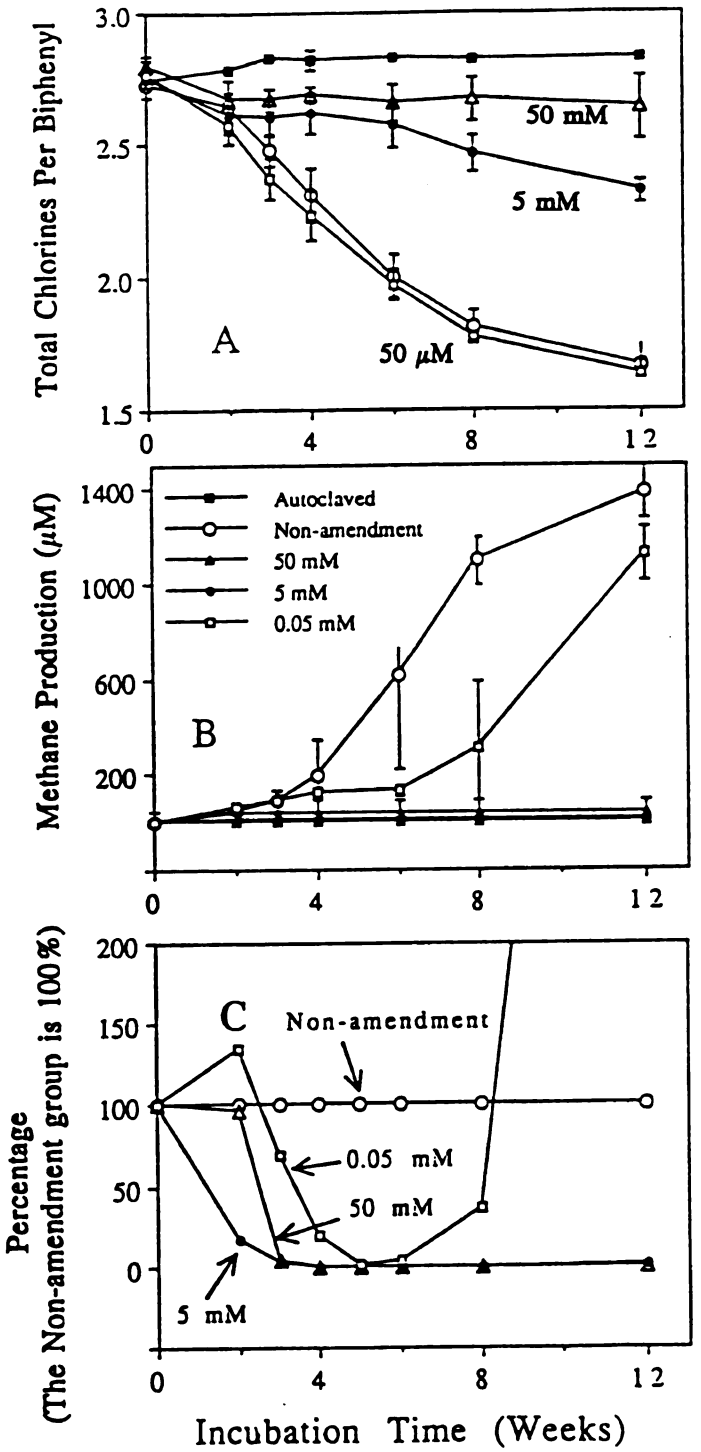

Fig. 5. Effects of BESA at 50 μM, 5, and 50 mM on dechlorination: (A) dechlorination of Aroclor 1242 by microorganisms eluted from Hudson River sediments as effected by BESA at different concentrations; (B) methane produced by different amendments; (C) methane produced by different amendments as percentages of the non-amendment group. Error bars are the standard deviation (SD) of triplicate samples; where not shown, the error bars are smaller than the symbols, except for Fig. (C).

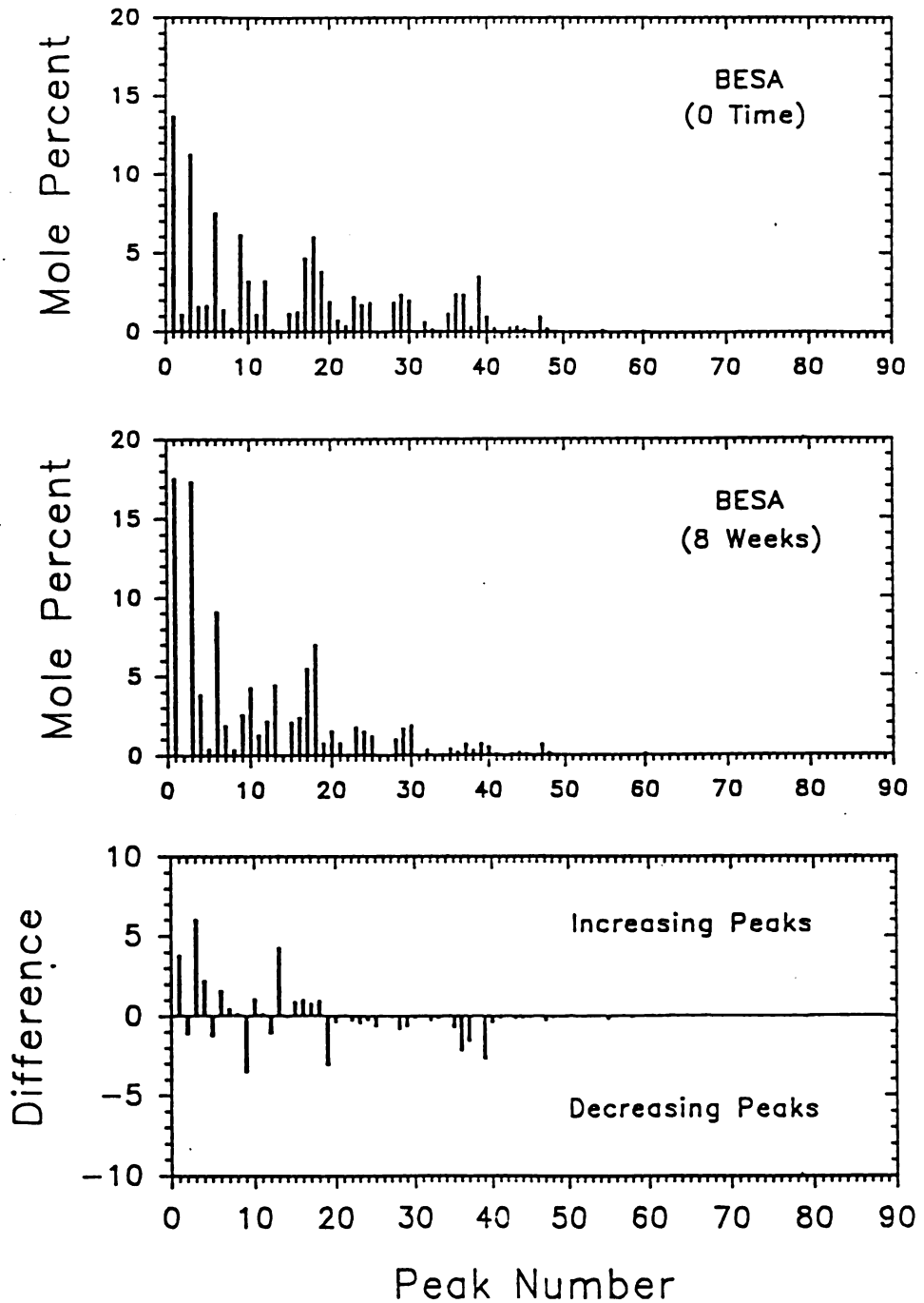

Fig. 6. Mole percentage of Aroclor 1242 peaks before and after 8 weeks of incubation with 5 mM BESA. For peak assignments, see reference (15).

after 3 weeks. Methane formation was also partially inhibited by 50 μM BESA between 2 and 8 weeks (Fig. 5B, 5C). The dechlorination pattern of the cultures amended with 5 mM BESA (Fig. 6) showed that this activity includes some dechlorination activity eliminated by pasteurization (23). The dechlorination pattern after 8 weeks, instead of 12 weeks, was plotted to facilitate comparison of this activity with those of sulfate and molybdate amended cultures (Fig. 4).

DISCUSSION

BESA, and sulfate completely inhibited PCB dechlorination in two separate experiments where Aroclor 1242 and the inhibitors were added together with the inoculum and where they were added to a culture which had already dechlorinated added PCBs. In the former case, molybdate delayed and reduced the extent of initial dechlorination, and in the latter case completely inhibited dechlorination. The different effects of molybdate on these two dechlorination experiments may be due to concentration effects. When molybdate was added along with Aroclor 1242 (400 ppm) to cultures that had already dechlorinated 200 ppm Aroclor 1242 (Fig. 1), a total amount of 25 mM of molybdate had been added. The cultures were continuously incubated with further supplements of molybdate (5 mM) at 1 and 2 weeks. Therefore, after 8 weeks, 35 mM of molybdate had been added. However, in the other dechlorination experiment (Fig. 2), at 8 weeks only 20 mM of molybdate had been added (5 mM added at 0 time and 2, 4, 6 weeks).

The results suggest that either sulfate-reducers or methanogens, or both, were involved in dechlorination. In these experiments, methanogensis and dechlorination were

simultaneously inhibited. Inhibition of methanogensis by BESA and sulfate was expected since BESA is a specific inhibitor of methanogens, and sulfate should stimulate sulfate-reducers and hence inhibit methanogens. Molybdate is generally regarded as a specific inhibitor of sulfidogens (21, 14) and thus is widely used in microbiology studies due to its specificity (14). However, inhibition of methanogensis by higher concentration of molybdate has been reported (9, 19) and also was observed in our experiments. All three anions also affected sulfate-reducers. As mentioned above, molybdate inhibited sulfate-reducers while sulfate stimulated sulfate reduction. The sulfonic acid moiety of BESA is a potential electron acceptor for sulfate-reducers (14). In our experiments the dark-black color, indicating the formation of metal sulfide, and the strong sulfide-smell of the BESA-amended cultures support this suggestion. Since molybdate, BESA, and sulfate affected both sulfate reducers and methanogens, and concomitantly inhibited dechlorination, the results suggest that either sulfate-reducers or methanogens, or both were involved in the dechlorination.

Inhibition of sulfate reducers by molybdate was evidenced by the following observations: (i) in presence of molybdate inhibition of sulfate consumption was observed (Fig. 3B). (ii) Compared to the non-amended cultures, the black color of sulfate-amended cultures indicated formation of metal sulfide, while the yellowish color of the molybdate-amended cultures probably suggested that reduction of sulfate was more or less inhibited. (iii) As shown in Fig. 3D, supplement of sulfate at 5 weeks suppressed methane production in the cultures amended only with sulfate, but not the cultures amended with sulfate plus molybdate, indicating that sulfate-reducers were less active in presence of molybdate.

Previously we have demonstrated that methanogens or methanogensis are not required for PCB dechlorination (23). Results reported here present another example that some non-methanogenic microorganisms which cannot survive the pasteurization are also capable of PCB dechlorination. When methanogensis was completely inhibited by 5 mM BESA after 3 weeks of incubation, partial dechlorination occurred in these cultures (Fig. 5A). The dechlorination activity (Fig 6) was different from that of pasteurized cultures, indicating that the responsible microorganism(s) is(are) non-methanogenic and cannot survive pasteurization. May et al. (11) also reported that dechlorination by a non-methanogenic culture produced a dechlorination pattern different from that produced by the pasteurized cultures. Comparison between the dechlorination patterns reported by May et al. and that shown here for the 5 mM BESA-amended cultures suggests that these two dechlorination activities are different. For example, the culture reported by May et al. only removed *para* chlorines from congeners containing adjacent *para* and *meta* chlorines (11). In contrast, the 5 mM BESA-amended cultures were able to dechlorinate 25-2-CB and 4-4-CB (peak 9). These results demonstrated that among the microorganisms eliminated by pasteurization, there are at least two different non-methanogenic populations capable of PCB dechlorination.

Our results demonstrate that PCB dechlorination can occur in the presence of $3 \sim 5$ mM sulfate. Inhibition of dehalogenation of many organic compounds (12) including PCBs (10, 13, 16) by sulfate have been reported in many investigations. Morris et al. (13) found that addition of 10 mM sulfate reduced the dechlorination activity by 50 % compared with CO_2 treatment. Rhee et al. (16) reported that dechlorination of PCB was inhibited partly by 10 mM sulfate, and May et al. (10) observed that a culture enriched

with 20 mM sulfate expressed no dechlorination activity until the sulfate was depleted (9 weeks). In contrast, Häggblom and Young (6) reported chlorophenol degradation coupled to sulfate reduction, with reductive dechlorination seeming to be the initial step. May et al. (11) also reported transfers from a sulfate-amended enrichment dechlorinated 2,3,4-CB after the addition of sulfate, and the activity was maintained for 15 weeks in presence of >20 mM sulfate. Our observation that partial dechlorination occurred in the presence of sulfate is in agreement with previous reports (11) that sulfate inhibits PCB dechlorination by microorganisms from Hudson River sediments, and also in agreement with the conclusion by May et al. (10) that despite the inhibitory effects of sulfate on dechlorination, different populations with ability to dechlorinate PCBs in presence of sulfate can be obtained.

The dechlorination activity of sulfate-amended cultures observed here is different from that observed by May et al.. This, together with different dechlorination activities expressed by the BESA- and molybdate-amended cultures, as well as those observed with our pasteurized cultures (23), methanogenic enrichment (Chapter 2), and other reported dechlorination patterns (4), indicate that diverse microorganisms in Hudson River sediments have the capacity to remove chlorines from certain positions of specific PCB congeners. The presence or absence of these active microorganisms may determine the actual PCB-dechlorination pattern observed *in-situ*.

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Chapter 2

Evidence for Dechlorination of Polychlorobiphenyls (PCB's) by Methanogenic Bacteria

ABSTRACT

When microorganisms eluted from the upper Hudson River (HR) sediment were cultured without any substrate except PCB-free HR sediment, methane formation was the terminal step of the anaerobic food chain. In this food chain, fermentative bacteria converted organic matter into substrates for methanogenic bacteria. In sediments containing Aroclor 1242, addition of eubacteria-inhibiting antibiotics, which should directly inhibit fermentative bacteria and thereby indirectly inhibit methanogens, resulted in no dechlorination activity nor methane production. However, when substrates for methanogenic bacteria were provided along with the antibiotics (to free methanogens from dependence on eubacteria), concomitant methane production and dechlorination of PCB's were observed. The dechlorination of Aroclor 1242 was distinctly different from, and more limited than, that observed with untreated or pasteurized inocula. Both methane production and dechlorination in the culture amended with antibiotics plus methanogenic substrates were inhibited by BESA, the specific inhibitor for methanogenic bacteria. These results suggest that methanogenic bacteria are probably among the physiological groups capable of anaerobic dechlorination of PCB's. *Ortho* dechlorination of 2-CB and 2-2-CB/2,6-CB by the transferred methanogenic cultures was observed, indicating that the Hudson River contains microorganisms with the potential for complete dechlorination of PCBs to biphenyl.

INTRODUCTION

Methanogenic bacteria play an important role in anaerobic destruction of organic compounds (4, 13). There are many observations of reductive dechlorination of chlorinated compounds either under methanogenic conditions (15, 16, 25), or by pure methanogenic strains (14, 18, 20).

In PCB dechlorination experiments when dechlorination of PCB occurred, methane production was always concomitantly observed (21, 22, 27). However, these studies could not distinguish whether methanogenic bacteria were among the microorganisms capable of PCB dechlorination or they functioned as the terminal step to maintain a continuing energy flow through the food chain, so that the dechlorination could proceed. Heat- and ethanol-treatments of mixed PCB dechlorinating populations eliminated non-sporeforming bacteria including methanogens, but a *meta*-preferential dechlorination activity was maintained (27), suggesting that involvement of methanogenic bacteria was not necessary for dechlorination. However, heat- and ethanol-treatments resulted in loss of *para* dechlorination activity perhaps due to elimination of some microorganisms. Methanogenic bacteria were among the microorganisms eliminated by the heat- and ethanol- treatments, however, the relationship between methanogens and the activities lost from the heat- and ethanol-treatments was unknown. It is important to identify the role methanogenic bacteria play in anaerobic reductive dechlorination of PCBs, so that an in-depth physiological and ecological understanding of the microbial community involved in anaerobic PCB dechlorination can be obtained. This understanding will improve PCB bioremediation strategies, and facilitate isolation of microorganisms capable of PCB dechlorination.

MATERIALS AND METHODS

Approaches. Methanogenic bacteria depend on fermentative bacteria for substrates. Addition of eubacteria-inhibiting antibiotics (penicillin-G plus D-cycloserine) will directly inhibit eubacteria and thus, indirectly inhibit methanogens. However, when substrates for methanogenic bacteria are provided along with the antibiotics to free methanogens from dependence on eubacteria, methanogens should be enriched. There are three kinds of archaebacteria: extreme halophiles, extreme thermophiles, and methanogens. Only methanogens can be enriched if the cultural conditions are not favorable for the other two groups. Therefore, if such a culture has any PCB dechlorination capacity, the responsible microorganisms are most likely methanogenic bacteria. Insight into the role methanogens play in the dechlorination can also be examined determining the effects of 2-bromoethanesulfonic acid (BESA), the specific inhibitor for methanogens (2), on PCB dechlorination by the methanogenic culture. The observation that BESA inhibits both dechlorination activity and methane production in the methanogenic culture, but does not inhibit dechlorination activity in the untreated culture would support the dechlorination capacity of methanogens.

Inoculum preparation. Inoculum was prepared with sediments collected from the upper Hudson River near Hudson River Falls, N.Y., and the inoculum preparation procedure was as previously described (23).

Preparation of experimental vessels. The medium for this experiment was Barker's medium (19) which is generally used for enrichment of methanogenic bacteria. The medium was modified with addition of $NaHCO_3$ (2.4 g/liter) and $Na_2S.9H_2O$ (0.28 g/liter)

For the initial methanogenic enrichments and subsequent transfers, 160-ml serum bottles were used. A preincubation procedure (23) was followed to ensure that the experimental vessels strictly anaerobic. The preincubated 160-ml serum bottles contained 25 g PCB-free air-dried Hudson River sediments, 15 ml of Barker's medium (19) containing 5% of inoculum and 0.1% of ethanol. After formation of methane was detected, all vessels were then autoclaved at 121°C for 1 h on 3 consecutive days with incubation at 37°C between each autoclaving.

After the third autoclaving, the following were added to each bottle while the bottle was being flushed with filter-sterilized O_2-free N_2/CO_2 (80:20, vol/vol) with a Hungate apparatus: 25 ml of Barker's medium, 25 ml of inoculum, 25 ml of sediment extract, 100 μl of 10% (wt/wt) Aroclor 1242 in acetone. Sediment extract was prepared by autoclaving PCB-free Hudson River sediment with deionized water (1:1, wt:vol) for 1 h, the mixture was then shaken for another hour, centrifuged and filtered. The final concentration of PCB's was 400 μg/g of dry sediment. Besides the reductant (sodium sulfide) in the Barker's medium titanium citrate (28) was also added at a final concentration of 0.2 mM. Penicillin-G (Sigma Co., St. Louis, Mo.) and D-cycloserine (Sigma Co., St. Louis, Mo.) were made as stock solutions, bubbled with O_2-free N_2/CO_2 (80:20, vol/vol) to drive out the dissolved oxygen, and then filter-sterilized (0.22 μm, Millipore, St. Louis, Mo.). The final concentration of Penicillin-G and D-cycloserine was 3340 U/ml, and 0.1 g/liter of liquid, respectively. The methanogenic substrates provided were methanol, formate, and acetate at final concentrations of 7 g each per liter of liquid. These substrates were autoclaved. The bottles were then sealed with Teflon-coated butyl rubber stoppers and aluminum crimps, and shaken thoroughly to completely

disperse the PCB's. The controls were autoclaved twice at 121°C for 1 h, with an interval of 5 h before PCB's were added.

For the BESA experiment, the same preparation procedure described above was followed with the following modifications: the experimental vessels were 60 ml serum bottles containing 10 g PCB-free dry sediments. The liquid portion was proportionally reduced based on the weight of sediment. Antibiotics were added at 0 time at the concentration described above and at 8 weeks at half that concentration. The PCB used was 3,4-2-CB, the methanogenic substrate provided was methanol only (7 g/liter of liquid). BESA (Aldrich Inc., Milwaukee, WI) was filter-sterilized and added at a final concentration of 2 mM; the same concentration of BESA was supplemented at 3 and 6 weeks.

Transfer. Cultures to be transferred were shaken for 30 min and the slurry was then allowed to settle for about 30 min. Supernatant (25 ml) was transferred into each 160-ml serum bottle containing 25 ml of Barker's medium, 25 ml of sediment extract, and 25 g of preincubated PCB-free Hudson River sediments. PCB, titanium citrate, and the antibiotics were then added as described above.

Analysis of methane production. Methane production was determined by gas chromatography using a flame ionization detector. Headspace gas was analyzed for determination of methane production after shaking the culture and before sampling the slurry for PCB analysis.

Other procedures. The incubation conditions, sampling, PCB analysis and data summation were as previously described (23).

RESULTS

Dechlorination of Aroclor 1242 by the initial methanogenic culture. No dechlorination nor methane production was observed in either the autoclaved control or the cultures amended with antibiotics without any substrate (the negative control, henceforth). However, dechlorination was observed in the cultures amended with both antibiotics and methanogenic substrates (the methanogenic culture, henceforth). After 8 weeks of incubation (Fig. 1), the major congeners that decreased were peak 19 (3,4-2-CB/2,3,4-CB/2,3-3-CB/2,5-2,6-CB), 35 (mainly 2,4,5-4-CB), 36 (2,5-3,4-CB/3,4,5-2-CB), 37 (2,4-3,4-CB/2,3,6-2,5-CB/2,4,5-2,6-CB), 39 (mainly 2,3-3,4-CB/2,3,4-4-CB) and 40 (2,4,5-2,5-CB/2,3,5-2,4-CB), with concomitant increases mainly in peak 5 (2-3-CB), 9 (2,5-2-CB/4-4-CB), 15 (2,5-3-CB) and 16 (2,4-3-CB). In terms of average chlorines removed from each biphenyl molecule, the dechlorination activity of the methanogenic culture was low. After 8 weeks of incubation, only 0.22 chlorines per biphenyl (Aroclor 1242) were removed however, for some specific congeners, the dechlorination activity was not low. As shown in Fig. 1, after 8 weeks of incubation, little change in peak 28 (23-25-CB) was observed and therefore this peak was used as an internal reference. Molar ratios of several selected peaks to peak 28 were plotted (Fig. 2) to show changes in the representative congeners after 8 weeks of incubation. The ratios for the methanogenic culture after 8 weeks incubation were compared to those at 0 time which was defined as 100% (Fig. 3). After 8 weeks of incubation, peak 19, 35, 36, 37, and 39 decreased 53, 70, 78, 65, and 64%, respectively, with peaks 5, 15, and 16 increased 263, 156, and 234, respectively.

The observation of decreases in peak 36 and peak 37 with concomitant increases

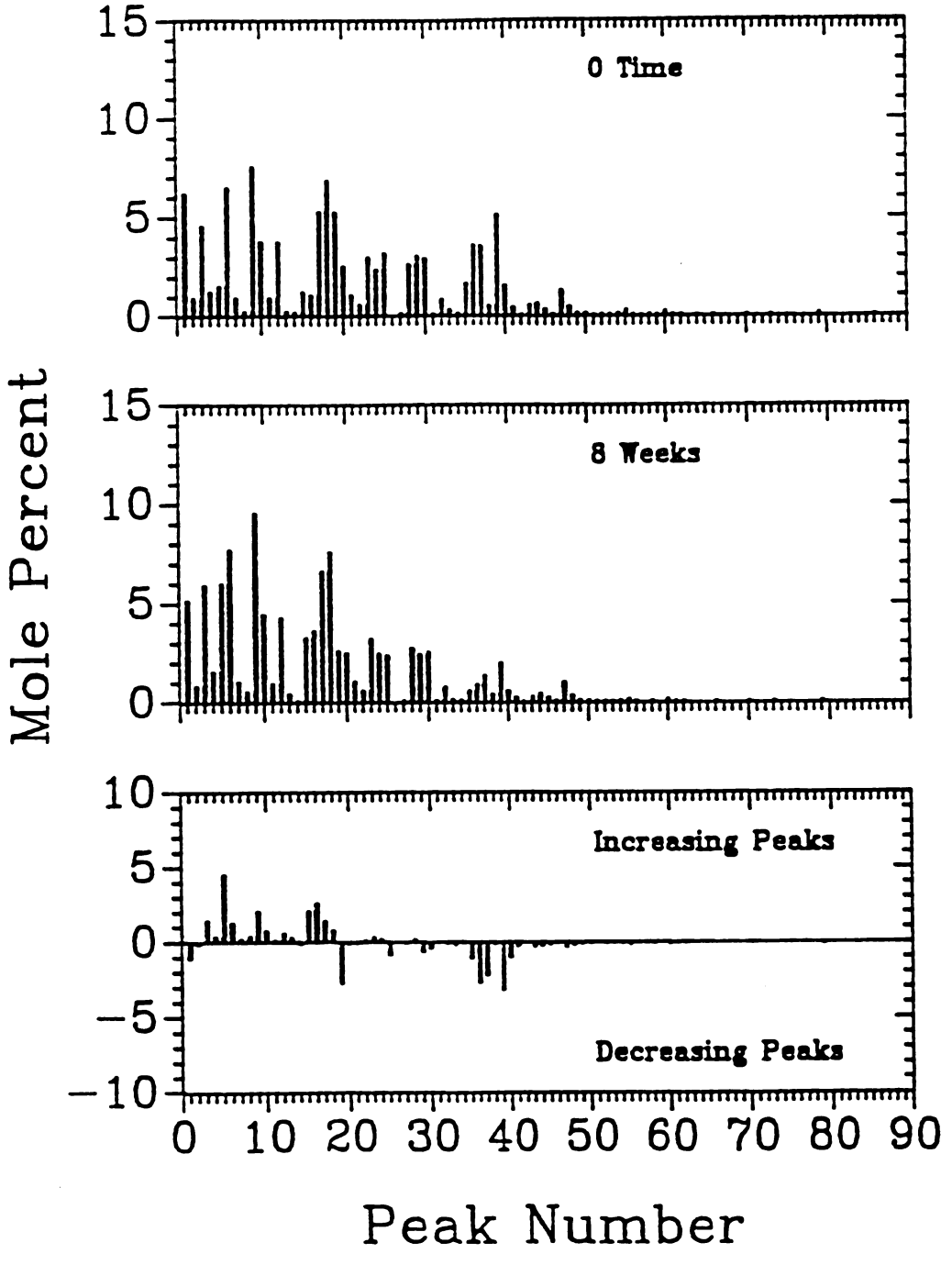

Fig. 1. Mole percentage of Aroclor 1242 represented by each chromatographic peak after 8 weeks incubation with the methanogenic culture. (A) 0 time (B) 8 weeks (C) difference between the 0 time and 8 weeks. For a complete list of the PCB congeners associate with each peak see reference (23).

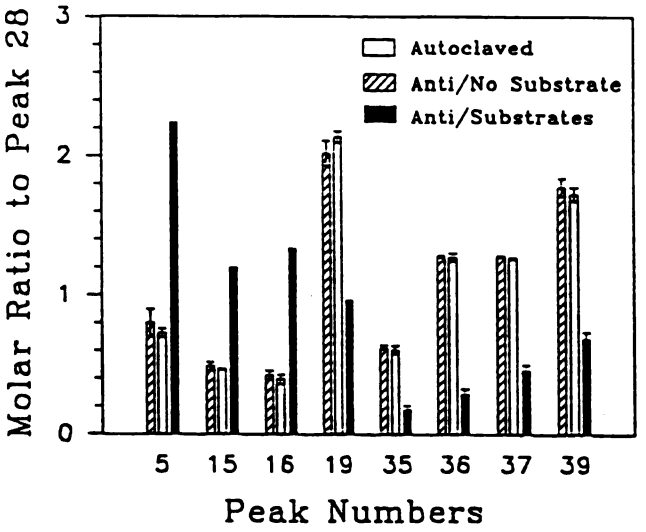

Fig. 2. Molar ratios of several selected peaks to peak 28 after 8 weeks of incubation with different amendments. Error bars are the standard deviation (SD) of duplicate samples; where not shown, the error bars are smaller than the symbols.

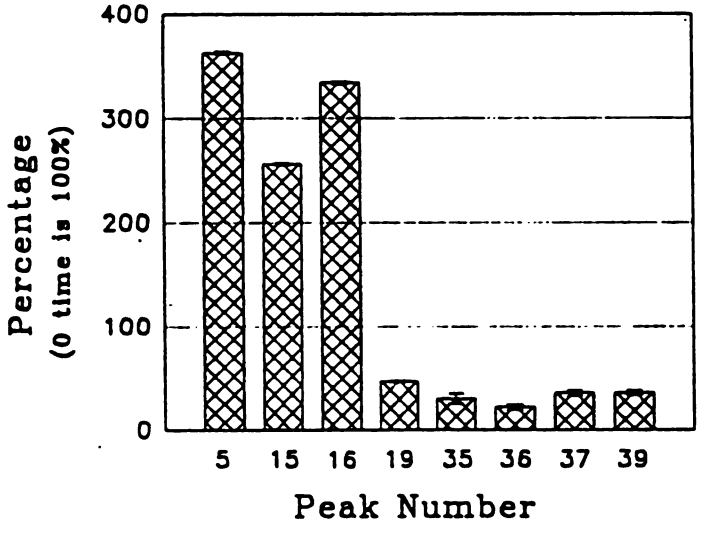

Fig. 3. Molar ratios of selected peaks to peak 28 (after 8 weeks incubation with the methanogenic enrichment) as percentages of those at 0 time (those at 0 time are defined as 100%). Error bars are the standard deviation (SD) of duplicate samples; where not shown, the error bars are smaller than the symbols.

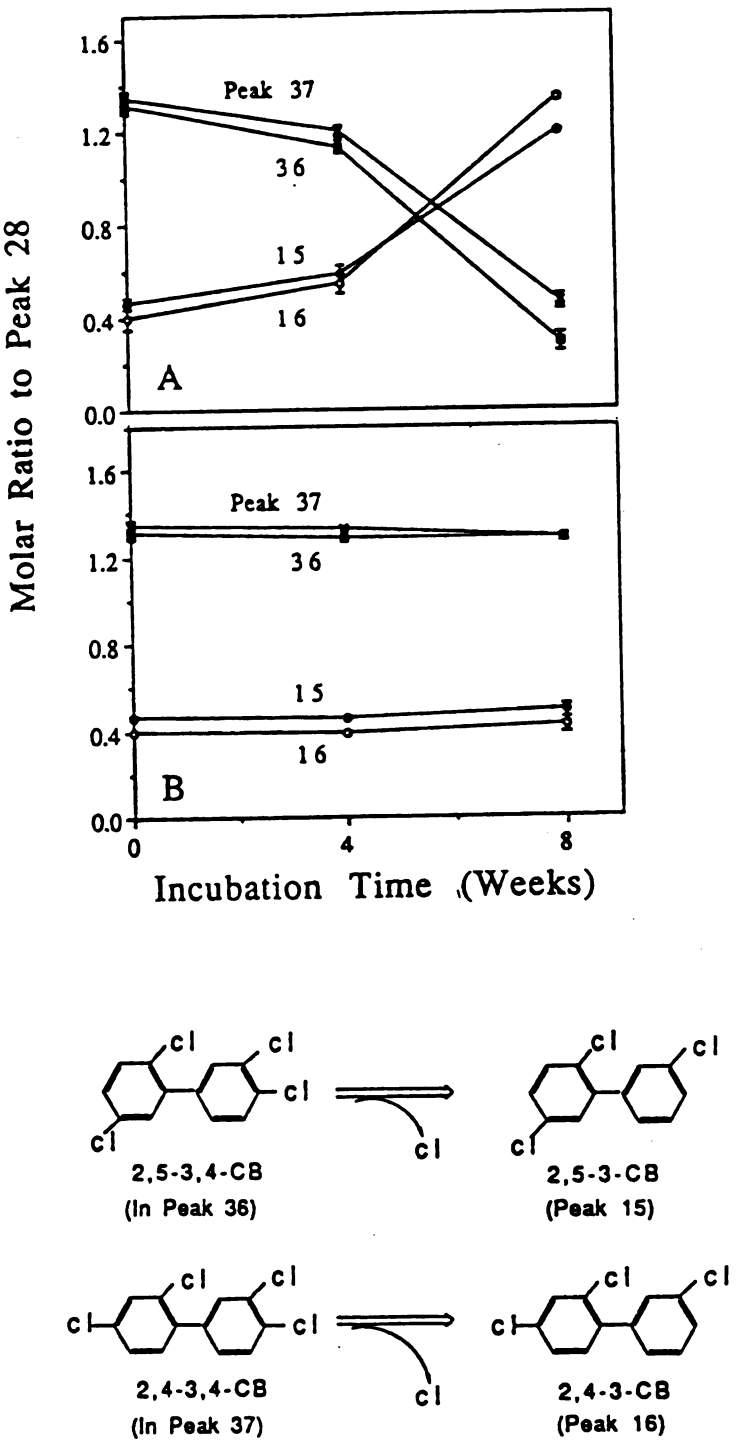

Fig. 4. Changes in Peak 15, 16, 36, and 37 after 8 weeks of incubation with (A) the methanogenic culture, and (B) the negative control group (with antibiotics, without the methanogenic substrates). (C) the proposed dechlorination pathway for congener peak 36, and 37. Error bars are the standard deviation (SD) of duplicate samples; where not shown, the error bars are smaller than the symbols.

in peak 15 and peak 16 (Fig. 4A, 4B) suggested that 2,5-3-CB (peak 15) and 2,4-3-CB (peak 16) were the dechlorination products of 2,5-3,4-CB (in peak 36) and 2,4-3,4-CB (in peak 37) (Fig. 4C). These represent *para* dechlorination, and this dechlorination activity was part of that lost from the heat- and ethanol-treated cultures (27).

Comparison of the dechlorination between the methanogenic culture and the heat- and ethanol-treated cultures. The dechlorination activity of the methanogenic culture was more limited than that of the heat- and ethanol-treated cultures (the treated culture, henceforth). After 8 weeks of incubation, only 0.22 chlorines were removed from each biphenyl molecule by the methanogenic culture, while 0.67 chlorines per biphenyl molecule were removed by the treated cultures.

The dechlorination pattern of Aroclor 1242 shown by the methanogenic culture was distinctly different from that of heat- and ethanol-treated cultures. The former pattern was characterized by accumulation of some *meta*-substituted congeners such as 2-3-CB (peak 5), 2,5-3-CB (peak 15), and 2,4-3-CB (peak 16) (Fig. 1). In contrast, the treated cultures accumulated some *para*-substituted congeners (Fig. 5) such as 2-4-CB/2,3-CB (peak 6), 2,4-2-CB (peak 10), 2,4-4-CB (peak 18), and 2,4-2,4-CB (peak 25) in addition to 2-CB (peak 1) and 2-2-CB/2,6-CB (peak 3) which were the primary products in the untreated culture (27). Additionally, one of the major characteristics of the chromatographic pattern of the treated cultures was that the abundance of peaks 35, 36, and 37 remained constant. However, in the methanogenic culture, these three peaks decreased significantly (GC chromatogram not shown).

Other major differences between the dechlorination activities of the methanogenic culture and the treated cultures were as follows: peak 9 (2,5-2-CB and 4-4-CB), which

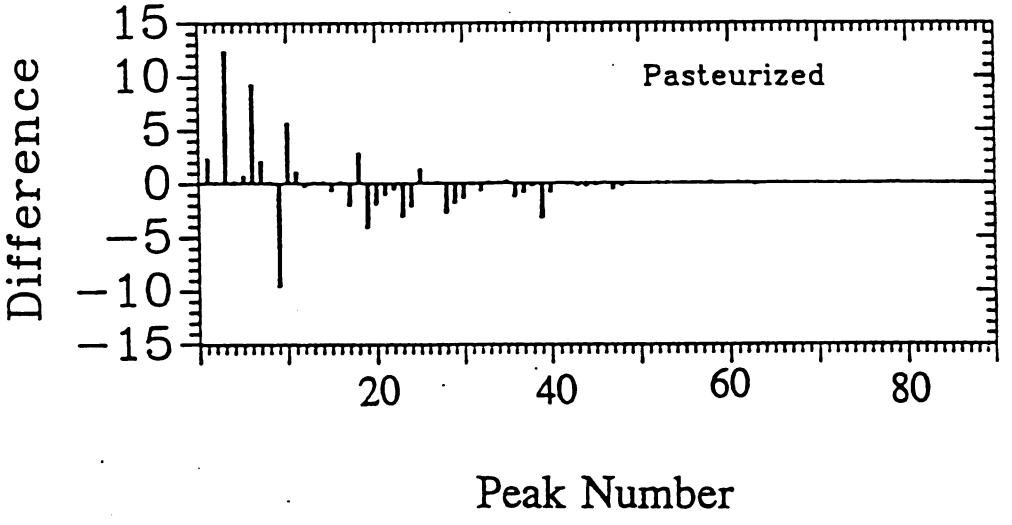

Fig. 5. Changes in mole percentage of Aroclor 1242 (represented by each chromatographic peak) after 8 weeks incubation with the heat- and ethanol-treated microorganisms from the Hudson River sediments.

represents the highest mole percentage of congeners in Aroclor 1242, completely disappeared in treated cultures with a major increase in peak 3 (2-2-CB), whereas in the methanogenic culture (Fig. 1), this peak did not decrease. Rather, it increased due to accumulation of the dechlorination products. Likewise, Peak 25 (2,4-2,4-CB) decreased in the methanogenic culture (Fig. 1), whereas in heat- and ethanol-treatments they increased slightly (Fig. 5).

Results of BESA experiment. 2-Bromoethanesulfonic acid (BESA) was used to examine the role methanogens play in the dechlorination. Considering that BESA is a potential electron acceptor for some anaerobic bacteria, it is possible that it might compete with PCB's for electrons and hence inhibit dechlorination. To distinguish if inhibition on dechlorination by BESA was due to competition for electrons or due to inhibition of methanogens, one group was amended with BESA, without addition of methanogenic substrate and antibiotics, to serve as a control. No methane production was observed in this amendment group while dechlorination did occur (Fig. 6). However, the same concentration of BESA inhibited both methane production and the dechlorination activity in the methanogenic culture (Fig. 6). Results are summarized in Table 1.

Dechlorination activity of the transferred cultures. In the initial cultural batch one group was amended with the same methanogenic substrates but without addition of the antibiotics. Dechlorination was also observed in this group (Fig 7). Both the methanogenic culture and the group amended with the same methanogenic substrates but without the antibiotics were transferred. Dechlorination activity increased in the subsequent transfer of the methanogenic culture, while little dechlorination was observed

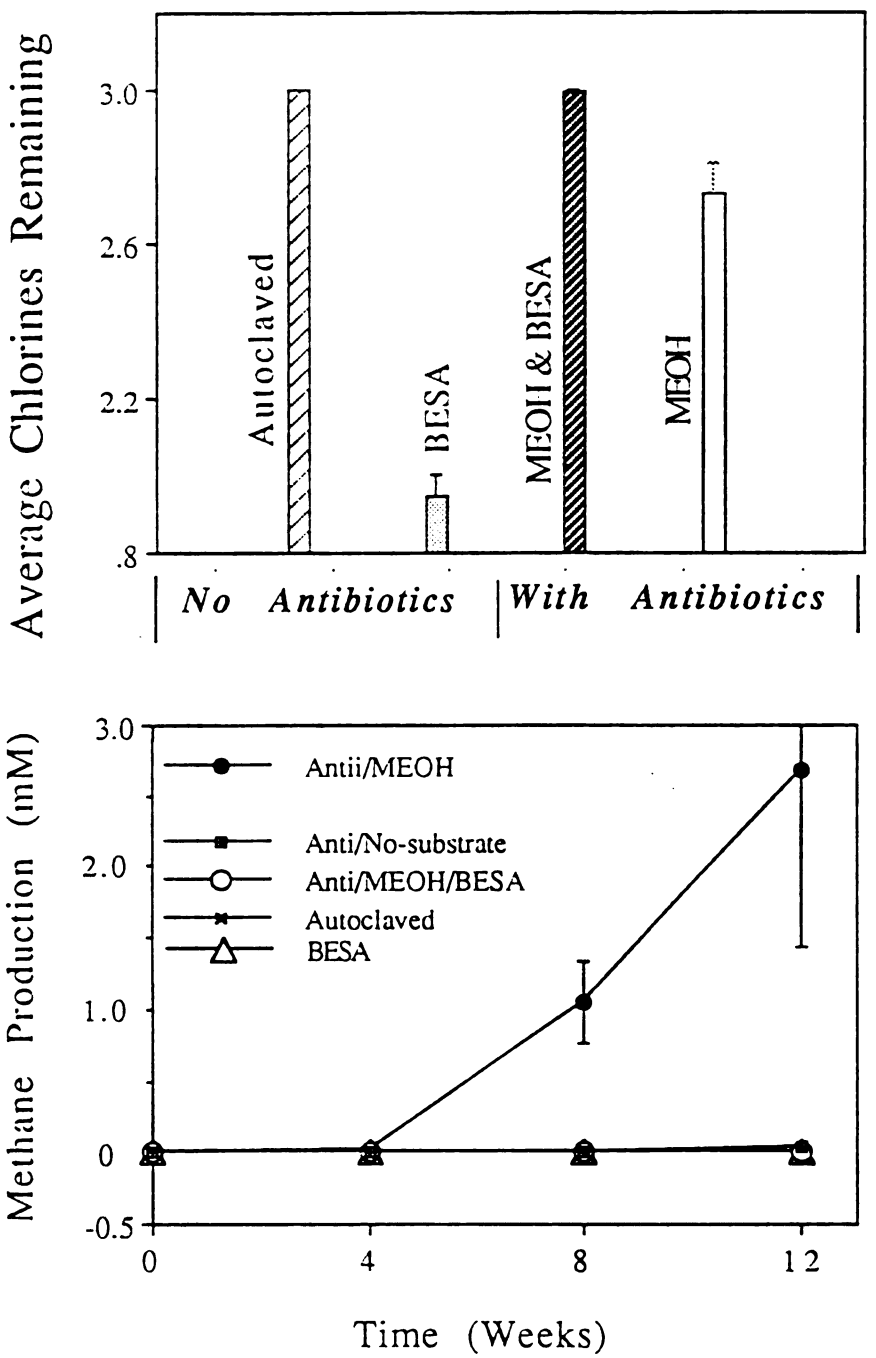

Fig. 6. Effects of BESA on (A) dechlorination; and (B) methane production; after 12 weeks of incubation. Error bars are the standard deviation (SD) of triplicate samples; where not shown, the error bars are smaller than the symbols.

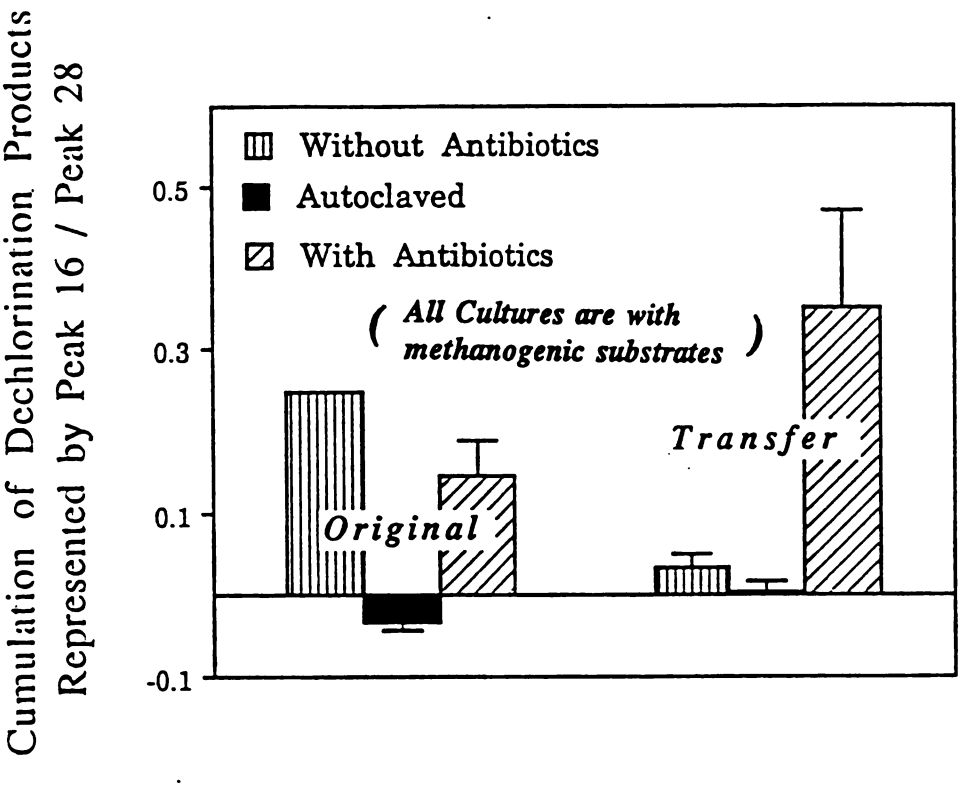

Fig. 7. Dechlorination by the initial cultures and the subsequent transfers of both the methanogenic culture (with both methanogenic substrates and antibiotics) and the group amended with methanogenic substrates but without antibiotics. The dechlorination activities are represented by cumulation of the dechlorination product, peak 16 (normalized to peak 28), after 4 weeks incubation. Error bars are the standard deviation (SD) of duplicate samples; where not shown, the error bars are smaller than the symbols.

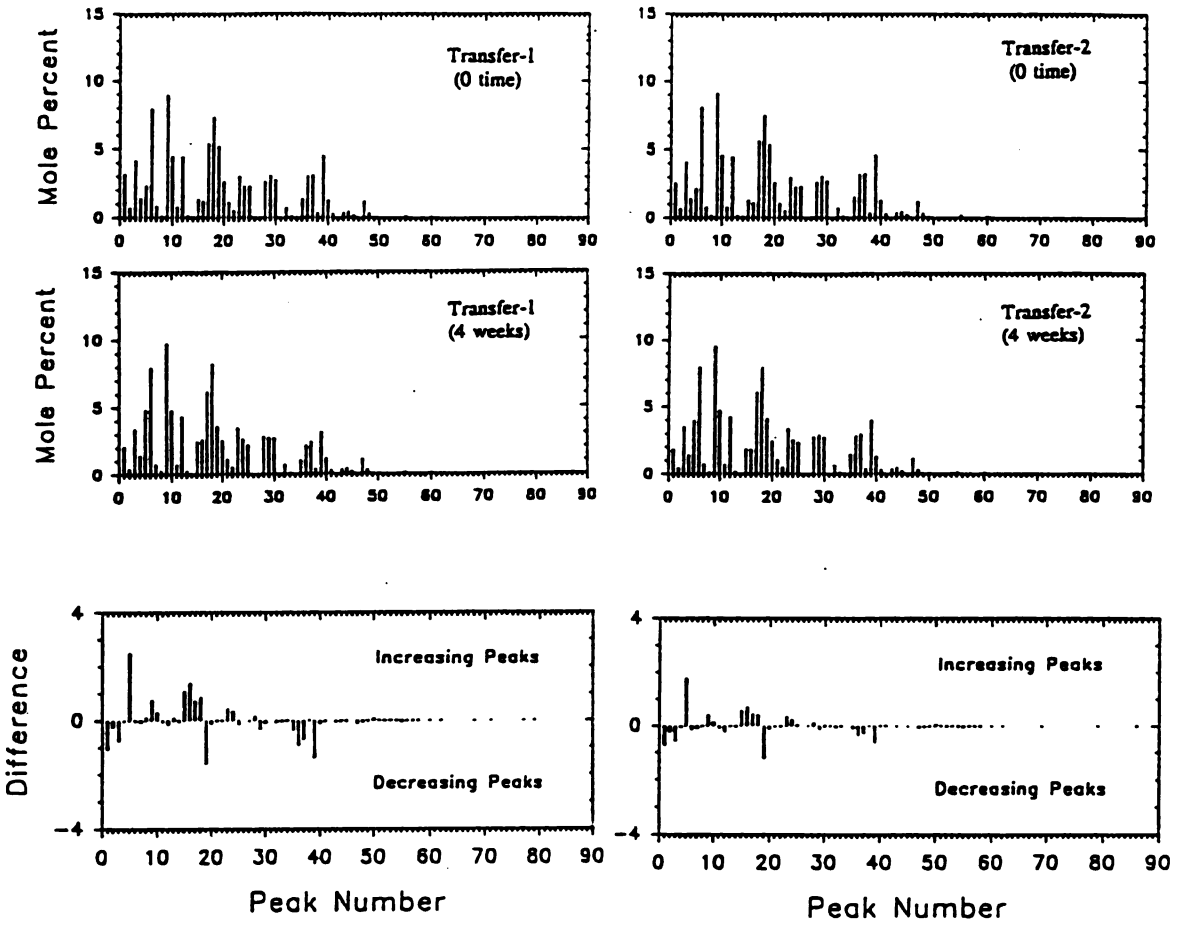

Fig. 8. Mole percentage of Aroclor 1242 represented by each chromatographic peak after 4 weeks of incubation with the transferred methanogenic cultures. For a complete list of the PCB congeners associate with each peak see reference (23).

Table 1. Effects of BESA on PCB Dechlorination and Methane Production

Amendments	Dechlorination	CH_4
Methanogenic Enrichment		
Antibiotics/no-substrate	-	-
Antibiotics/substrate	+	+
Antibiotocs/substrate/BESA	-	-
Control		
BESA only, no antibiotics/substrate	+	-

+ Dechlorination/CH_4 production

- No dechlorination/CH_4 production

in the cultures transferred from the group without addition of the antibiotics (Fig 7).

***Ortho* dechlorination activity exhibited by the transferred cultures.** After 4 weeks of incubation, 2-CB (peak 1) and 2-2-CB/2,6-CB (peak 3) in the transferred methanogenic cultures decreased (Fig. 8), indicating that *ortho* dechlorination occurred.

DISCUSSION

Methane production is typically observed in our PCB-dechlorination assays using PCB-free Hudson River sediments and microorganisms eluted from PCB contaminated sediments (21, 22, 27), suggesting that methane formation is the terminal step of the anaerobic food chain. In this anaerobic food chain, fermentative microorganisms converted the sediment organic matter into substrates for methanogens. Therefore, addition of eubacteria-inhibiting antibiotics should directly inhibit eubacteria, and hence methanogens. This was confirmed by the negative control group in which addition of penicillin-G and D-cycloserine, the eubacteria-inhibiting antibiotics, resulted in no dechlorination nor methane production. When eubacteria are inhibited with antibiotics, only archaebacteria could survive. There are three groups of archaebacteria: extreme thermophiles, extreme halophiles, and methanogens. Our culture conditions were not favorable for either extreme halophiles or extreme thermophiles, therefore, when eubacteria were inhibited and methanogenic substrates were provided, the enriched microorganisms were most probably methanogenic bacteria.

Penicillin-G and D-cycloserine specifically inhibit cell wall synthesis of eubacteria and by different mechanisms (26). Both antibiotics do not inhibit archaebacteria with the exception that *Methanococcus vannielii* is sensitive to D-cycloserine (6). Zinder et al.

(29) used combination of penicillin (3340 U/ml) and D-cycloserine (0.1 g/ml) to inhibit eubacteria in the isolation of a thermophilic strain of *Methanosarcina*; the antibiotics successfully eliminated all eubacteria. In our experiment the same concentration of the antibiotics was used. Since the cultural temperature for Zinder's enrichment was 55°C while in our culture it was only 25°C, the antibiotics should be effective longer in our culture than in that of Zinder's because these antibiotics are more labile as temperature increases. The effects of the antibiotics were evidenced by two observations: first, addition of antibiotics without providing methanogenic substrate (the negative control) resulted in no dechlorination activity nor methane production, indicating that the eubacteria were effectively inhibited. Second, the original cultures of Barker's medium with methanogenic substrates showed dechlorination activity in presence and absence of antibiotics. The dechlorination activity in the cultures with antibiotics increased in its subsequent transfer, while the dechlorination activity in the original cultures without antibiotics decreased with time (data not shown) and was lost from the subsequent transfer. This may be because in the absence of antibiotics microorganisms responsible for the dechlorination activity were outcompeted by some non-dechlorinating eubacteria stimulated by the methanogenic substrates.

Both dechlorination activity and methane production were inhibited when BESA was introduced to the methanogenic culture. BESA also showed inhibitory effects on dechlorination by pasteurized cultures containing no methanogens, and the cultures concomitantly turned black indicating formation of some metal sulfide (data not shown). Additionally, these cultures gave-off sulfide smell when they were sampled while being flushed with N_2-CO_2. This observation suggested that BESA was probably used as an

electron acceptor, and that the inhibitory effects might due to the competition of BESA with PCBs for electrons. Similar inhibitory effects of BESA on PCB dechlorination (21) and on anaerobic dechlorination of other compounds (17) has been reported. For the reason mentioned above, in this experiment a control group was set up to determine whether the inhibitory effects of BESA on dechlorination by the methanogenic culture were due to the inhibition on methanogens or due to the possible competition for elections. As shown in Table 1, in this control group no methane production was observed while PCB dechlorination did occur. This ensured that BESA at this concentration effectively inhibited methanogens without inhibiting dechlorination by other microorganisms. This control proved that inhibition of BESA on the dechlorination by the methanogenic culture was due to the direct inhibition on methanogens instead of competition with PCB for electrons. The high concentration of the added methanol (218 mM) should also rule out the possibility of competition by BESA for electron, because this concentration was high enough to effectively scavenge BESA in case BESA was used as electron acceptor. These results confirmed that it was most probably methanogens that was responsible for the dechlorination activity in the culture.

The dechlorination activity of the methanogenic culture was limited to some specific congeners. However, it is unclear whether the actual dechlorination capacity of methanogens in the natural environment is limited or not. Methanogens depend on eubacteria not only for substrates, but also some nutrients and growth factors (5, 19). In our methanogenic culture eubacteria were inhibited, consequently, even though methanogenic substrates were provided, those methanogens requiring some nutrients or/and growth factors from eubacteria still could not grow. Moreover, in order to

enhance the suppressive effects of antibiotics on eubacteria, no trace metals nor vitamins were added to our methanogenic medium, and this also certainly eliminated some fastidious methanogens. With the harsh selective pressure, only a portion of methanogenic bacteria was expected to be enriched. Additionally, inhibition of eubacteria might also result in eliminating some possible cooperation from eubacteria. For example, lecithinase was produced when two *Pseudomonas* strains grown together, while neither was able to produce the active enzyme alone (3). It is possible that with nutrients, growth factors, and some cooperation from eubacteria, methanogens might have a more broad dechlorination spectrum instead of the narrow one. Nevertheless, the actual dechlorination capacity of methanogenic bacteria in natural environment remains to be elucidated. On the other hand, different microbial populations may share a similar dechlorination pattern. Our results do not rule out the possibility that under other enrichment conditions some other microorganism(s) may also exhibit a similar dechlorination pattern to the one reported here.

The transferred methanogenic cultures exhibited the *ortho* dechlorination activity on specific congeners. Dort and Bedard (12) reported *ortho* dechlorination of 2,3,5,6-tetrachlorobiphenyl (2,3,5,6-CB) by microorganisms from methanogenic pond (Wood Pond, Lenox, Mass.) sediment. However, *ortho* dechlorination by microorganisms from Hudson River sediment has never been observed. 2-CB, 2-2-CB, and 2,6-CB are the major products of PCB dechlorination by Hudson River microorganisms (23). Our results demonstrate that the Hudson River contains microorganisms with the potential for complete dechlorination of PCBs to biphenyl.

May (19a.) et al observed a very similar *para* dechlorination activity with a

enrichment amended with 20 mM sulfate. The enrichment expressed no dechlorination activity until after the sulfate was depleted. The pattern suggested that *para* chlorine removal only with congener containing *para* and *meta* chlorines that were adjacently positioned. The enrichment was than subcultured on solid media containing no sulfate but sterilized river sediment. The PCB-dechlorinating colonies that had been grown on solid medium for a second or third time were not methanogenic in the sediment slurries, and the dechlorination activity concomitantly changed from mainly *para*-removal to *meta*-removal. The concomitant disappear of methanogensis and the congener-specific *para* dechlorination activity provided another supportive evidence to correlate methanogens to this *para* dechlorination activity.

Different environmental PCB dechlorination patterns have been reported (7, 8, 9, 10) and also observed under laboratory experimental conditions (1, 27). The dechlorination pattern by the methanogenic enrichment was close to pattern H (8, 11). The different dechlorination patterns observed have been explained by Abramowitz et al. (1), Brown et al. (9), and Quensen et al. (23) as due to different microbial dechlorinating populations that may exist in the Hudson River sediment. Results of the heat- and ethanol-treatments support the suggestion that anaerobic sporeformers are at least one of the PCB-dechlorinating physiological groups (27). Results of the methanogen experiment reported here provide additional evidence for different dechlorinating populations. Thus, in addition to anaerobic sporeformers, methanogenic bacteria are also probably among the physiological groups capable of anaerobic PCB dechlorination.

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Chapter 3

Anaerobic Dechlorination of Polychlorobiphenyls (Aroclor 1242) by Pasteurized and Ethanol-Treated Microorganisms from Hudson River Sediments

ABSTRACT

A polychlorobiphenyl (PCB)-dechlorinating inoculum eluted from upper Hudson River sediments was treated with either heat or ethanol or both. The treated cultures retained the ability to dechlorinate PCBs (Aroclor 1242) under strictly anaerobic conditions. The dechlorination activity was maintained in serial cultures inoculated with transfers of 1% inoculum when the transferred inoculum was treated each time in the same manner. No methane production was detected in any treated culture, although dechlorination of PCBs in the untreated cultures was always accompanied by methane production. All treated cultures preferentially removed *meta* chlorines yielding a dechlorination pattern characterized by accumulation of certain *ortho*- and *para*-substituted congeners such as 2-4-chlorobiphenyl (2-4-CB), 2,4-2-CB, and 2,4-4-CB. In contrast, the untreated cultures showed more extensive dechlorination activities, which almost completely removed both *meta* and *para* chlorines from Aroclor 1242. These results suggest that microorganisms responsible for the dechlorination of PCBs in the upper Hudson River sediments can be grouped into two populations according to their responses to the heat and ethanol treatments. Microorganisms surviving the heat and ethanol treatments preferentially remove *meta* chlorines, while microorganisms lost from the enrichment mainly contribute to the *para* dechlorination activity. These results indicate that anaerobic sporeformers are at least one of the physiological groups responsible for the reductive dechlorination of PCBs. The selection of a dechlorinating population by such treatments may be an important step in isolation of PCB-dechlorinating microorganisms.

INTRODUCTION

During the past 20 years intensive studies on microbial degradation of polychlorobiphenyls (PCBs) have been reported (for reviews, see references 1 and 15). These include studies with soil communities, isolates from nature, and recombinant organisms. The results indicate that the microbial degradation of PCBs leading to biphenyl ring cleavage is generally limited to lightly chlorinated congeners, leaving highly chlorinated congeners unaltered (1, 15).

Recently, the microbially mediated anaerobic reductive dechlorination of PCBs has been described. This process converts a large array of highly chlorinated PCBs into lesser chlorinated congeners, predominately *ortho*-substituted mono- and dichlorobiphenyls (2, 3, 13, 14). This is especially noteworthy because the dechlorination products are less toxic and more readily degraded by aerobic microorganisms. However, despite its environmental significance, research on the anaerobic reductive dechlorination of PCBs has, until now, failed to identify any physiological group(s) of dechlorinating microorganisms. Therefore, a PCB-dechlorinating inoculum was treated with either heat or ethanol or both to determine whether anaerobic sporeformers are responsible for PCB dechlorination.

MATERIALS AND METHODS

Inoculum preparation. Sediments were collected in September, 1989 from the upper Hudson River near Hudson River Falls, N.Y. (site H7 in reference 3) and shipped under anaerobic conditions to the laboratory as described previously (13). To make the inoculum, sediments were transferred into sterile Erlenmeyer flasks and mixed with an

equal volume of reduced anaerobic mineral medium (RAMM) (16) while flushing with filter-sterilized O_2-free N_2-CO_2 (80:20 vol/vol) using a Hungate apparatus. The flasks were then sealed and incubated at 25°C in the dark for two weeks to allow the facultative anaerobic microorganisms to consume residual oxygen. Anaerobic conditions were indicated by the detection of methane. The slurry was vigorously shaken by hand for 3 min and then allowed to settle for 30 min. Supernatant containing the eluted microorganisms was used as the inoculum.

Pasteurization. Inoculum was anaerobically introduced into sterile N_2-CO_2 (80:20, vol/vol)-flushed anaerobic culture tubes (Bellco Glass Inc., Vineland, N.J.) (20 ml per 28-ml tube). The tubes were then sealed with teflon-coated butyl rubber stoppers (West Co., Phoenixville, Pa.) and incubated in a water bath at 80, 85, or 90°C. The temperature increase was monitored with another tube containing the same amount of inoculum and a thermometer. When the thermometer reached 78, 83, or 88°C (for pasteurization at 80, 85, or 90°C, respectively) (7), the tubes were incubated for 15 min (80 and 85°C) or 10 min (90°C) and then immediately cooled to room temperature.

Treatment with ethanol or combination of ethanol and heat. A 70-ml inoculum was anaerobically transferred into a sterile N_2-CO_2 (80:20, vol/vol)-flushed 160-ml serum bottle and mixed with an equal volume of autoclaved ethanol (200 proof, dehydrated; Quantum Chemical Co., Tuscola, Ill.); the bottle was then sealed. The mixture was incubated at room temperature (20°C) for 35 min and occasionally shaken by hand. The serum bottle was then centrifuged at 3,000 × *g* for 30 min at 20°C. The total time for the inoculum to contact ethanol was about 1 h. After the serum bottle was centrifuged, the liquid portion was removed and the remainder was washed three times

with autoclaved 0.9% NaCl to remove the residual ethanol. Another 140 ml of inoculum without ethanol was centrifuged, and the supernatant was anaerobically filter sterilized (0.22 μm; Millipore Co., Bedford, Mass.), and then used to re-suspend the ethanol-treated inoculum. Oxygen contamination of the re-suspended inoculum did not occur as indicated by resazurin test.

For the combined treatment, the inoculum was first treated with ethanol, then anaerobically transferred to the culture tubes and heated at 80°C as described above.

Preparation of experimental vessels. Assay vessels were prepared as described previously (13) with the following modifications. A preincubation procedure was used to ensure anaerobic conditions in the assay vessels prior to initiation of the actual dechlorination assay. For this, serum bottles (60 ml) received 10 g of the PCB-free dry sediment and 10 ml of inoculum prepared from site H7 sediment. The PCB-free sediment was collected from the Hudson River at River Mile 205 (13). The H7 inoculum was added to moisten the dry sediment and to introduce additional microorganisms to reduce the preincubation time. After methane was detected in the head space, the bottles were autoclaved at 121°C for 1 h on three consecutive days, with incubation at 37°C between each autoclaving.

After the third autoclaving the following were added to each preincubated serum bottle while it was being flushed with filter-sterilized O_2-free N_2-CO_2 (80:20, vol/vol) with a Hungate apparatus: 10 ml of inoculum, 10 ml of RAMM, 100 μl of 10% autoclaved solution of cysteine, and 80 μl of 10% (wt/vol) Aroclor 1242 (Monsanto Co., St. Louis, Mo.) in acetone. The final concentration of cystine was 330 mg/liter of liquid, and the final total PCB concentration was 800 μg/g of sediment (dry weight).

The bottles were then sealed with teflon-coated butyl rubber stoppers and aluminum crimps and shaken thoroughly to completely mix the PCBs. The controls were autoclaved twice at 121°C for 1 h, with an interval of 5 h before PCBs were added.

The experiments were performed three times: in May, July, and September (1990). A flow diagram (Fig. 1) summarizes the experimental procedure. The inoculum for the three experiments was established in April 1990. In the May and July experiments, the treated cultures were only pasteurized at 80°C for 15 min. However, in the September experiment, all treatments were performed; heating at 80°C or 85°C for 15 min, treatment with 50% ethanol for 1 h, and treatment with the combination of heat and ethanol. Cultures from experiments initiated in the September were serially transferred, and the transferred inocula were treated each time.

All treatments were in triplicate except those in the May experiment and the 90°C pasteurized transfer (the third serial culture of the September experiment), which were done in duplicate.

Transfer. Cultures that were to be transferred (heat treated and heat plus ethanol treated), were shaken for 30 min and then the slurry was allowed to settle for about 30 min. The supernatant was used as the inoculum and transferred directly or treated again in the same manner as described above.

For the 1% transfer of the culture treated with the combination of ethanol and heat, 4 ml of the inoculum was treated again in the same manner as described above except that the vessel was a 10-ml serum bottle, the time exposed to ethanol was 1 h, the centrifugation was performed at 8,000 x *g* for 10 min, and the heating temperature was

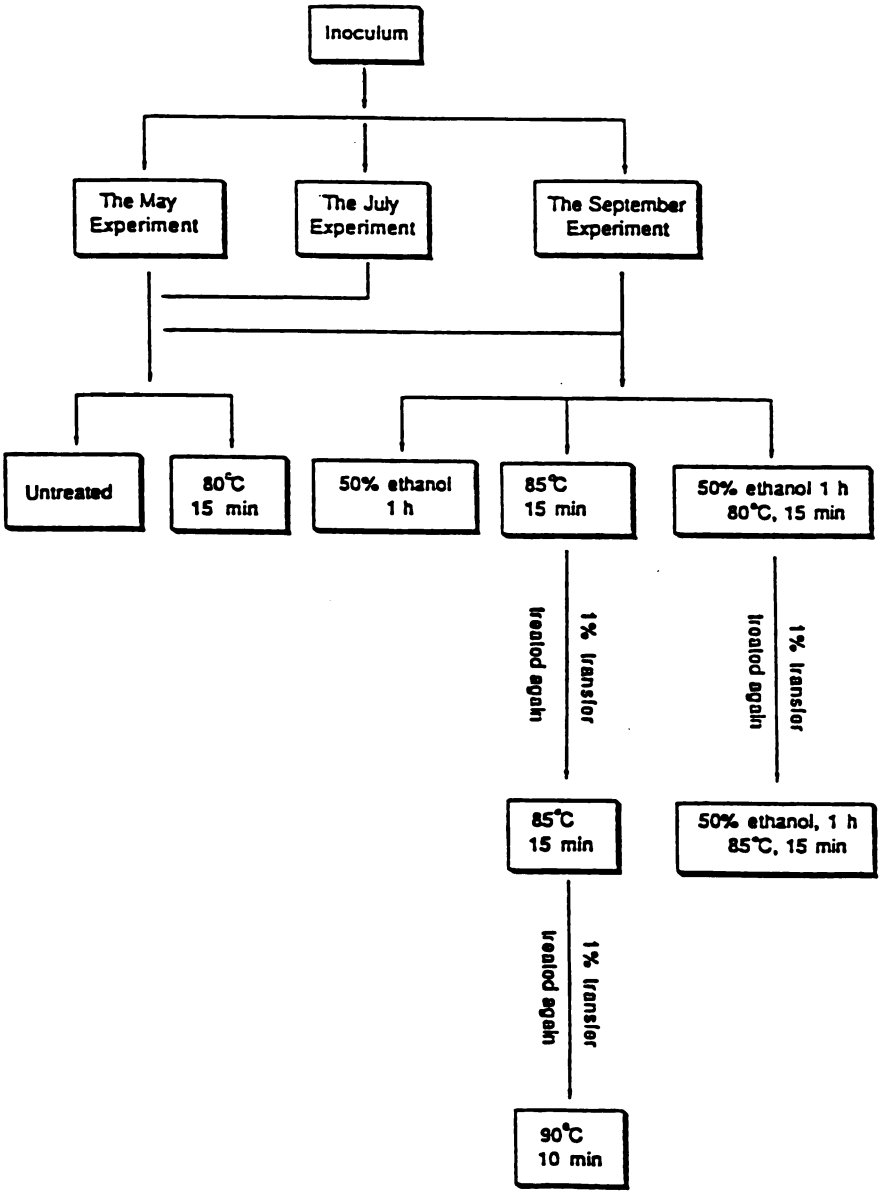

Fig. 1. A brief flow diagram of the experiment procedure.

increased to 85°C.

The experimental vessels for the transferred cultures were prepared as described above except that only 0.3 ml of the treated inoculum was inoculated to each bottle containing 29.7 ml of RAMM to make a 1% transfer.

Other procedures. The incubation conditions, sampling, extraction, and analysis procedures, and data summation have been described previously (13).

RESULTS

Dechlorination of Aroclor by heat- and ethanol-treated cultures. In all three experiments (May, July, and September), the treated cultures showed dechlorination activities (Fig. 2, data for two experiments shown). Higher dechlorination rates for the treated cultures occurred before 4 weeks and then slowed between 4 and 8 weeks. After 8 weeks, no further dechlorination was observed. Despite the fact that the treatments were different and the same inoculum was used over a 5-month period, the treated cultures shared a common dechlorination pattern, as illustrated in Figure 3B. As dechlorination proceeded, the levels of most highly chlorinated congeners decreased, with concomitant major increases in 2-chlorobiphenyl (2-CB) (peak 1), 2-2-CB and 2,6-CB (peak 3), 2-4-CB and 2,3-CB (peak 6), 2,4-2-CB (peak 10), 2,4-4-CB (peak 18) and 2,4-2,4-CB (peak 25) as dechlorination products.

Dechlorination of PCBs in the untreated cultures was always accompanied by methane production. No methane was detected in any treated culture.

The acetone used as a carrier for PCB additions is a potential substrate. However, it does not appear to be directly important to the dechlorinating microorganisms because

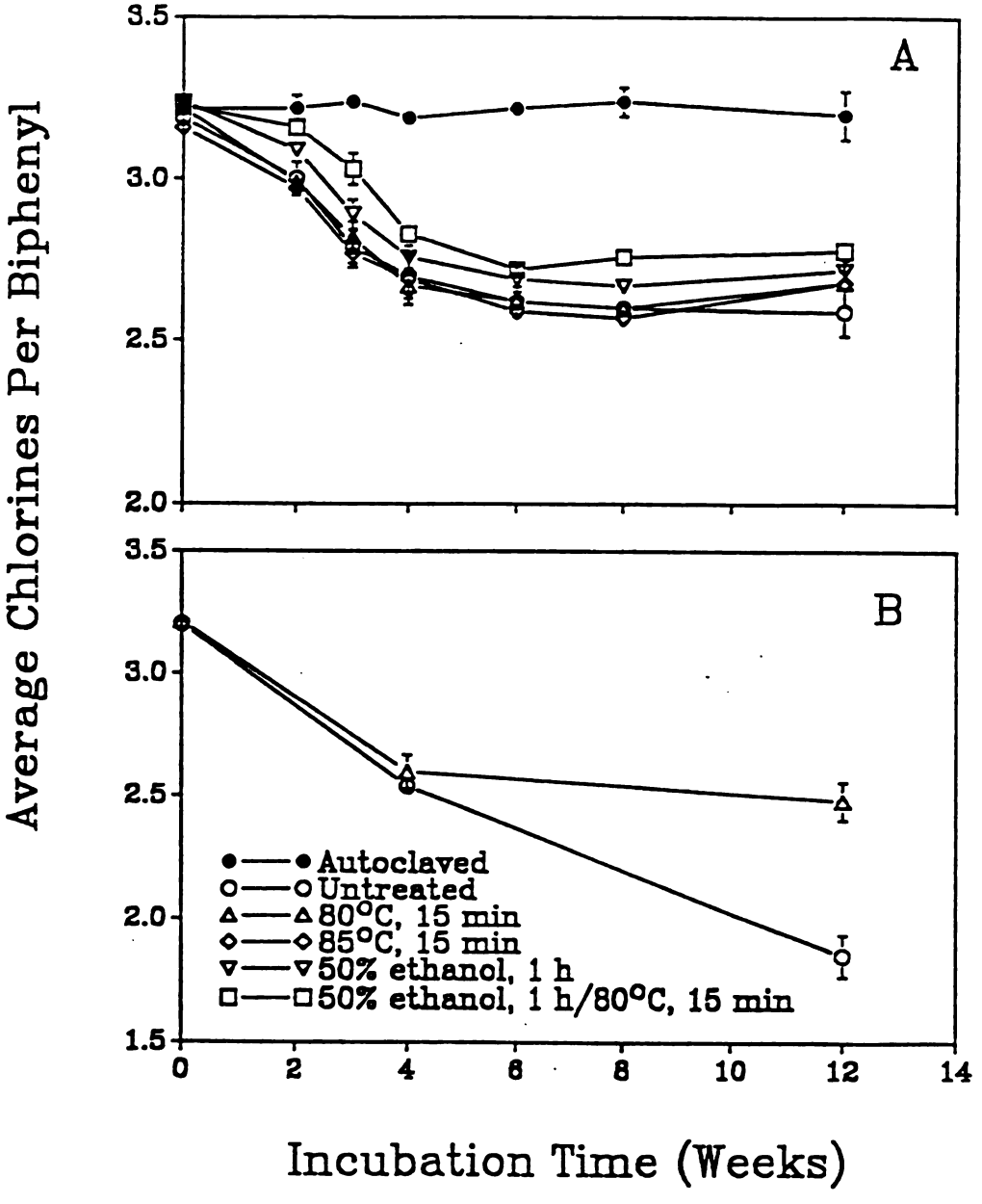

Fig. 2. Dechlorination of Aroclor 1242 by heat and/or ethanol treated microorganisms eluted from the Hudson River sediments by experiments performed in (A) September, and (B) May. Error bars are the SD of triplicate (A) or duplicate (B) samples, where not shown, the error bars are smaller than the symbols.

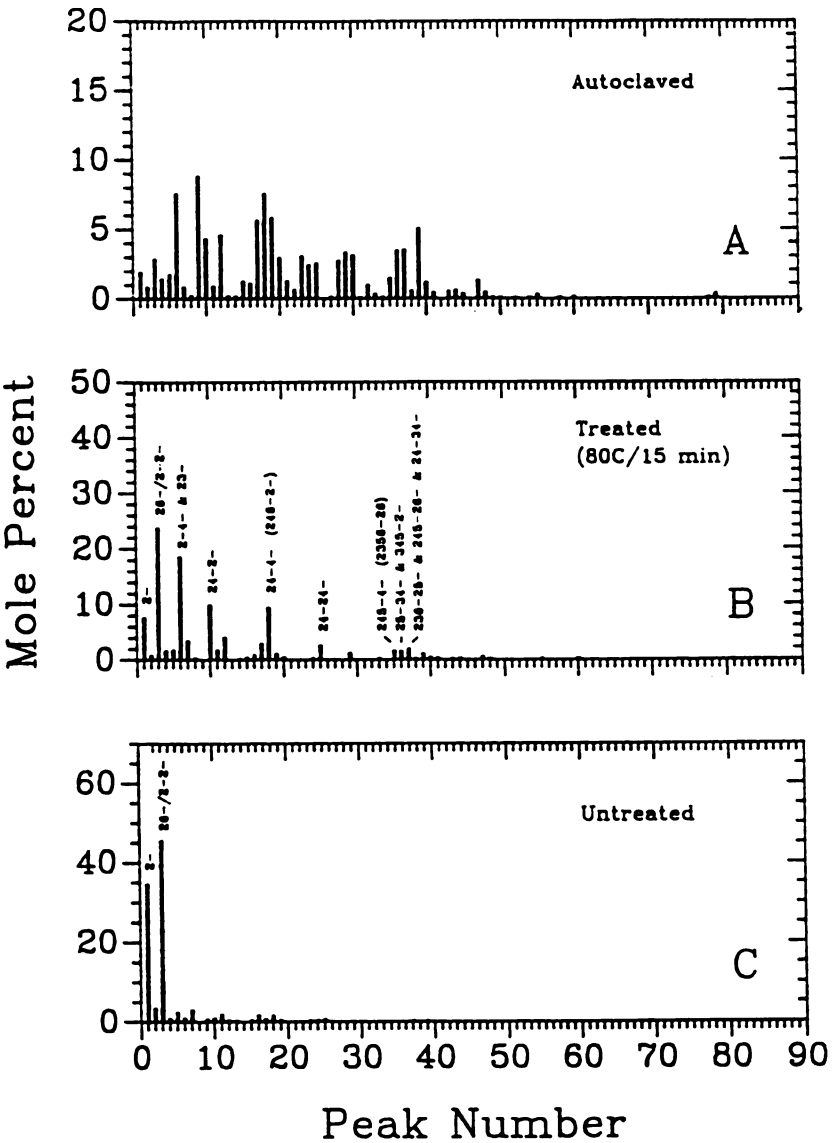

Fig. 3. Mole percentage of Aroclor 1242 represented by each chromatographic peak after 12 weeks incubation in the May experiment with (A) Autoclaved (B) Treated (80°C, 15 min), and (C) Untreated microorganisms eluted from the Hudson River sediments. For a complete list of the PCB congeners associate with each peak see (13).

we failed to enhance PCB dechlorination by using higher levels of acetone (data not shown).

Dechlorination of Aroclor 1242 by the untreated cultures. In contrast to the stable dechlorination activities of the treated cultures, the dechlorination activities of the untreated cultures varied among the three experiments. The highest dechlorination activity was observed in the May experiment when the inoculum was relatively fresh (Fig. 2B) compared to July (not shown) and September (Fig. 2A). In the May experiment the major dechlorination products were 2-CB and 2,6-CB and/or 2-2-CB; these congeners accounted for 79% of the total PCBs recovered after 12 weeks incubation. In the July and September experiments, lesser amounts of these dechlorination products were formed (23% for July; 22% for September). The same *ortho*-substituted products plus some *ortho*- and *para*-substituted products (2-4-CB, 2,4-2-CB, 2,4-4-CB) tended to accumulate.

Comparison of the dechlorination patterns between treated and untreated cultures. The common dechlorination pattern shared by all treated cultures in the three experiments (May, July and September) is very different from that of the untreated culture in the May experiment (Fig. 3). The essential difference is that the treated cultures accumulated dechlorination intermediates containing *para*-substituted chlorine(s) (Fig. 3B) such as 2-4-CB and 2,3-CB (peak 6), 2,4-2-CB (peak 10), 2,6-4-CB (peak 12), 2,5-4-CB (peak 17), 2,4-4-CB (peak 18), and 2,4-2,4-CB (peak 25), in addition to the 2-CB (peak 1) and 2-2-CB and 2,6-CB (peak 2) which were the primary products in the untreated (May) culture (Fig. 3C). Additionally, the chromatographic patterns of the treated and the untreated cultures are easy to distinguish by the apparent difference in the

heights of peaks 35, 36 and 37 (the responses of electron capture detector for these peaks is high), which were evident in the treated cultures but not in the untreated cultures (gas chromatograms not shown).

The dechlorination patterns obtained with the treated inocula were similar to those of the untreated inocula in the July and September experiments.

Analysis of the homolog distribution (Fig. 4) showed that at 4 weeks the distribution in both treated and untreated cultures was similar (Fig. 4A). After 4 weeks, dechlorination slowed in the treated cultures, but continued in the untreated culture, resulting in mono- and dichlorinated biphenyls as the final products (Fig. 4B). At 4 weeks, the mole percentage of monochlorinated biphenyls in both cultures was almost equal. From 4 weeks to 12 weeks, the mole percentage of monochlorinated biphenyls in the treated cultures remained the same, while in the untreated cultures it increased from 9 to 33%. The increase in monochlorinated biphenyls is mainly due to 2-CB. Figure 4B also illustrates that after 12 weeks incubation, the tetra-, penta-, and hexachlorinated congeners have decreased much more in the untreated culture than in the treated culture. Although the mole percentage of dichlorinated congeners in both cultures was comparable, their compositions were different. In the untreated culture, the major dichlorinated biphenyls were 2-2-CB and 2,6-CB (peak 3), while in the treated cultures the major dichlorinated biphenyls were 2-2-CB and 2,6-CB (peak 3) plus 2-4-CB and 2,3-CB (peak 6) (Fig. 3).

Dechlorination of Aroclor 1242 by transferred cultures. The treated culture in the May experiment was transferred at concentrations of 25, 10, and 1%. All transferred cultures showed similar dechlorination activities (data not shown). Two treated cultures

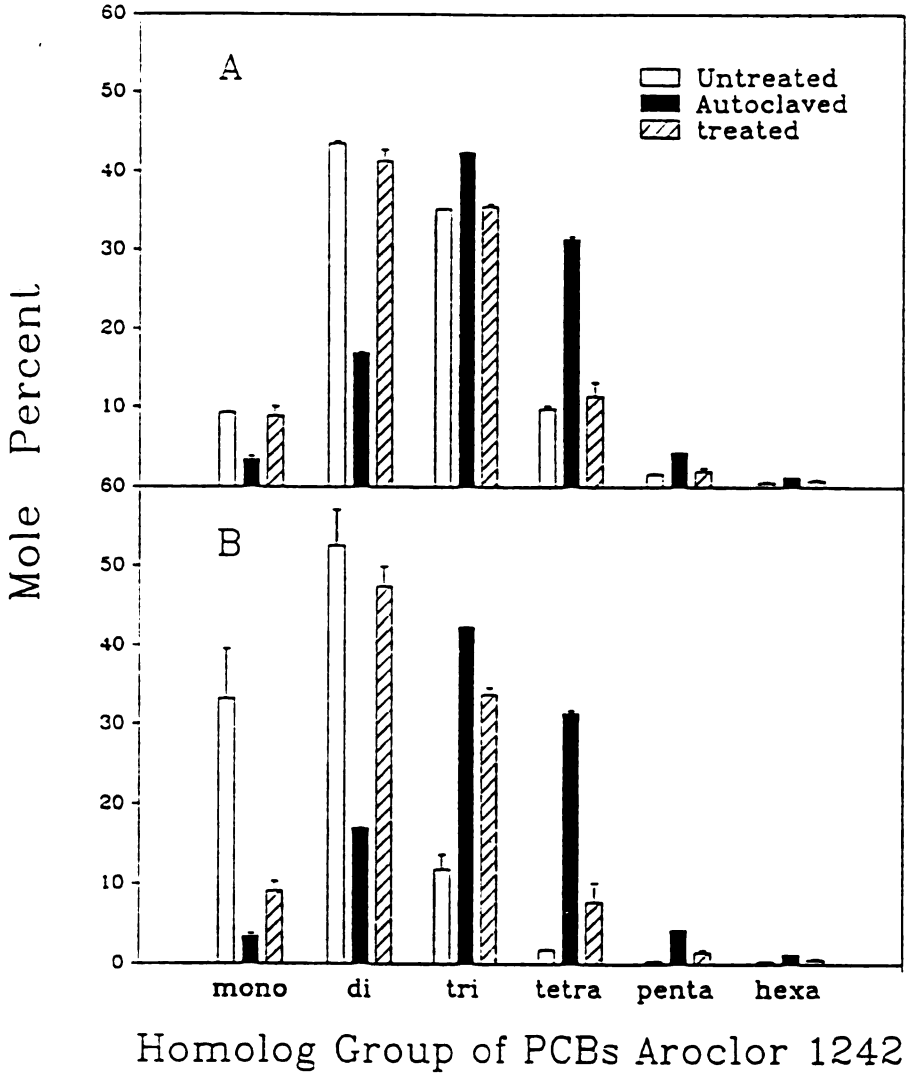

Fig. 4. Homolog distribution of Aroclor 1242 after (A) 4 and (B) 12 weeks of incubation with treated (80°C, 15 min) and untreated microorganisms (May experiment) eluted from the Hudson River sediments. The hepta-, octa-, nona- and decachlorinated homologs are not plotted because their mole percentages in Aroclor 1242 are too low. Error bars are the SD of duplicate samples, where not shown, the error bars are smaller than the symbols.

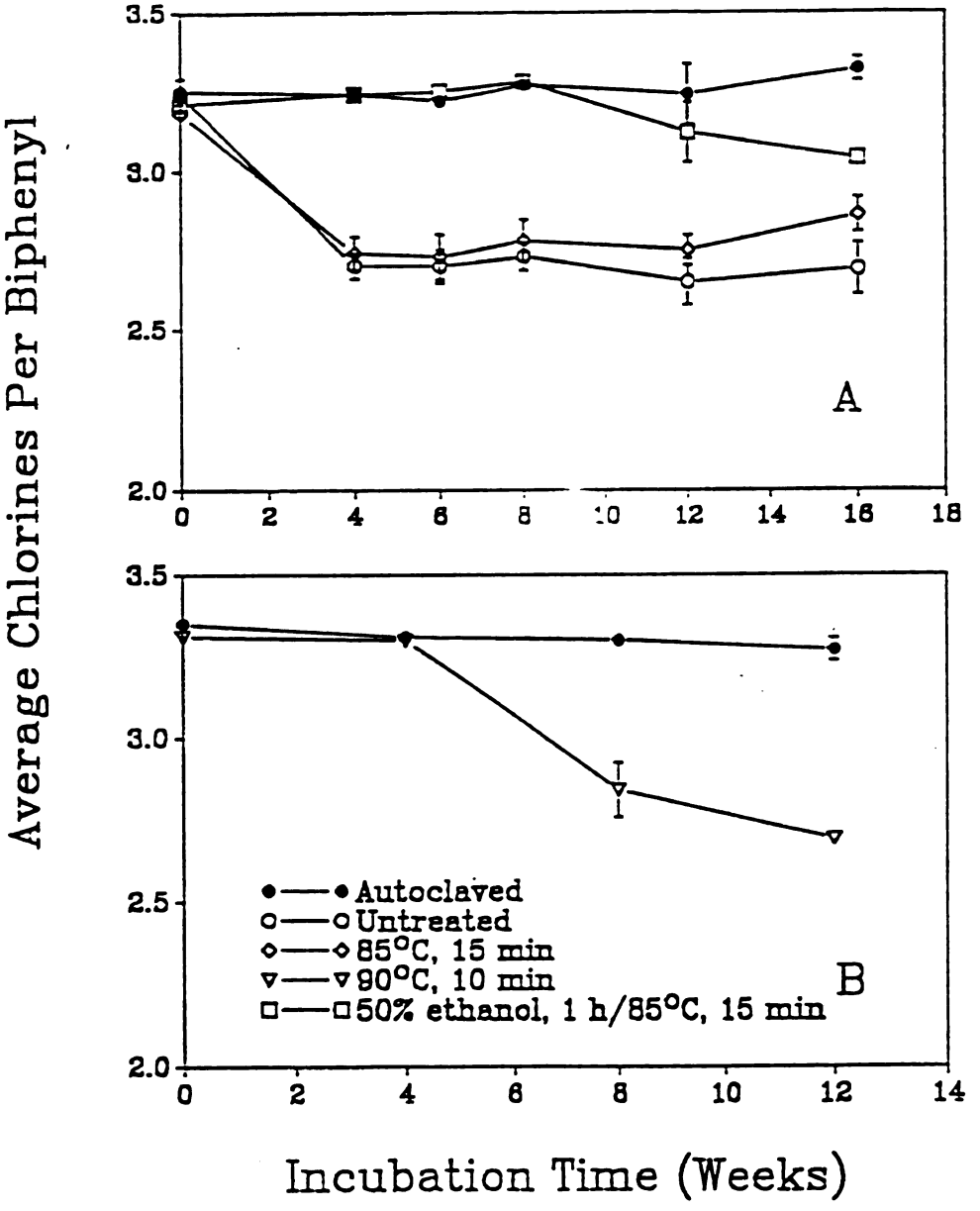

Fig. 5. Dechlorination of Aroclor 1242 by the cultures transferred from the September experiment (1% transfer). (A) The second serial culture (85°C, 15 min). (B) The third serial culture (90°C, 10 min). Error bars are the SD of triplicate (A) or duplicate (B) samples, where not shown, the error bars are smaller than the symbols.

from the September experiment were transferred to the second serial culture at concentration of 1% following treatment of the inocula; one was originally pasteurized at 85°C, and the other was originally treated with the combination of ethanol and heat. When the inoculum from the combined treatment was treated again, the temperature was increased from 80 to 85°C. The transferred pasteurized cultures exhibited dechlorination activities similar to the original cultures, whereas the transferred cultures that received the combined treatment showed a lag time of 12 weeks (Fig. 5A). After 4 weeks, the inoculum taken from the second serial culture (Fig. 5A) was heated at 90°C and transferred (1%) to a third serial culture. The dechlorination activity was still evident (Fig. 5B) even though the pasteurization temperature was raised to 90°C.

DISCUSSION

Heat- and ethanol-treated microorganisms eluted from upper Hudson River sediments were able to dechlorinate Aroclor 1242 under anaerobic conditions, as evidenced by a decrease in the highly chlorinated congeners and a corresponding accumulation of lesser- chlorinated congeners.

In previous studies in which PCB dechlorination was observed, the cultures also produced methane. This correlation, along with the observed dechlorination of other compounds in methanogenic samples (6, 8, 17) and by pure cultures of methanogens (5, 12), has led to speculation that methanogens may be required or responsible for dechlorination of PCBs. However, the pasteurized cultures produced no methane while dechlorinating the PCBs, indicating that methanogens or methanogensis is not required for PCB dechlorination.

Gottschalk et al. (7) pointed out that heating at 70°C or 80°C for 10 min is generally regarded as sufficient for elimination of non-sporeformers, including most thermophilic nonsporeformers. Koransky et al. (11) and Johnston et al. (10) reported that treatment with 50% ethanol for 1 hour is also an effective technique for selective isolation of sporeforming bacteria from mixed cultures. In our experiments, the dechlorinating microorganisms survived both heat and ethanol treatments, and the selective conditions we used were even more strict, including heating at 85°C for 15 min or at 90°C for 10 min, timing from temperatures 3°C higher than those Gottschalk et al. (7) recommended, and combining both heat and ethanol treatments. The dechlorination does not appear to be catalyzed by thermophiles, because in our temperature profile experiment, no dechlorination by the pasteurized cultures was observed when the bottles were incubated at 37, 50, or 65°C (data not shown). Furthermore, the dechlorinating microorganisms withstood not only high temperatures but also 50% ethanol for 1 h, which should eliminate thermophiles. Spores were observed microscopically in the untreated and treated cultures, and were more prevalent in the latter. Because the dechlorination does not appear to be due to thermophiles and the vegetative cells of nonthermophilic microorganisms should have been eliminated by the heat and ethanol treatments, the dechlorinating microorganisms are most likely sporeformers.

The treated cultures had a common *meta* preferential dechlorination activity regardless of the type of treatment. This activity was not only resistant to the heat and ethanol treatments but also stable in the inoculum over time and through serial transfers. This indicates that the responsible microbial population was stably maintained. This stability is consistent with the survival capability of sporeformers.

Comparison of the dechlorination patterns showed that the major difference between the treated and untreated cultures was that the treated cultures lost some of the *para* dechlorination activity present in the fresh untreated inoculum (Fig. 3). Besides this, in contrast to the stable *meta* preferential dechlorination activities in the treated inoculum, the *para* dechlorination activity of the untreated inoculum changed with time. As the inoculum aged, more *para* dechlorination activity was lost, and the dechlorination pattern of the untreated culture approached that of the treated one. The differences in surviving the heat and ethanol treatments and in maintaining the activity over time between *meta* and *para* dechlorination activities may reflect the physiological characteristics of the different responsible microbial populations.

It seems that Hudson River microorganisms responsible for dechlorination of Aroclor 1242 can be grouped into two populations according to their responses to the heat and ethanol treatments: one group can survive such treatments, while the other will be eliminated. The surviving population is responsible for the *meta* preferential dechlorination activities, whereas the eliminated population mainly contributes to *para* dechlorination activities. The untreated cultures in the May experiment consisted of both populations and thus had both activities.

Even though the eliminated population could not withstand the heat and ethanol treatments, it might include some sporeformers as well, because spores normally show widely varying levels of heat and ethanol sensitivity. For example, some bacterial spores can survive at 100°C or more for several hours (9), while some are known to be almost as heat sensitive as the vegetative cell (10).

In the May experiment, when the fresh inoculum was used, dechlorination of

Aroclor 1242 by the Hudson River microorganisms in the untreated culture occurred almost exclusively at the *meta* and *para* positions (Fig. 3), as previously observed by Quensen et al. (13). This dechlorination pattern is very close to the pattern C of Brown et al. (2-4), while the dechlorination pattern of the treated cultures is close to pattern M (1a). Quensen et al. (13) and Abramowicz et al. (1a) suggested that dechlorination pattern C is the result of two separate and partially complementary dechlorination activities. One is pattern Q, characterized by *para* dechlorination activities; the other is pattern M, which is *meta* preferential. They also suggested that two PCB-dechlorinating populations may exist in the Hudson River sediments. The results of this study support this suggestion. Culture conditions and the particular batch of sediment used may both contribute to determining whether one or both dechlorinations are expressed in a particular experiment.

According to our experimental results, it appears that the anaerobic sporeformers are at least one of the physiological groups responsible for the reductive dechlorination of PCBs.

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Chapter 4

Anaerobic Dechlorination of Polychlorobiphenyls (PCB's) by Pasteurized Microorganisms as Affected by Molybdate, 2-Bromoethanesulfonic Acid (BESA), and Sulfate

ABSTRACT

Anaerobic dechlorination of Aroclor 1242 by the pasteurized cultures was completely suppressed during 12 weeks of incubation when either 5 mM molybdate, 20 mM sulfate, or 25 mM 2-bromoethane sulfonic acid (BESA) was added at 0 time and the same amounts of molybdate and sulfate, and half as much BESA were re-fed either at 2, 4, 6, and 8 weeks (molybdate and BESA) or 4, and 8 weeks (sulfate). Effects of these anions on dechlorination at different concentrations were screened. BESA and sulfate completely inhibited dechlorination at their lowest screened concentrations, 1 mM and 2 mM, respectively. Molybdate partially inhibited the dechlorination at 2, 4, and 8 mM, and completely inhibited the dechlorination at 16 mM. Methanogens were eliminated by the pasteurization as evidenced by the fact that no methane was detected in any pasteurized culture. Since methanogens were eliminated, BESA probably inhibited the dechlorination by acting as a sulfuroxy anion. It seems that the inhibition of dechlorination by molybdate, sulfate, and BESA is not due to general toxicity but due to the effects on sulfidogens. Since molybdate should not inhibit microorganisms competing with sulfidogens, while sulfate should not inhibit microorganisms depending on sulfidogens, it appeared that microorganisms capable of anaerobic dechlorination of PCBs in the pasteurized cultures were probably sulfidogens. Molybdate probably directly inhibited sulfidogens, while both sulfate and BESA probably inhibited the dechlorination by competing with PCB as electron acceptors. Only sporeforming bacteria were expected to survive the harsh pasteurization, and among sulfidogens the only known genus forming spores is *Desulfotomaculum*. Therefore, the experimental results suggest that

microorganisms responsible for the dechlorination of PCBs in our pasteurized enrichment are probably *Desulfotomaculum*-like sporeforming sulfate-reducing bacteria. However, isolation of the microorganism(s) and demonstration of PCB dechlorination activity with the isolate(s) are required in order to make a final conclusion.

INTRODUCTION

Characterization of the PCB dechlorination activities in an undefined microbial community is an important step in the identification of the responsible microbial population(s). Information from these studies can provide a basis for further enrichment, and may facilitate the identification and isolation of microorganisms capable of PCB dechlorination.

Microbial group specific inhibitors can sometimes be used to distinguish which group is responsible for a specific activity (18). We previously investigated the effects of molybdate (a specific inhibitor of sulfate-reducing bacteria), 2-bromoethanesulfonic acid (BESA, a specific inhibitor of methanogenic bacteria) on the anaerobic dechlorination of PCBs in non-pasteurized cultures (Chapter 3). We also tested the effect of sulfate on PCB dechlorination by the same cultures. All of these anions inhibited both dechlorination and methanogensis. But because the non-pasteurized inoculum contained a high diversity of microorganisms, the results alone did not specifically define the microbial group responsible for PCB dechlorination activity.

Pasteurization of the inoculum used above eliminates methanogens which play an important role in anaerobic microbial processes (2, 29), but a partial dechlorination activity was still retained (28). Pasteurization also decreased the microbial diversity in

the system since only spore-forming bacteria maybe expected to survive the treatment. Therefore, the effects of molybdate, BESA, and sulfate on PCB dechlorination in pasteurized cultures were investigated to further characterize the dechlorination activity in the pasteurized culture, and to try to correlate the activity to a certain type(s) of microorganism(s).

MATERIALS AND METHODS

Inoculum and pasteurization. Sediments were collected from the upper Hudson River, N.Y. (site H7 in reference 3). Inoculum preparation and pasteurization were as described elsewhere (28). The inoculum for the general inhibition experiment was prepared directly from Hudson River sediment and was pasteurized at 85°C for 15 min (timing began when the thermometer in the monitor tube reached 83°C). The inoculum for the concentration profiles experiment was taken from a PCB dechlorinating culture previously pasteurized at 90°C for 15 min and this culture exhibited retained a *meta*-preferential dechlorination activity. The inoculum from this culture was re-pasteurized at 90°C for 10 min prior to use.

Preparation of the experimental batch. 60-ml serum bottles were used as the experimental vessels for the general inhibition experiment. Each serum bottle received 10 g of PCB-free Hudson River sediments. The bottles containing sediments were evacuated and refilled with N_2 in a glove box lock, then flushed with N_2-CO_2 (80:20, vol/vol) with a Hungate apparatus, and received 10 ml of RAMM (21) containing 0.1% methanol and 10% freshly prepared non-pasteurized inoculum. The serum bottles were then incubated at 37°C for 1 week. After methane was detected, the bottles were

autoclaved for 1 h on 3 consecutive days with incubation at 37°C between each autoclaving. After the third autoclaving, 10 ml of the pasteurized inoculum, 10 ml of sterile RAMM, 100 μl of 10% autoclaved solution of cysteine, and 80 μl of 10% PCBs (Aroclor 1242, Monsanto Co., St. Louis, Mo.) in acetone were added. The stock solutions of molybdate (sodium salt), sulfate (sodium sulfate), and BESA were bubbled with N_2, autoclaved and then introduced. The initial concentration of molybdate and BESA were 5 mM, and 50 mM, respectively. The same amounts of molybdate and half as much BESA were re-fed at 2, 4, 6, and 8 weeks. The initial concentration of sulfate was 20 mM, and the same amount was added at 4 and 8 weeks. The controls were autoclaved twice, 1 h each time with an interval of incubation at 37°C for 5 h before PCBs were added. The samples were shaken for 1 h and then incubated at 25°C in dark.

For the concentration profiles experiment, the experimental vessels were 28-ml serum tubes. The experimental batch of the concentration profiles experiment was prepared in the same way as that of the general inhibition experiment, except that the 28-ml serum tube received 1 g of sediment, 4.5 ml of RAMM, and 0.5 ml of inoculum, and 5 μl of 10% Aroclor 1242 in acetone. The cultures were incubated at 30°C instead of 25°C, and molybdate, sulfate, as well as BESA were added only at the beginning of the experiment.

Analysis of methane, sulfate, and molybdate. Methane was assayed on a gas chromatograph (Varian 3700) with a flame ionization detector.

Before being sampled for molybdate and sulfate, the culture tubes were vortexed for 3 min. After the sediments settled, and while the tubes were flushed with N_2, 1 ml of the liquid portion was taken, acidified with 1 N HCl, and bubbled with N_2 for 5 min

to drive out H_2S. The samples were then centrifuged, filtered (0.22 μm, Millipore Co., Bedford, Mass.), and analyzed for sulfate and molybdate. Sulfate and molybdate were analyzed with a high pressure ion chromatograph (Dionex) equipped with a HPIC AS4A column (20 cm) and a conductivity detector. The mobile phase was 1.7 mM $NaHCO_3$/1.8 mM Na_2CO_3, and the flow rate was 2.3 ml/min.

PCB analysis and data summation. These procedures were as previously described (20).

RESULTS

Results of the general inhibition experiment. Dechlorination was completely inhibited by molybdate, sulfate, and BESA (Fig. 1) during 12 weeks of incubation. The color of the cultures amended with either sulfate or BESA turned dark black after two weeks of incubation. Additionally, these cultures gave a strong sulfide smell when they were sampled while being flushed with N_2-CO_2. In contrast, those cultures amended with molybdate turned yellowish, and there was no sulfide odor when these cultures were sampled. No methane production was detected in any of these pasteurized cultures as previously reported (28), indicating that methanogens were eliminated by the pasteurization.

Concentration effects of molybdate, BESA, and sulfate on PCB dechlorination by the pasteurized cultures. Both BESA and sulfate completely inhibited dechlorination at all concentrations tested. The lowest concentrations for BESA and sulfate were 1 mM and 2 mM, respectively (Fig. 2). Molybdate partially inhibited dechlorination at 2, 4 and 8 mM, and completely inhibited dechlorination at 16 mM (Fig. 2).

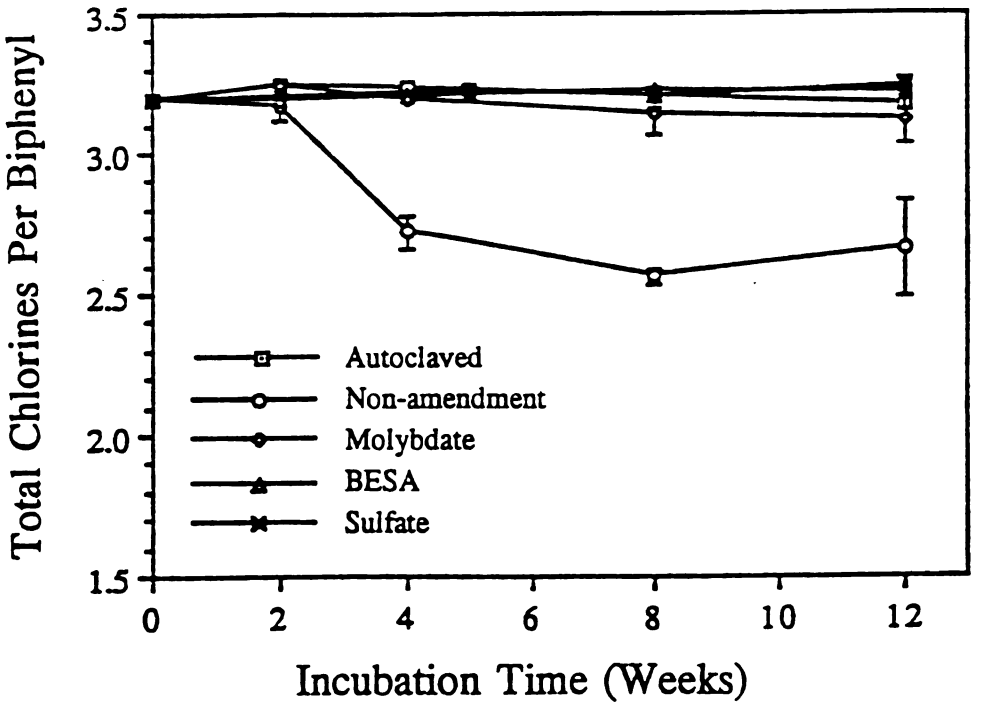

Fig. 1. Dechlorination of Aroclor 1242 by the pasteurized microorganisms as affected by molybdate, sulfate, and BESA. The microorganisms were eluted from Hudson River sediments before the pasteurization. Error bars are the standard deviation (SD) of triplicate samples; where not shown, the error bars are smaller than the symbols.

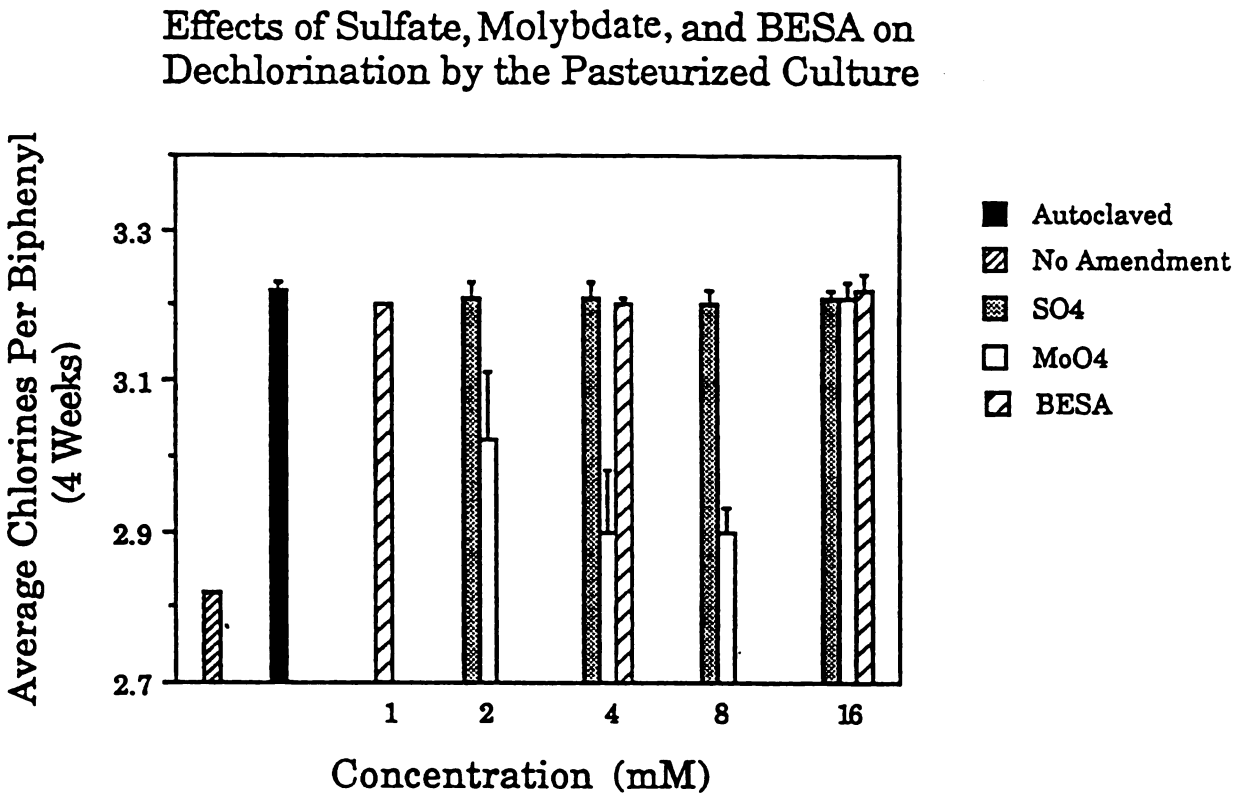

Fig. 2. Effects of molybdate, sulfate and BESA at different concentrations on dechlorination by the pasteurized microorganisms. Error bars are the standard deviation (SD) of triplicate samples; where not shown, the error bars are smaller than the symbols.

Consumption of both sulfate and molybdate was confirmed by chromatographic analysis. After 4 weeks of incubation, sulfate and molybdate in the 4 mM amendment groups decreased 23.1, and 27.7%, respectively. Differences in the color of the cultures amended with different concentrations of molybdate, BESA, and sulfate were also observed. Compared with the non-amended cultures, the color of the cultures amended with 8 or 16 mM sulfate or 16 mM BESA turned black, while the molybdate-amended cultures exhibited a slight yellowish color. These colors were consistent with those observed in the general inhibition experiment and in our similar experiment with the non-pasteurized cultures (Chapter 3). No methane production in any culture in this experiment was detected.

DISCUSSION

Molybdate, sulfate, and BESA all inhibited dechlorination of PCBs by the pasteurized cultures. In the general inhibition experiment, after 12 weeks of incubation, dechlorination was still completely blocked by all of the three anions at the experimental concentrations. However, it was unclear whether molybdate, sulfate, or BESA at their experimental concentrations might have had general toxic effects. Furthermore, in order to examine the sensitivity of the dechlorination process to the inhibitors , it was necessary to lower the experimental concentrations. Therefore, concentration profiles of molybdate, BESA, and sulfate were screened. As shown in Fig. 2, the dechlorination by the pasteurized cultures was more sensitive to both BESA and sulfate than to molybdate, since both BESA and sulfate completely inhibited dechlorination at their lowest experimental concentrations, 1 mM, and 2 mM, respectively, while molybdate did

not completely inhibit dechlorination at 8 mM.

BESA is a specific inhibitor of methanogens (1, 18). However, methanogens were eliminated by the pasteurization as evidenced by the fact that no methane production was detected in any of the pasteurized culture. Therefore, BESA probably functioned as a sulfuroxy anion due to its sulfonic moiety, which is a potential electron acceptor for sulfate-reducing bacteria (SRB). The black color and these cultures and the sulfide smell support this hypothesis. This, coupled with the facts that molybdate is a specific inhibitor of SRB and sulfate is a electron acceptor of SRB, suggests that in the pasteurized cultures the effects of BESA, molybdate, and sulfate are all related to SRB.

In order to facilitate the discussion, we here divide all microorganisms in the pasteurized cultures into two groups based on their relationship to SRB: one group is composed of microorganisms not related to SRB and is designated as Group I, whereas Group II consists of SRB and those microorganisms depending on or competing with SRB. Microorganisms capable of dechlorinating PCBs (DCM, henceforth) in the pasteurized cultures may belong to either group or both.

As mentioned above, in the pasteurized culture the effects of molybdate, sulfate, and BESA are all related to SRB, while Group I only consists of microorganisms not related to SRB. Therefore, if there were DCM in Group I, general toxicity is the only possible explanation.

It appears that inhibition of dechlorination by BESA was not due to toxicity. Sparling et al. (23) examined the effect of 25 mM BESA on a wide variety of microorganisms, including the anaerobic/facultative anaerobic spore-formers *Clostridia* and *Bacillus*, and found that BESA had no significant side effect. Other investigators

also found that 25 mM BESA had no undue influence upon bacterial growth of eight different types of anaerobes (18). The addition of 2 and 5 mM BESA to salt marsh sediments caused moderate but discernible decrease in the rate of sulfate reduction (16 and 22%, respectively) when compared with sediments lacking inhibitor, this was probably because SRB used the sulfonic acid moiety as an electron acceptor in preference to sulfate (6). In our experiment, BESA completely blocked dechlorination by the pasteurized cultures at concentration of 1 mM, which is much lower than 25 mM.

It is unlikely that inhibition of dechlorination by 2 mM molybdate was due to a general toxic effect. Molybdate is generally regarded as a specific inhibitor of SRB (24) and thus widely used in microbiology studies due to its specificity (18). Molybdate at high concentrations may have a toxic effect on some microorganisms since it can bind free sulfide ions to form a molybdosulfide complex (25), and this could influence microorganisms requiring sulfide ions (18) e.g. methanogens (2, 29). However, this toxic effect depends on experimental concentration. It is reported that $\geq$20 mM inhibit microorganisms that require sulfide such as methanogens (9, 22), and also inhibited PCB dechlorination in non-pasteurized cultures (Chapter 3). However, Lovely and Klug (11) reported that a concentration at $\sim$2 mM did not inhibit methanogensis in sediments. In our experiment 2 mM molybdate partially inhibited the dechlorination. The effective concentration in our cultures should have been even lower because some molybdate should adsorb onto clay surfaces (19) present in the sediment slurries. Additionally, molybdate is a component of nitrogenase and nitrate reductase, therefore, any anaerobic nitrogen-fixers (such as some *Clostridia*) and denitrifiers present would consume some of the molybdate, further reducing its effective concentration. Therefore, at this low

concentration, general toxic effect is unlikely.

In summary, it is possible that some microorganism(s) in group I might be inhibited by either 1 mM BESA, 2 mM sulfate, or 16 mM molybdate, and the sensitive microorganism(s) happened to be the DCM. However, it is improbable that it(they) was(were) completely suppressed by all three of these anions. Therefore, it appeared that Group I did not contain DCM. Hence all DCM in the pasteurized culture were in Group II.

Microorganisms in Group II can be subdivided into three categories: (i) those competing with SRB, (ii) those dependent on SRB, and (iii) SRB. The DCM may belong to one or all three categories. It is possible to distinguish which category they belong to based on the effects of sulfate, molybdate, and BESA.

Sulfuroxy anions may inhibit anaerobic dechlorination by competing for electrons if the DCM are either SRB or SRB-competing microorganisms. Inhibition of dechlorination by sulfuroxy anions have been reported in many studies (7, 11, 14, 16, 17). When the DCM are SRB, sulfuroxy anions may inhibit dechlorination by diverting electron flow to sulfuroxy-anion-reduction instead of dechlorination (10); if the DCM are not SRB but SRB-competing microorganisms, sulfuroxy anions may also inhibit dechlorination by diverting the electron flow away from the DCM to SRB, however, in this case, relief of the inhibition by molybdate is expected. For example, Madsen and Aamand (12) observed that dechlorination of chlorophenol by an enriched culture was inhibited by sulfur oxyanions, but this inhibition could be relieved by molybdate. The dechlorinating microorganism (DCB-2) in the enrichment was later isolated and identified as *Clostridium* sp. (13). Since DCB-2 is not SRB, the inhibition was obviously due to

competition between DCB-2 and SRB for electrons. As a second example, Gibson and Suflita reported stimulation of the dechlorination of 2,4,5,-trichlorophenoxyacetic acid by molybdate in a methanogenic aquifer amended with sulfate (8).

In our experiment, if the DCM were SRB-competing microorganisms, addition of molybdate should enhance PCB dechlorination. Since molybdate inhibited dechlorination by the pasteurized cultures, the results indicate that SRB-competing microorganism in the pasteurized cultures are not among DCM.

The possibility that the DCM depend on SRB is inconsistent with the fact that sulfate completely inhibited dechlorination at all experimental concentrations. RAMM medium does not contain sulfate, and the sediment used in the culture was freshwater sediment (Hudson River sediment) containing little low sulfate. Therefore, the culture conditions are sulfate-limited, and addition of sulfate should stimulate growth of SRB and thus benefit SRB-dependent microorganisms. If any DCM were SRB-dependent, stimulation, instead of complete inhibition, would be expected. Integration of the results of the BESA, molybdate, and sulfate experiments suggests that both SRB-dependent and SRB-competing microorganisms are not responsible for the dechlorination activity in the pasteurized cultures.

One exception to the above discussion is that the SRB were not the DCM but were part of an obligate syntrophy associated with the DCM. Then both molybdate and sulfate would inhibit dechlorination activity. Molybdate would inhibit dechlorination by inhibiting SRB, while sulfate would inhibit dechlorination by driving SRB from fermentation to anaerobic respiration, and hence disrupting the obligate syntrophy relationship. This could be true if SRB functioned as the only H_2-producer and the DCM

an H_2-consumer. This is possible, but not likely. Our cultures contained a mixed community, and many anaerobic spore-formers such as some *Clostridium* (27) are well-known versatile H_2-producers. Therefore, if the DCM are H_2-consumers, inhibition of SRB would not be expected to completely block the dechlorination, since other H_2 sources should be readily available.

From the above line of reasoning, it appears that the DCM in the pasteurized cultures is (are) probably SRB. The inhibitory effects of the amendments on PCB dechlorination can be explained by molybdate's directly inhibition of SRB, while sulfate and BESA inhibited dechlorination by competing with PCB as electron acceptors. This is similar to the experiment reported by Linkfield and Tiedje (10). In that case dechlorination by a sulfidogen, *D. tiedjei*, was inhibited by sulfuroxy anions, suggesting that when grown with sulfuroxy anions, *D. tiedjei* prefers these sulfuroxy anions as electron acceptors, and may divert the flow of electron from dechlorination to sulfuroxy anion reduction (10). However, whether sulfate inhibits dechlorination by sulfate-reducers depends on the culture conditions. After the culture medium had been modified, dechlorination by *D. tiedjei* occurred in the presence of sulfate (4). The above discussion is summarized by Table 1.

The hypothesis that sulfate and BESA inhibited dechlorination by competing for electrons is consistent with the observation of the dark black color which probably indicated the formation of sulfide, and the sulfide smell of the sulfate- and BESA-amended cultures. Consumption of sulfate was confirmed by HPIC analysis. Despite that it was unclear whether the consumption was dissimulatory or assimilatory, it nevertheless provided another possible evidence. However, we did observe inhibition

Table 1. The Possible Interpretation for the Results

Inhibited by	Possible dechlorinating microorganism(s)		
	Depending on SRB*	Competing with SRB	SRB
Sulfate	No	Yes	Yes
Molybdate	Yes	No	Yes
BESA**	No/Yes	Yes/No	Yes

* Sulfate-reducing bacteria

* The effects of BESA on sulfate-reducing bacteria (SRB) may vary. It may enhance/inhibit SRB and hence may promote/suppress microorganisms depending-on/competing-with SRB depending on the culture conditions (e.g. balance between the available electron donor and sulfate concentration of the culture).

of SRB by molybdate in our similar experiment with untreated cultures. In that experiment, supplementation with sulfate (to inhibit methanogens by promoting SRB) inhibited methanogensis, but failed to inhibit methanogensis in presence of molybdate (Chapter 3).

The inhibitory effects resulting from competition for electrons may depend on the balance between the sulfuroxy anions and the available electron donors. In some studies addition of electron donors to deplete available sulfate allowed dechlorination to start (10, 17). May et al (17) reported that when 20 mM sulfate was added to anaerobic sediment slurries spiked with PCBs, dechlorination was not observed until after 15 weeks when sulfate was depleted. In our experiment the culture medium was pre-incubated to ensure a strictly anaerobic condition, and was not supplemented with electron donor(s). The pre-incubation procedure should consume some electron donor(s). Additionally, pasteurization should eliminate some fermentative microorganisms, decreasing the potential of the microbial community to utilize electron donors from the sediment contained in the culture. These differences may explain why concentrations of BESA and sulfate as low as 1, and 2 mM completely inhibited dechlorination by the pasteurized cultures.

Our pasteurization conditions should eliminate non-sporeformers, and the possibility of thermophiles can also be ruled out as previously discussed (28). Therefore, the DCM in the pasteurized cultures should be sporeformers. Among SRB the only known genus forming spores is *Desulfotomaculum*. Therefore, these results suggest that microorganisms responsible for dechlorination activity in our pasteurized cultures are most probably *Desulfotomaculum*-like sporeforming SRB. Attempt to isolate the

responsible dechlorinating microorganism(s) did not succeed. To current knowledge genus *Desulfotomaculum* is diverse (5), the dechlorinating microorganism(s) in our pasteurized cultures may be very different from those have been described. However, isolation of the dechlorinating *Desulfotomaculum*-like sporeforming SRB (pure strain(s) or in obligatory syntrophy) and demonstration of the dechlorination by the isolate(s) are required in order to make a final conclusion. On the other hand, we do not extrapolate this proposition to other enrichments. Sporeforming bacteria are a diverse group, and different enrichments may have different sporeforming populations depending on inoculum source, pasteurization technique, and culture conditions. The proposition that the DCM in our pasteurized cultures is(are) *Desulfotomaculum*-like sporeforming SRB does not rule out the possibility that other sporeformer(s) may also have PCB dechlorination capability.

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Chapter 5

Differentiation of Three Classes of Congener-specific Dechlorination Activity on Aroclor 1242

ABSTRACT

Two classes of congener specific microbial dechlorination activity on Aroclor 1242 in Hudson River sediments have been reported previously, one mainly removes *meta* chlorines, while the other mainly removes *para* chlorines. Results of temperature experiments presented here indicate three (one *meta*, two *para*) classes of dechlorination activity on Aroclor 1242 by microorganisms from the Hudson River sediments. The previously recognized *para* dechlorination activity (Pattern Q activity) actually consists of two activities: one that dechlorinates *para*-substituted congeners without *meta* chlorines (p_o), and the other mainly dechlorinates *para* chlorines from congeners containing *meta* chlorines (p_m). The p_o dechlorination activity was lost from the untreated cultures when the temperature was raised from 25°C to 30°C perhaps because the responsible microorganisms were unable to successfully compete for nutrients, and this *para* dechlorination activity was nutrient-dependent. The p_m dechlorination activity is very similar to that exhibited by our methanogenic enrichment, which is very close to environmental dechlorination process H seen in the tidal Hudson. Decrease in the lag time of the *meta*-preferential dechlorination by heat shock was observed probably due to the activation effects of heat shock, and this observation supports the previous suggestion that anaerobic sporeformers are among the *meta*-preferential dechlorinating microorganisms. Compared with the two *para* dechlorination activities,the *meta*-preferential dechlorination is a fast precess. Despite the fact that the p_o activity lost at 30°C, 30°C was more favorable than 25°C for both *meta*-preferential and the p_m dechlorination activities.

INTRODUCTION

Temperature is an important factor affecting biodegradation (8, 9). Temperature is one of the selective factors in determining the dominant populations and resultant metabolic activities in dynamic microbial communities (9, 11, 17). In anaerobic microbial dechlorination research, isolation of pure strains capable of aryl dechlorination is difficult often due to the complex trophic relationship among the involved microbial populations. As a result, indirect methods with different selective pressures including incubation temperature have been used to differentiate the involved microorganisms and their dechlorination capacities (10, 19, 20). Kohring et al. (10) screened anaerobic biodegradation of 2,4-dichlorophenol (2,4-DCP) in freshwater lake sediments at different temperatures and observed two peak activities in different temperature ranges. Based on this observation they proposed that at least two different microorganisms were involved in the transformation of 2,4-DCP to 4-CP. Wu et al. (19) examined the effect of temperature on anaerobic transformation of 2,3,4,6-tetrachlorobiphenyl (2,3,4,6,-CB) in methanogenic pond sediment slurries, and observed that the fastest dechlorination occurred at 30°C. A higher rate of dechlorination was observed at 50, 55, and 60°C than at 37, 40, and 45°C, indicating two dechlorinating communities.

Tiedje et al. (16) investigated dechlorination of Aroclor 1242 at 12, 25, 37, 45, and 60°C by microorganisms eluted from Hudson River sediments, and found that the optimal temperature for the dechlorination activity was 25°C. No dechlorination activity was observed at 37°C and above, suggesting that the microorganisms involved have similar optimal and tolerant temperature ranges. Despite the fact that only one peak activity was found , however, two dechlorination activities have been reported (one is *meta-*

preferential, the other is mainly *para*-removal) (2), and different dechlorination activities usually reflect the involvement of different microorganisms which may have different temperature preferences.

Heat- and ethanol-treatments were used to eliminate microorganisms from a mixed PCB dechlorinating population. The resulting simplified population retained a *meta*-preferential dechlorination activity (20), providing a way to separate microorganisms capable of *meta* dechlorination from the mixed community. It thus became possible to characterize the temperature requirement of the *meta*-preferential dechlorination activity. By comparing the dechlorination at different temperatures by both the pasteurized and the untreated cultures, information about the temperature requirement of the *para* dechlorination activity can also be obtained. Because both incubation temperature and pasteurization are powerful selective forces, the combination of both should more effectively "fractionate" the diverse microbial populations in the community, providing information about the composition of the mixed community. The results presented here indicate three distinct PCB (Aroclor 1242) dechlorination activities by microorganisms from Hudson River sediments.

MATERIALS AND METHODS

Inoculum preparation and pasteurization. Sediments were collected from the upper Hudson River near Hudson River Falls, N.Y., and shipped under anaerobic conditions to the laboratory as previously described (13). Inoculum preparation and the pasteurization technique were as previously described (20) except that the pasteurization temperature was 85°C, the pasteurization time was 15 min, and the pasteurization was

timed after the temperature in the monitor tube reached 83°C.

Experimental batch preparation. Anaerobic culture tubes (28-ml, Bellco Glass Inc., Vineland, N.J.) containing PCB-free dry Hudson River sediment (1 g per tube) were sealed with rubber stoppers in an anaerobic chamber. The PCB-free sediment was collected from the Hudson River at river mile 205. The sediment was air-dried and sieved with a 2-mm-mesh screen. The sediment was then moistened with 1 ml of Reduced Anaerobic Mineral Medium (RAMM) (15) containing 0.1% methanol and 10% inoculum. The tubes were then pre-incubated (20). After formation of methane was confirmed, all tubes were autoclaved at 121°C for 1 h on 3 consecutive days with incubation at 37°C between each autoclaving.

After the third autoclaving the following were added to each preincubated culture tube while flushing with filter-sterilized (0.22 μm, Millipore Co., Bedford, Mass.) O_2-free N_2-CO_2 (80:20, vol/vol) using a Hungate apparatus: 1 ml of inoculum, 2 ml of RAMM, 6 μl of 10% (wt/vol) Aroclor 1242 (Monsanto Co., St. Louis, Mo.) in acetone. The final concentration of PCBs was 600 μg/g sediment (dry weight). Autoclaved 10% solution of cysteine was added at a final concentration of 250 mg/L. The tubes were then sealed with Teflon-coated butyl rubber stoppers (West Co., Phoenixville, Pa.) and aluminum crimps, and the contents were mixed with a Vortex mixer to completely disperse the PCBs. The controls were autoclaved twice at 121°C for 1 h with an interval of 5 h before PCBs were added. For the pasteurized treated cultures the inocula was pasteurized before being introduced into the incubation tubes.

Incubation. The tubes were incubated in the dark at 10, 25, 30, 37, 50, and 65°C. To confirm the existence of O_2 free conditions of the different temperatures, triplicate

monitor tubes containing 5 ml of RAMM, cystine (250 mg/L) and resazurin, without any sediments, were prepared and incubated at each temperature along with the experimental tubes.

Analysis of methane production. Headspace gas of the samples was analyzed for determination of methane production before the samples were extracted for PCB analysis. Methane was detected by gas chromatography using a flame ionization detector.

Sampling, extraction, analysis, and data summation. These were as previously described (13).

RESULTS

Anaerobic conditions over the experimental temperature range. During 12 weeks of incubation all O_2-monitor tubes retained their black color indicating no O_2 contamination except for one of the triplicates incubated at 50°C which turned pink after 7 weeks of incubation. Inspection showed that there was a tiny breach at the mouth of the serum tube, and this was probably the reason for the oxygen contamination in this tube. These results confirmed that all cultures were under strictly anaerobic conditions at the different incubation temperatures.

Temperature profiles of the dechlorination activities. Dechlorination only occurred in those cultures incubated at either 25 or 30°C, no dechlorination was observed when cultures were incubated at 37, 50, 65, and 10°C (Fig.1). As previously described (20), no methane production was observed by the pasteurized cultures, while all untreated cultures produced methane. The highest methane production occurred at 25°C (Fig. 2). Temperature profiles for the dechlorination activities of both the treated and the untreated

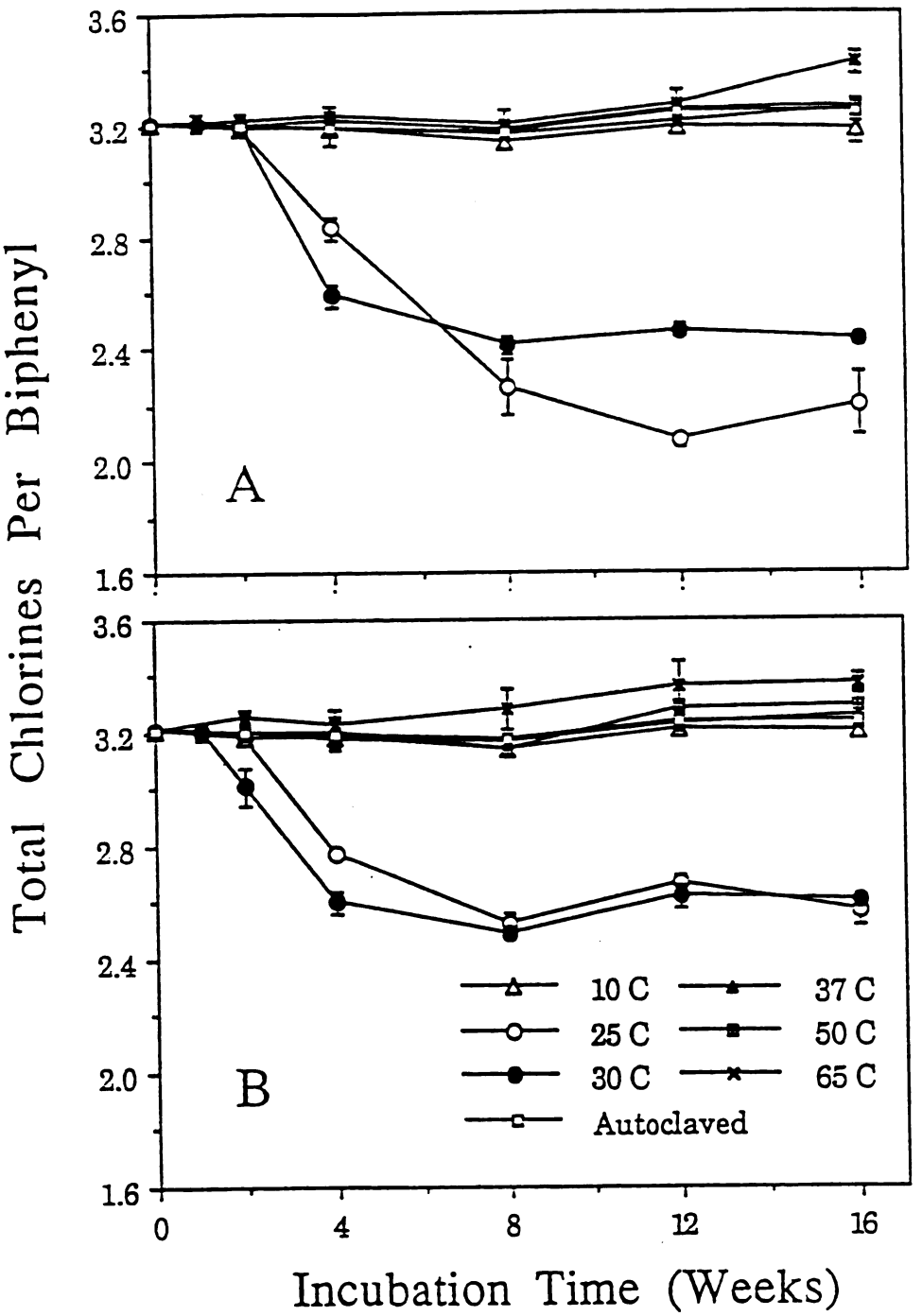

Fig. 1. Dechlorination of Aroclor 1242 by (A) untreated, and (B) pasteurized microorganisms eluted from Hudson River sediments at different temperatures. Error bars are the standard deviation (SD) of triplicate samples; where not shown, the error bars are smaller than the symbols.

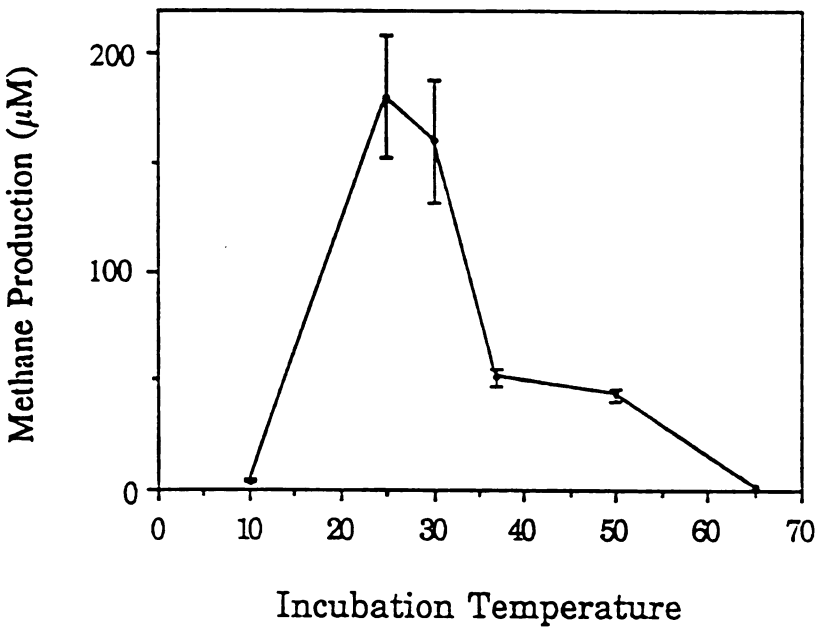

Fig 2. Methane production in the untreated cultures after 16 weeks of incubation. Error bars are the SD of triplicate samples; where not shown, the error bars are smaller than the symbols.

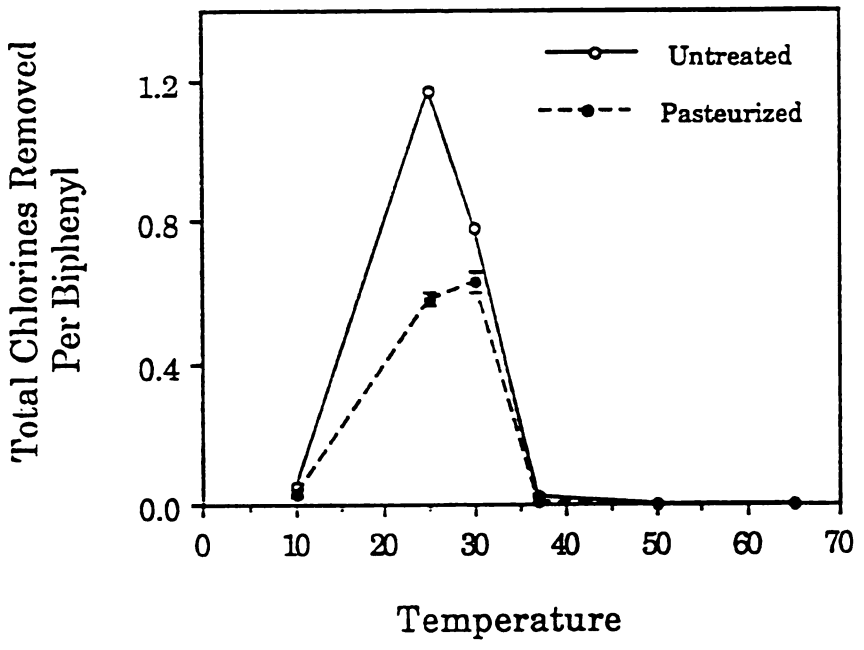

Fig. 3. Temperature profiles of dechlorination of Aroclor 1242 by microorganisms eluted from Hudson River sediments after 12 weeks incubation. Error bars are the SD of triplicate samples; where not shown, the error bars are smaller than the symbols.

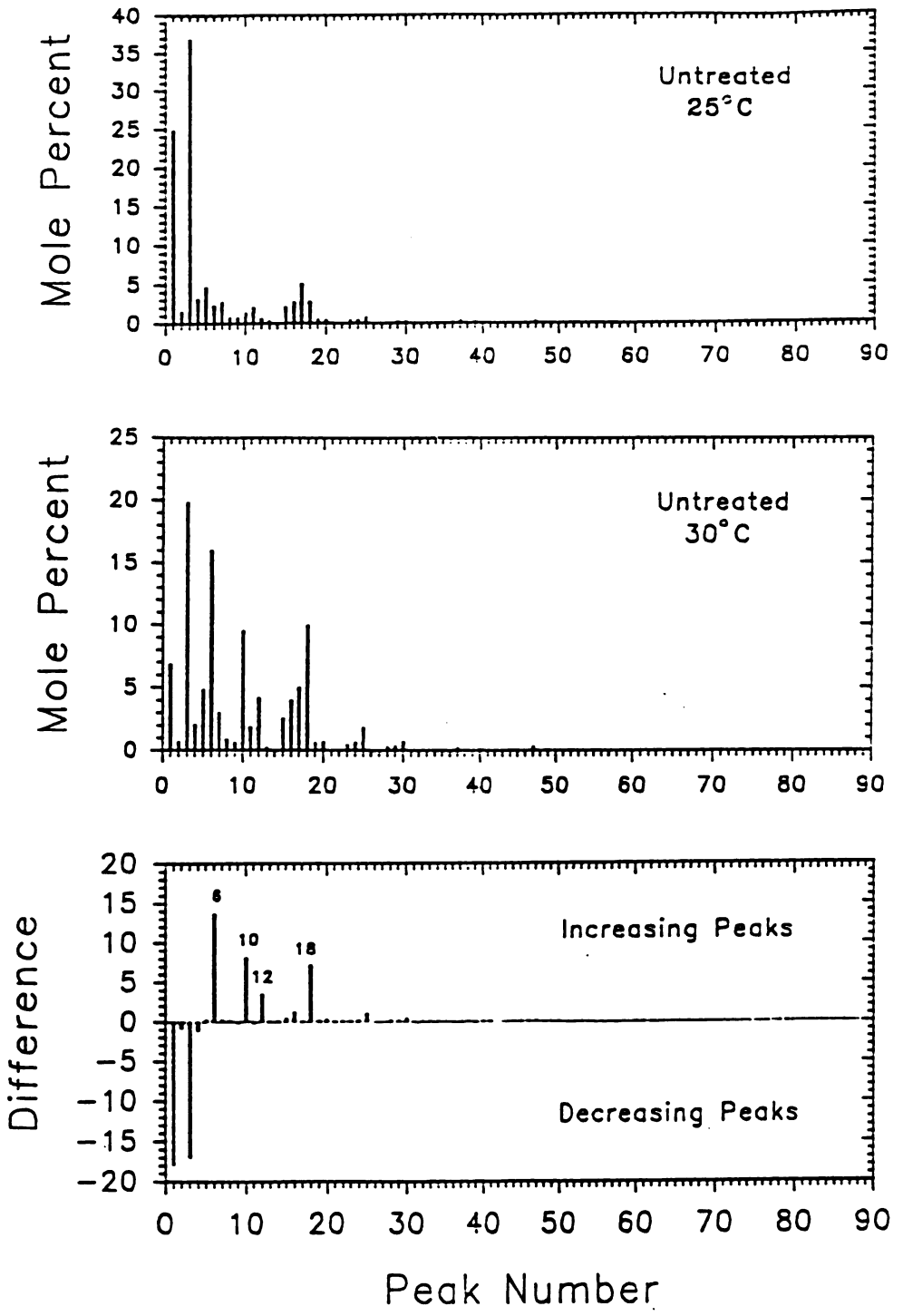

Fig. 4. Mole percentage of Aroclor 1242 represented by each chromatographic peak after 12 weeks incubation with the untreated cultures at 30°C and 25°C. For peak assignments, see reference (13).

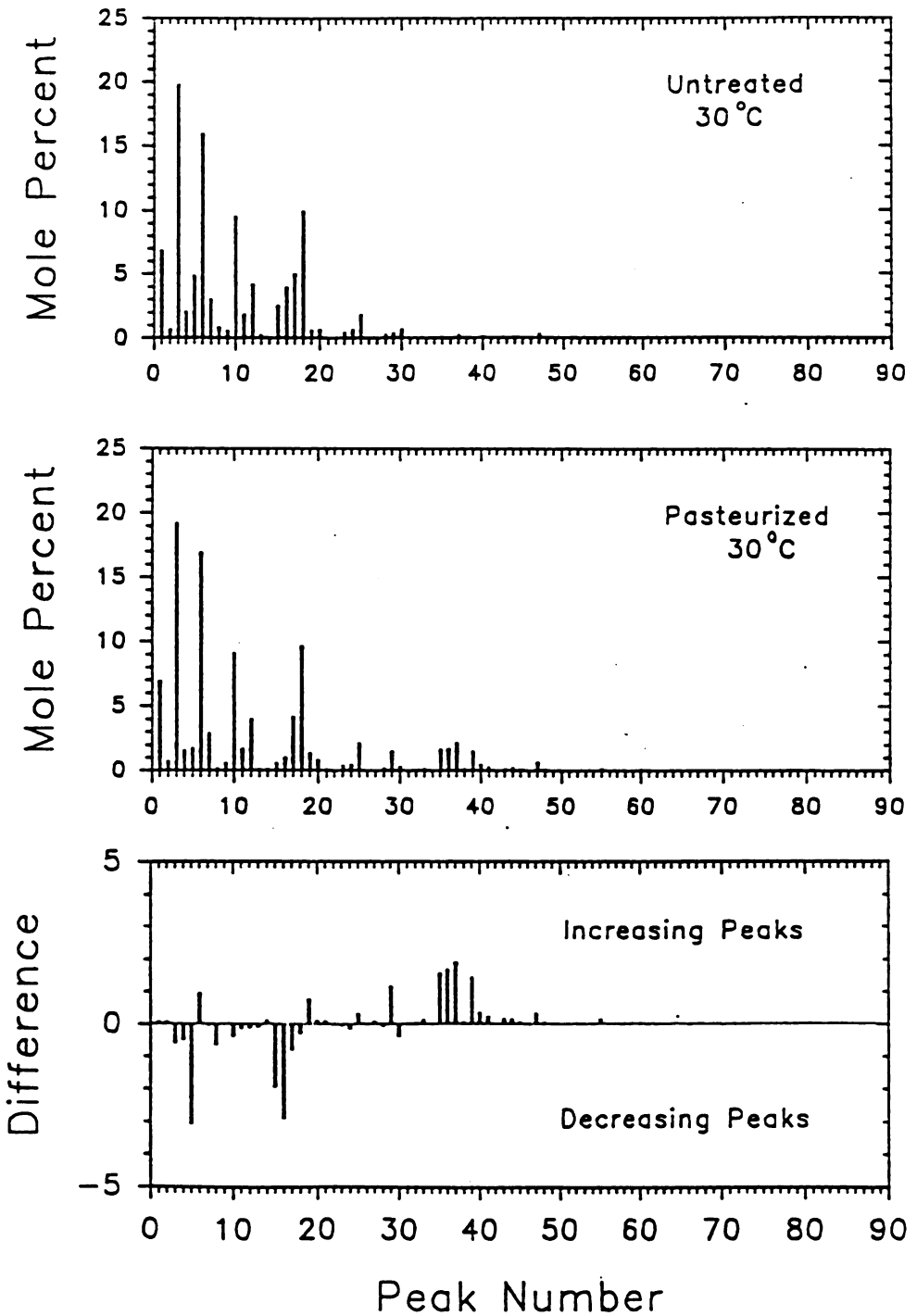

Fig. 5. Mole percentage of Aroclor 1242 represented by each chromatographic peak after incubation at 30°C for 12 weeks with untreated and pasteurized cultures. For peak assignments, see reference (13).

cultures are illustrated in Fig. 3.

Differences in the dechlorination patterns at 25°C and 30°C. There was no difference in dechlorination pattern shown by the pasteurized cultures at 25 or 30°C. However, when incubated at 30°C, the untreated cultures lost some dechlorination activity on 2-4-CB/2,3-CB (peak 6), 2,4-2-CB(peak 10), 2,6-4-CB (peak 12), and 2,4-4-CB (peak 18). As shown in Fig 4, comparison between untreated cultures incubated at 25°C and 30°C showed that the 30° cultures accumulated more congeners associated with peaks 6, 10, 12, and 18, and less congeners associated with peaks 1 (2-CB) and peak 3 (2-2/2-6-CB), which are the *para* dechlorination products of peaks 6, 10, 12, and 18. The congeners associated with peaks 6, 10, 12, and 18 are *para*-substituted without *meta* chlorines and with at least one *ortho* chlorine attached to the biphenyl rings. Despite the loss of this *para* dechlorination activity, however, 0.24 more chlorines were removed from each biphenyl molecule after 4 weeks when the untreated cultures were incubated at 30°C than when they were incubated at 25°C (Fig. 1A). Similar enhancement effect was also observed with the pasteurized cultures (Fig. 1B). There was a difference between the dechlorination extension after 8 weeks of incubation between at 25 and at 30°C by the untreated cultures (Fig. 1A), this was due to losing the *para* dechlorination activity at 30°C.

Differences in the 30°C dechlorination patterns between pasteurized and untreated cultures. Comparison of the 30°C dechlorination patterns between the pasteurized and the untreated cultures showed that the pasteurized cultures lost some dechlorination activities on congener peaks 35 (2,4,5-4-CB), 36 (2,5-3,4-CB), 37 (2,4-3,4-CB/2,4,5-2,6-CB/2,3,6-2,5-CB), 39 (2,3-3,4-CB/2,3,4-4-CB), 19 (3,4-2-CB/2,3,4-

CB/2,3-3-CB/2,5-2,6-CB), and 29 (2,3-2,4-CB/2,3,6-3-CB/3,4-4-CB) (Fig.5). These are all *para*-substituted congeners with *meta* chlorines.

Differences in the 25°C dechlorination patterns between pasteurized and untreated cultures. We previously reported the differences in the dechlorination patterns between the untreated and the pasteurized cultures at 25°C (20), and in the present experiment identical results were obtained. After 12 weeks of incubation, the untreated cultures showed both *meta* and *para* dechlorination activities while the pasteurized cultures lost some *para* dechlorination activity and thus, showed a *meta*-preferential dechlorination activity. The *para* dechlorination activities lost from the pasteurized cultures were actually the activities lost from the untreated cultures when temperature changed from 25°C to 30°C, plus those *para* activities remaining at 30°C but eliminated by not being able to withstand the pasteurization

Decrease in the lag time of the dechlorination by heat shock. In our previous experiment (the July experiment mentioned in Chapter 3) decrease in the lag time of the dechlorination by the heated cultures was observed (Fig. 6). After 2 weeks of incubation dechlorination occurred in the pasteurized cultures but not the untreated cultures.

Timecourse of different dechlorination activities. Analysis on the dechlorination patterns showed that during 4 weeks of incubation with either pasteurized or untreated cultures, only *meta* dechlorination activity was observed and this dechlorination was a rapid processes. The two kinds of *para* dechlorination activities were observed in the untreated cultures only after 4 weeks of incubation, and reached the maximum at 12 weeks.

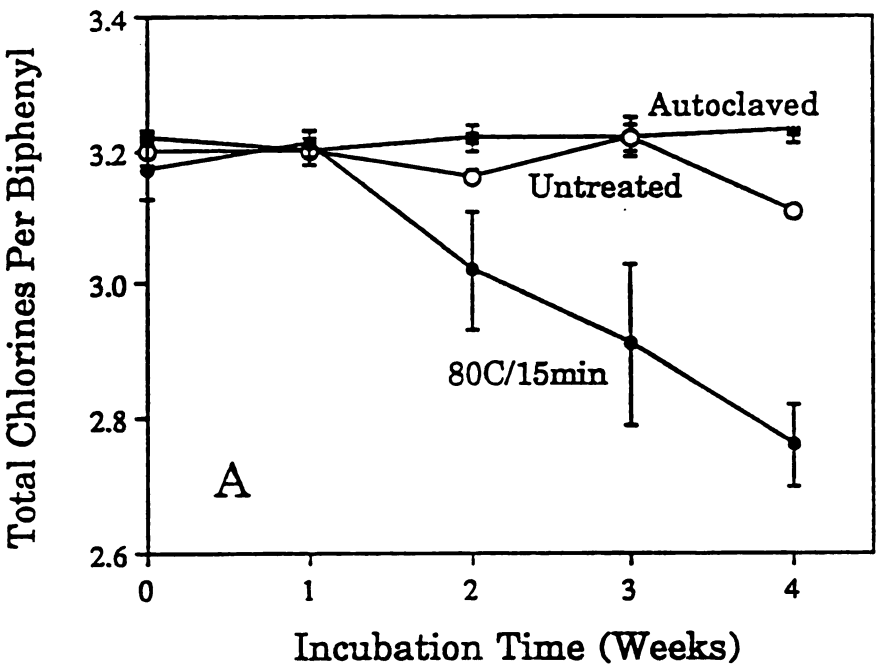

Fig. 6. Dechlorination of Aroclor 1242 by microorganisms from Hudson River sediments. Error bars are the SD of triplicate samples; where not shown, the error bars are smaller than the symbols.

DISCUSSION

No dechlorination of Aroclor 1242 by microorganisms from Hudson River sediments was observed at temperatures of 37°C and above, consistent with results reported by Tiedje et al. (16) but in contrast to those of Wu et al. (19) for the dechlorination of 2,3,4,6-CB in methanogenic pond (Wood Pond, Lenox, Mass.) sediment. It appears that the microorganisms capable of 2,3,4,6-CB dechlorination at thermophilic temperatures reported by Wu et al. (19) were not present in the Hudson River inocula used in our experiments.

Two classes of dechlorination activities on Aroclor 1242 in the Hudson River sediments have been reported previously (2). In the present study, comparison of the dechlorination patterns between the pasteurized and the untreated cultures at different temperatures showed three classes of dechlorination activity on Aroclor 1242 by microorganisms from the Hudson River sediments. The previously (2) recognized *para* dechlorination activity (Pattern Q) actually consists of two activities: one on *para*-substituted congeners without *meta* chorines and with at least one *ortho* chlorine attached to the biphenyl ring (designated as p_o activity), and the other on *para* chlorines from congeners containing *meta* chlorines (p_m activity). The dechlorination activities exhibited by the untreated cultures incubated at 30°C is actually composed of both the *meta*-preferential dechlorination activity (m_t activity) and the p_m activity.

Both the p_o and the p_m dechlorination activities could not survive the pasteurization, however, the p_o activity was more unstable than the p_m activity as by the observation that it was lost from the untreated cultures when incubated at 30°C. A temperature increase from 25°C to 30°C may directly inhibit the microorganisms responsible for the p_o

activity. However, competition between the microorganisms may be a more reasonable explanation. Microbial metabolic rate changes as temperature increases, and those more stimulated by the increasing temperature may out compete more slow-growing populations. This is especially in nutrient-limiting cases like our culture conditions in which the medium contains no substrate except the limited sediment organic matter.

In our previous experiment (20), and here again, we observed that the *para* dechlorination activity of the inoculum changed with time; as the inoculum aged, some *para* (mainly the p_o) dechlorination activities was lost (Fig. 7). It was also noted that timely transfers significantly increased the *para* (mainly the p_o) dechlorination activities (Fig. 8). Both observations support the idea that the p_o activity is nutrient-dependent. This is further supported by the effects of preincubation on dechlorination. We have observed that non-preincubated cultures showed all three dechlorination activities (Pattern C) (2), while those preincubated lost the p_o activity probably because during the preincubation procedure some nutrients in the medium were consumed (unpublished data). These results not only suggest that the p_o activity was nutrient-dependent, but also that the relationship between microorganisms capable of the m_t and the p_o dechlorination is most likely competitive. Abramowitz et al. (2) reported that addition of cysteine hydrochloride at 1 mg/liter resulted in *para*-dominated dechlorination activity (pattern Q). The exact cause of this effect is unclear but it does provide additional evidence that nutrients play a key role in determining the expression of dechlorination activities. Considering these points it seems reasonable that at 30°C the p_o activity was lost from the untreated cultures because microorganisms responsible for this activity lost competition for nutrients.

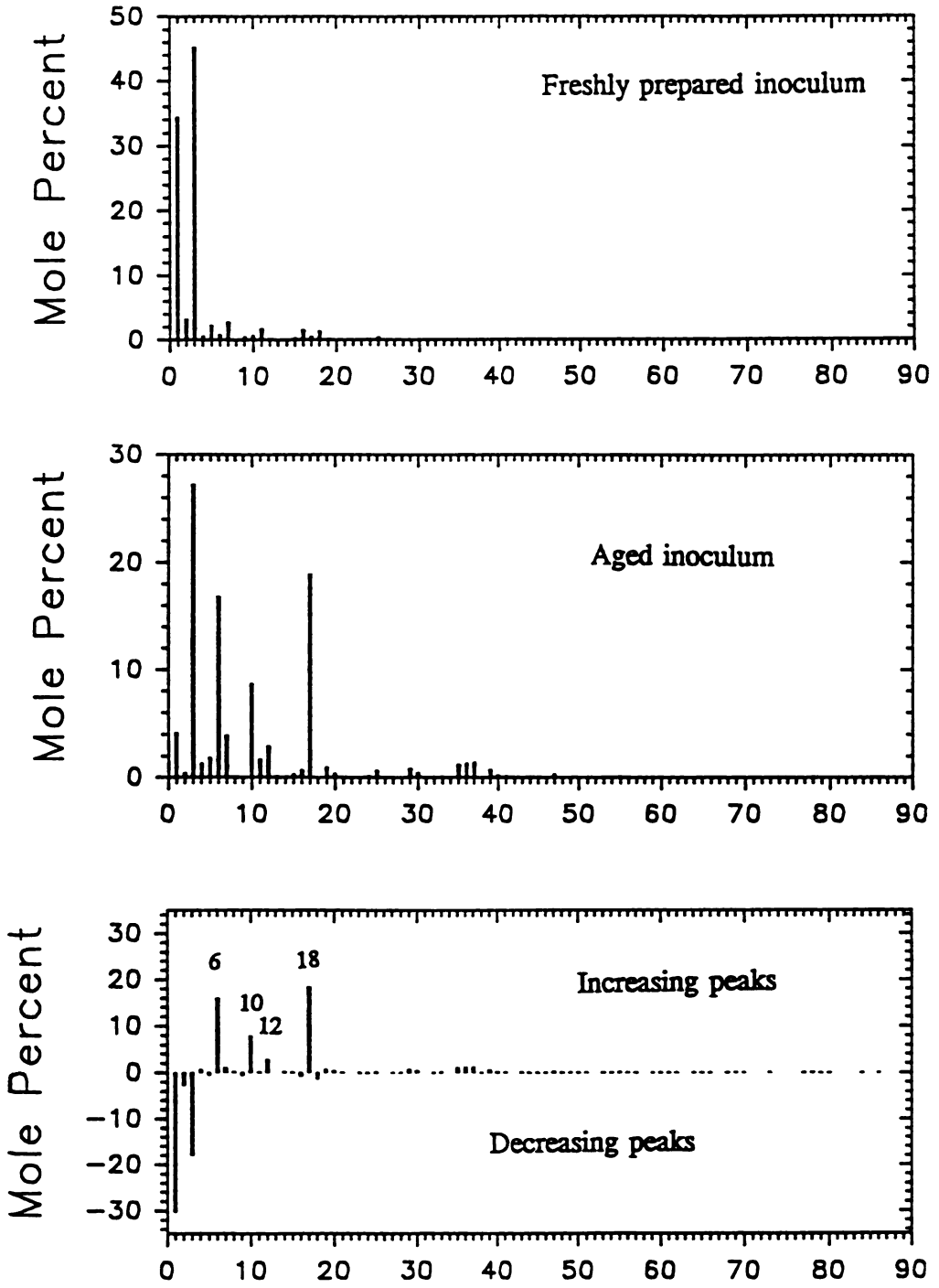

Fig. 7. Mole percentage of Aroclor 1242 represented by each chromatographic peak after 12 weeks incubation at 25°C with freshly prepared and aged Hudson River inoculum. For peak assignments, see reference (13).

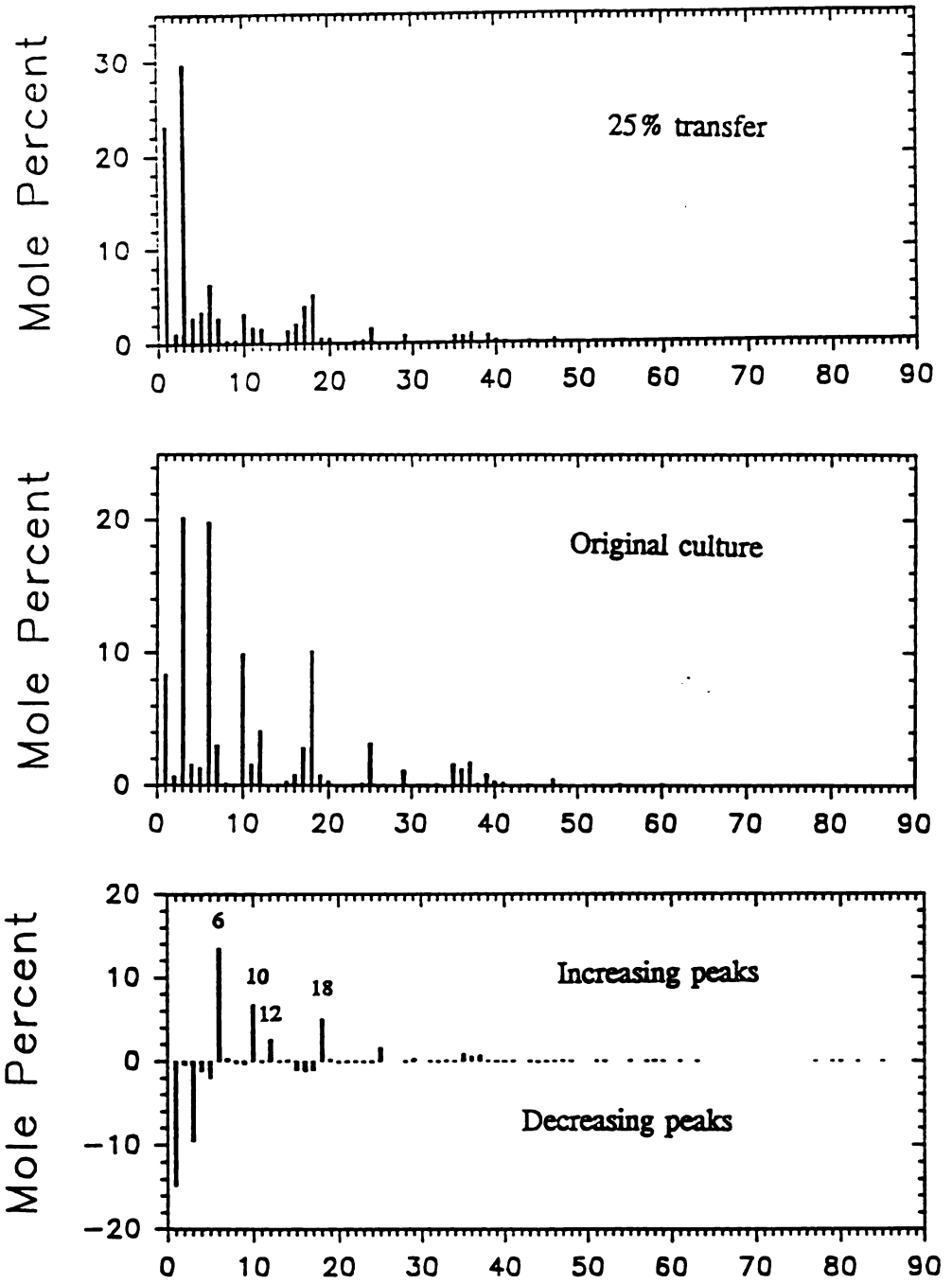

Fig. 8. Mole percentage of Aroclor 1242 represented by each chromatographic peak after 12 weeks incubation at 25°C with original cultures and the transferred (25%) cultures. For peak assignments, see reference (13).

In contrast to the unstable p_o dechlorination activity, the m_t activity was not only resistant to the heat- and ethanol-treatments, but also stable over time and through serial transfers (20). Moreover, after being heated at 85°C for 15 min, the m_t activity was not eliminated, instead, it was stimulated. There are two possible explanations: i) heat eliminated some competitors of the dechlorinating microorganisms, and ii) heat stimulated spore germination. The first possibility is not likely as evidenced by the following facts: first, such stimulative effect was never observed in the ethanol-treated cultures (including same batch). Microorganisms eliminated by heat should also be eliminated by the ethanol-treatment, and if the stimulative effect was due to elimination of competing microorganisms, in the same experimental batch the stimulative effect should also be observed in the ethanol-treated cultures. Second, the activation effect varied from one inoculum batch to another, probably depending on whether most sporeforming bacteria in the inoculum were in the form of vegetative cells or bacteria spores. It appears that the stimulative effect is probably because that heat shock stimulates spore germination (14). The activation results provided additional evidences supporting the previous conclusion (20) that anaerobic sporeformers are among the physiological groups responsible for the *meta*-preferential dechlorination activity in the pasteurized cultures. Morris et al. (12) also suggested that *meta* dechlorinating microorganisms may be more robust or more broadly distributed among microbial groups.

The p_m dechlorination activity was very similar to the dechlorination activity expressed by our methanogenic enrichment. This dechlorination is very close to the environmental alteration process H seen in the tidal Hudson (7, 7a).

While the p_o activity was eliminated when cultures were incubated at 30°C, the rates of both the m_t and the p_m dechlorination accelerated when temperature increased from 25°C to 30°C. In general, higher temperatures that do not kill the microorganisms result in higher metabolic activities (3). Enzymes usually have Q_{10} value near 2 (3), that is, when temperature increased 10°C within the tolerance range, the activity is roughly doubled. As to microbial population, for example, it was reported that sulfate reducers in marsh sediment had a Q_{10} of 3.5 for sulfate reduction (1).

At 30°C enhancement of the m_t activity did not suppress the p_m activity in our nutrient-limiting cultural conditions, suggesting that the microorganisms involved in these two dechlorination activities either had different nutrient requirements, or had a somewhat cooperation relationship. It is well known that methanogens depend on fermentative bacteria for substrates, and that the consumption of the fermentation products by methanogens is beneficial, or even necessary for fermentative bacteria (4, 5).

When both *meta* and *para* dechlorination expressed, *meta* dechlorination activity always occurred first. Compared with the two *para* activities, the m_t dechlorination process usually completed within a short time, suggesting that this dechlorination is a rapid process. Boyle et al. (6) also reported a strict *meta* dechlorination of 2,3,6-CB by the cultures enriched from Hudson River sediments and concluded that it was a rapid dechlorination. There are three possible explanations for the different timecourse between the *meta* and *para* dechlorination activities. (i) The *para*-removal microorganisms were relatively slow-growing, while microorganisms responsible for the m_t activity were fast-growing. (ii) The initial population size of the *para*-removal

microorganisms was smaller, and hence it took time to increase the population size to a threshold level necessary for detectable activity, while microorganisms comprising the m_t activity were dominant in the inoculum. (iii) accumulation of *para*-substituted congeners from the *meta* dechlorination induced the production of the *para*-dechlorination enzyme(s). However, the *para*-dominant dechlorination activity (pattern Q) indicated that accumulation of *para*-substituted congeners was not required for the *para* dechlorination activity, and thus ruled out this possibility. It seems that both the first and the second explanations are possible.

Tiedje et al. reported dechlorination at 12°C. Wu et al. also observed dechlorination of 2,3,4,6-CB starting from 4°C. In this experiment those cultures incubated at 10°C showed little dechlorination activity within 16 weeks. Prolonged incubation time may be needed at these lower temperatures.

Results of the temperature experiment demonstrated and partially characterized three anaerobic PCB dechlorination activities by microorganisms from Hudson River sediments. These results indicate that isolation of different microorganisms capable of anaerobic PCB dechlorination and enhancement of different *in situ* microbial dechlorination activities are possible.

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SUMMARY

Addition of molybdate, BESA, and sulfate inhibited concomitantly PCB dechlorination and methane formation, suggesting that either sulfate-reducing bacteria or methanogenic bacteria, or both, were involved in anaerobic PCB dechlorination.

Further investigations were then conducted to examine whether methanogenic bacteria are capable of PCB dechlorination. The results showed that methanogenic bacteria are probably among the physiological groups capable of anaerobic PCB dechlorination. This conclusion is based on the following observations: (i) In sediments containing Aroclor 1242, addition of eubacteria-inhibiting antibiotics, which should directly inhibit fermentative bacteria and thereby indirectly inhibit methanogens, resulted in no dechlorination activity nor methane production. However, when substrates for methanogenic bacteria were provided along with the antibiotics (to free methanogens from dependence on eubacteria), concomitant methane production and dechlorination of PCB's were observed. (ii) Both methane production and dechlorination in the culture amended with antibiotics plus methanogenic substrates were inhibited by BESA, the specific inhibitor of methanogenic bacteria. The inhibitory effects of BESA on the dechlorination was due to the direct inhibition on methanogens because BESA at the experimental concentration did not inhibit PCB dechlorination by other microorganisms.

The dechlorination of Aroclor 1242 by the methanogenic culture was mainly *para*-removal, and was distinctly different from, and more limited than, that observed with untreated or pasteurized inocula. This dechlorination process was very similar to the

environmental dechlorination process H.

Anaerobic sporeformers are another physiological group responsible for the reductive dechlorination of PCBs. The pasteurized and ethanol-treated cultures retained a partial dechlorination activity, suggesting that the responsible microorganisms survived both pasteurization and the ethanol treatment. The dechlorinating microorganisms were neither methanogens nor thermophiles since no methane production was detected in any treated culture, and no dechlorination was observed when the cultures were incubated at 37°C and above. Additionally, the dechlorinating microorganisms withstood not only high temperatures but also 50% ethanol for 1 h, which should eliminate thermophiles. Besides the above results, the dechlorination activity of the treated cultures was stable in the inoculum over time and through serial transfers, indicating that the responsible microbial population was stably maintained, and this stability is consistent with the survival capability of sporeformers.

The treated cultures had a common dechlorination activity regardless of the type of treatment. This activity preferentially removed *meta* chlorines yielding a dechlorination pattern characterized by accumulation of certain *ortho*- and *para*-substituted congeners. In contrast, the untreated cultures showed more extensive dechlorination activities, which almost completely removed both *meta* and *para* chlorines from Aroclor 1242. These results suggest that microorganisms surviving the heat and ethanol treatments preferentially remove *meta* chlorines, while the *para* dechlorination activities are mainly contributed from microorganisms eliminated by such treatments.

The dechlorinating microorganisms in the pasteurized and ethanol-treated cultures are probably *Desulfotomaculum*-like sulfate-reducing bacteria. The dechlorination by the

pasteurized cultures was inhibited by both molybdate, BESA, and sulfate. As discussed in Chapter 4, it appeared that the inhibition was not due to general toxicity but due to the effects on sulfidogens. Molybdate probably directly inhibited sulfidogens, while both sulfate and BESA probably inhibited the dechlorination by competing with PCB as electron acceptors. Only sporeforming bacteria are expected to survive the harsh pasteurization, and among sulfidogens the only known genus forming spores is *Desulfotomaculum*. Therefore, microorganisms responsible for the dechlorination of PCBs in our pasteurized cultures are probably *Desulfotomaculum*-like sulfate-reducing bacteria. However, isolation of the microorganism(s) and demonstration of PCB dechlorination activity with the isolate(s) are required in order to make a final conclusion. On the other hand, anaerobic sporeformers are a diverse group, different enrichments may have different sporeforming populations depending on inoculum source, pasteurization technique, and culture conditions. The proposition that the dechlorinating microorganisms in our pasteurized cultures are *Desulfotomaculum*-like sulfate-reducers does not rule out the possibility that other sporeformer(s) may also have PCB dechlorination capability.

Temperature experiments demonstrated and partially characterized three classes of dechlorination activity. One was *meta*-preferential, while the other two were mainly *para*-removal. The major difference between the two *para* dechlorination activities is that one removes *para* chlorines from congeners without *meta* chlorines (the p_o activity) whereas the other mainly removes *para* chlorines from congeners containing *meta* chlorines (the p_m activity). The p_m activity is very similar to that exhibited by the methanogenic cultures, and this dechlorination process is very close to the environmental

dechlorination process H. In contrast to the stable *meta* dechlorination activity, both the p_o and the p_m activities could not survive the pasteurization. The p_o activity was more unstable than the p_m activity and appeared to be nutrient-dependent.

In presence of 3 ~ 5 mM sulfate, a distinct dechlorination activity characterized by the ability to attack 2,4-4-chlorobiphenyl (2,4-4-CB) and 2,4,6-2-CB was observed. This activity is part of the p_o activity.

Ortho dechlorination of 2-CB and 2-2-CB/2,6-CB by the transferred methanogenic cultures was observed, suggesting that the Hudson River contains microorganisms with the potential for complete dechlorination of PCBs to biphenyl.

The different dechlorination activities observed under different cultural conditions are summarized in Table 1. These observed dechlorination activities, together with other reported dechlorination activities, indicate that diverse microorganisms in Hudson River sediments may have the capability to remove chlorines from certain positions of specific PCB congeners. The presence or absence of these active microorganisms may determine the actual PCB-dechlorination pattern observed *in-situ*.

Table 1. The Observed Dechlorination Activities in This Study

Activity	Culture condition
remove *meta* chlorines ethanol	inocula treated with heat or/and
remove *para* chlorines with adjacent *meta* chlorines	methanogenic
mainly attack 2,4,4-CB/2,4,6,-2-CB	in presence of 3 ~ 5 mM sulfate
remove *ortho* chlorines from 2-CB, 2-2-CB/2-6-CB	methanogenic

Appendix

I. Effects of More Selective Pasteurization Treatments

1) Different pasteurization time (90°C)

The inoculum for the 1st serial culture was prepared with the upper Hudson River sediment, was originally pasteurized at 85°C for 15 min and then was serially transferred using a 1% inoculum. The inoculum for all subsequent transfers was re-pasteurized. The pasteurization temperature and time were changed from 85°C/15 min to 90°C/10 min from the 3rd serial transfer. A lag time for dechlorination activity of 4 weeks was observed in the 3rd serial culture, however, no such a lag time was observed (data not shown) in the all subsequent transfers. The inoculum from the 5th serial transfer was pasteurized at 90°C for 10, 15, 20, 25, and 30 min to increase selective pressure. The transfers heated for 10, 15, and 20 min retained the dechlorination activity. In contrast, no dechlorination was observed in those cultures heated for 25 and 30 min even after 6 months of incubation (Fig. 1).

Besides the cultures heated at 85°C for 15 min in the 1st serial culture observed above, there were other two experimental groups, one was pasteurized at 90°C for 10 min, the other was pasteurized at 100°C for 10 min. Only one of the 90°C-treated triplicates showed dechlorination activity, and that was after an 8 week lag time (data not shown). The more active PCB dechlorination by the 5th serial culture compared to the 1st serial culture indicates that the heat-resistant dechlorinating microorganisms were enriched through the serial transfers.

2) Pasteurization at temperatures above 90°C

In one batch of the BESA experiments reported in Chapter 1, one of the triplicate autoclaved controls showed dechlorination activity (Fig. 2). After the contamination was

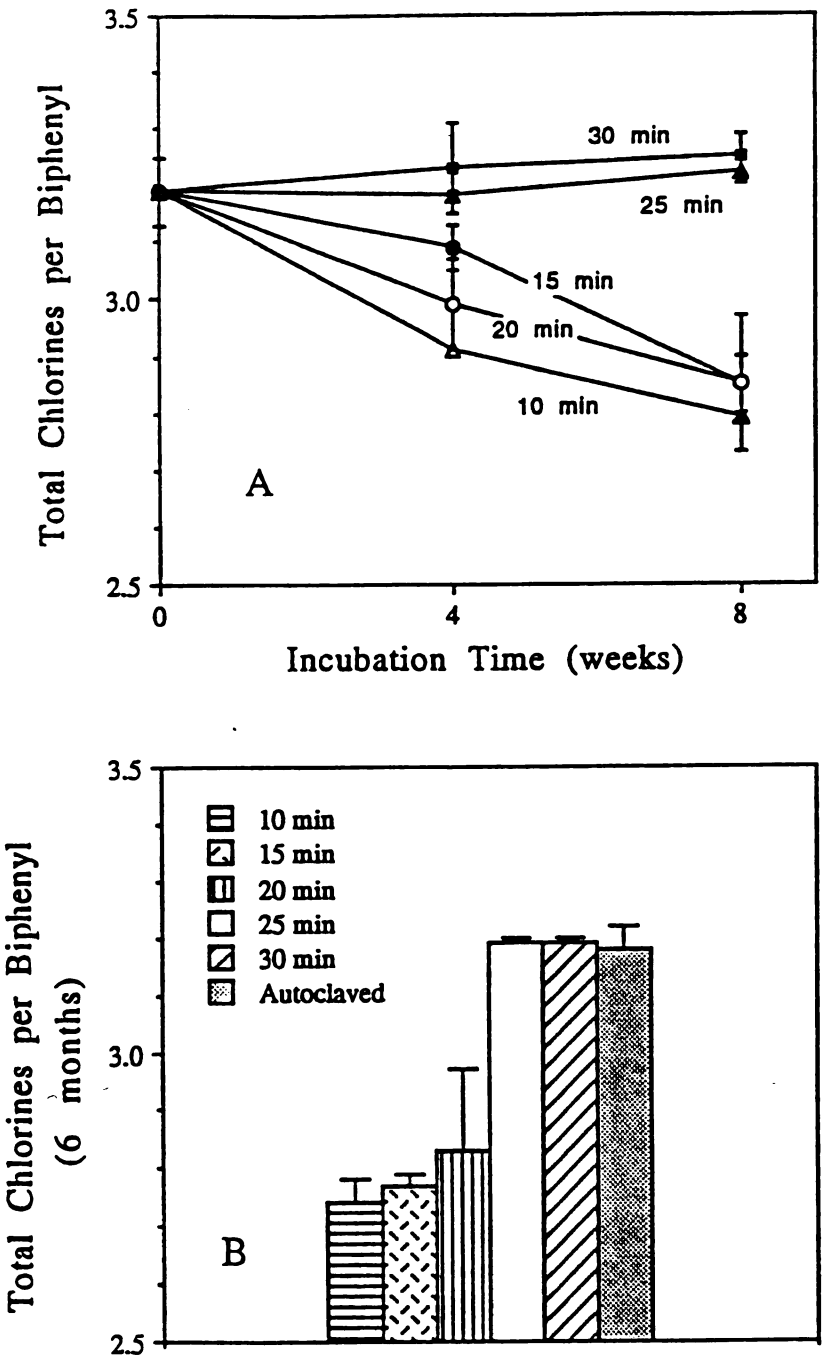

Fig. 1. Dechlorination of Aroclor 1242 by microorganisms pasteurized at 90°C for different time after (A) 4 and 8 weeks, and (B) 6 months of incubation. Error bars are the standard derivation (SD) of duplicate samples; where not shown, the error bars are smaller than the symbols.

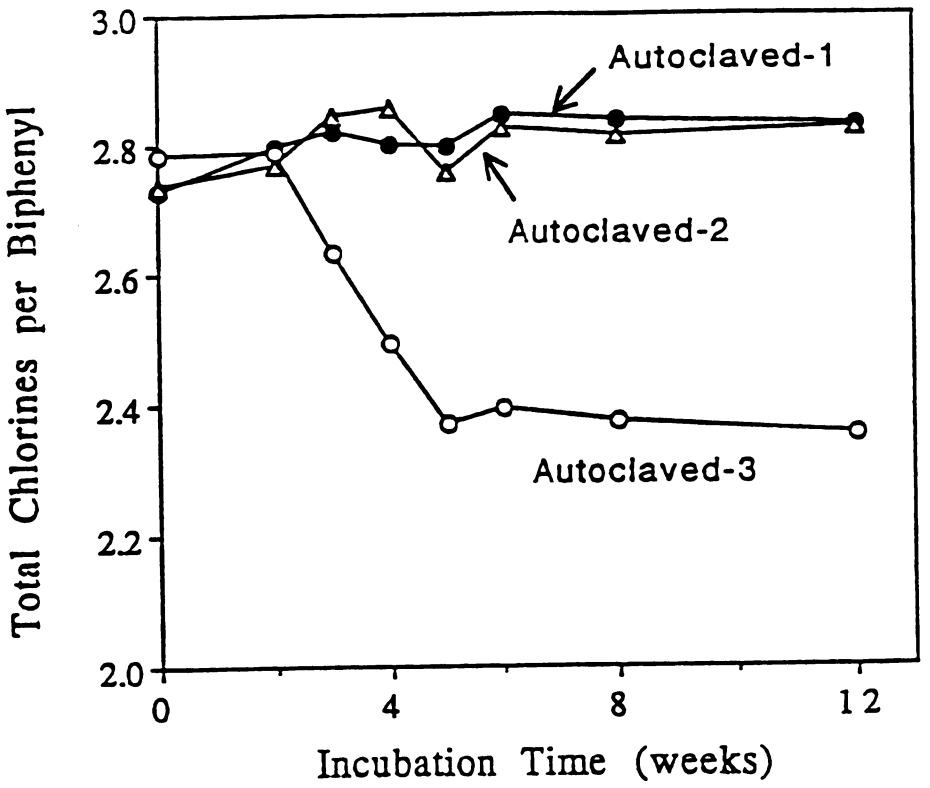

Fig. 2. Dechlorination of Aroclor 1242 by autoclaved microorganisms eluted from the Hudson River sediments. The experimental vessels were 160-ml serum bottles containing 25 g PCB-free sediments and 75 ml RAMM with microorganisms. The serum bottles were autoclaved at 121°C for 1 hour.

carefully examined and ruled out, this unexpected result suggested that some dechlorinating microorganism(s) was(were) particularly heat-resistant. It was this result that prompted me that initiated the pasteurization and ethanol-treatment experiments. Some details of the above-mentioned controls are as follows: The autoclaved controls were 160-ml serum bottles containing 25 g PCB-free Hudson River sediment, 75 ml RAMM (7) with eluted microorganisms, and 50 μl of 10% (wt/vol) Aroclor 1242 in acetone. These controls were part of an experiments to study the on-set dechlorination, and therefore, had been incubated for 4 weeks, and were autoclaved after dechlorination was confirmed. Then 100 μl of 10% Aroclor 1242 in acetone was re-spiked to each culture. The cultures were then incubated at 25°C in the dark, with this time designated as 0 time. Despite the fact that the controls had been autoclaved (121°C) for 1 hour, after an additional 3 weeks of incubation, dechlorination was observed in one of the triplicates. (Fig. 2) (this experiment was set up in March, 1989, data of 0 time and 5 weeks were summarized in May, 8 weeks in June).

The active culture was then transferred. The inoculum concentration was 1/3 to repeat the original inoculum size (1/3 of the total liquid portion). Inoculum from this culture was split and re-heated at 100°C, and 121°C, respectively, for 10 min. The pasteurization was performed in 28-ml serum tubes instead of 160-ml serum bottles as previously described. No dechlorination was observed during 12 weeks of incubation. After 4 months of incubation, one of the duplicate 100°C-heated cultures showed dechlorination activity (Fig. 3). Since the cultures were not sampled between 12 weeks and 4 months, no information about the lag time after 12 weeks was obtained, nevertheless the dechlorination occurred sometime between 12 weeks and 4 months.

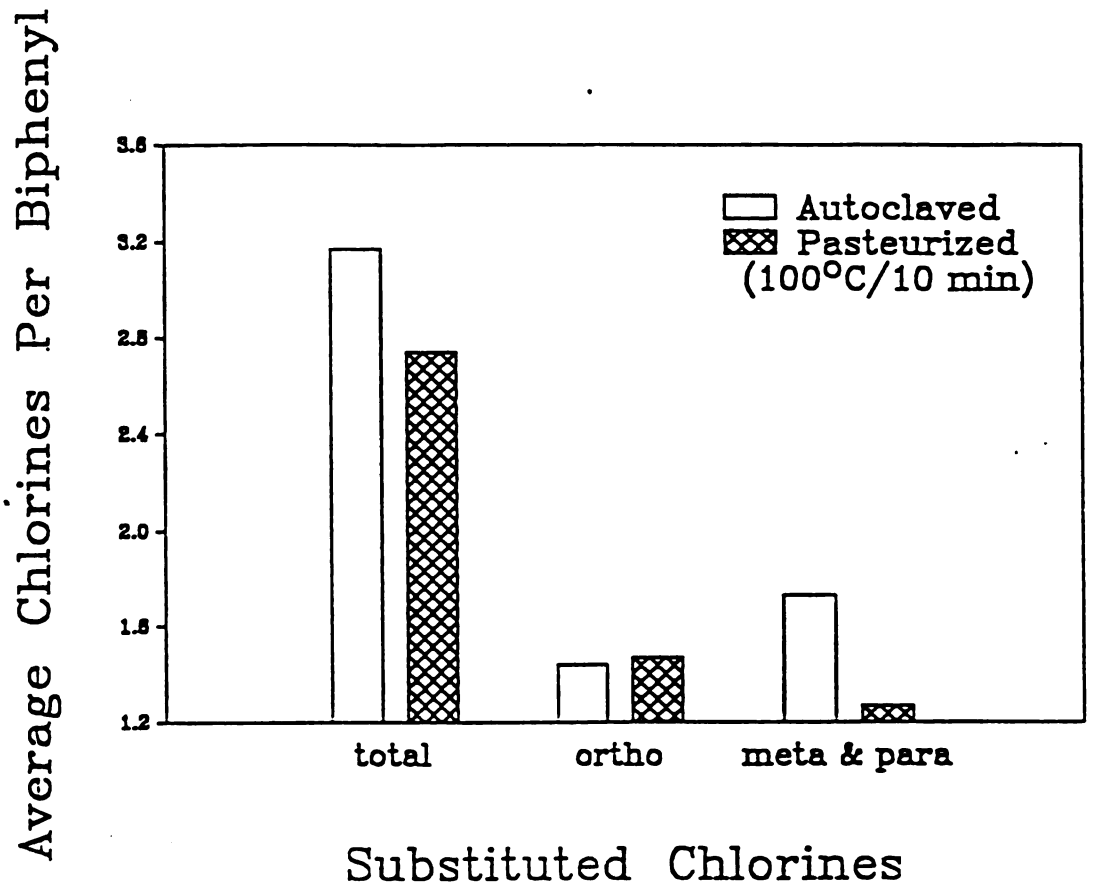

Fig. 3. Dechlorination of Aroclor 1242 by microorganisms transferred from the culture retaining the dechlorination activity after autoclaving (see Fig. 2). The inoculum was re-pasteurized at 100°C for 10 min upon transfer, and the pasteurization was performed with a 28-ml serum tube (refer to reference 8).

Effects of pasteurization temperature on the 1st and the 2nd serial cultures were different. The 1st serial cultures were heated at 121°C for 1 h and one of the triplicates still retained the dechlorination activity, and the lag time was only 3 weeks. However, those 2nd serial cultures re-pasteurized at 121°C for 10 min lost dechlorination activity, and only one of the two 2nd serial cultures re-pasteurized at 100°C for 10 min retained the dechlorination activity with a lag time longer than 12 weeks. The difference might be because: (i) the 1 serial cultures were heated in 160-ml serum bottles, while the inoculum for the 2nd serial cultures was heated in 28-ml serum tubes, (ii) the 1st serial cultures were heated in presence of sediments, however, for the 2nd serial cultures only the supernatant from the 1st serial culture was taken as the inoculum and only this inoculum, not the whole culture, was pasteurized, (iii) the status of the heat-resistant microorganisms in the 1st and the 2nd serial cultures might be different (vegetative cells or spores).

It is unclear whether pasteurization of inoculum in the 28-ml serum tubes or in the 160-ml serum bottles is important, or pasteurization of the inoculum with presence or absence of sediment is important. However, pasteurization with the serum tubes, and without presence of sediment should be more effective.

3) **Serial dilution experiment**

Inoculum taken from the 4 serial cultures was re-pasteurized at 90°C for 10 min, and was diluted. Figure 4 shows the dechlorination after 4 weeks. After 8 weeks of incubation, dechlorination occurred in one of the triplicates of the 10^{-6} dilution (Table 1). No dechlorination was observed in a dilution higher than 10^{-6} as measured at 6 months (Table 2). HPLC analysis showed that in both dechlorination-positive and

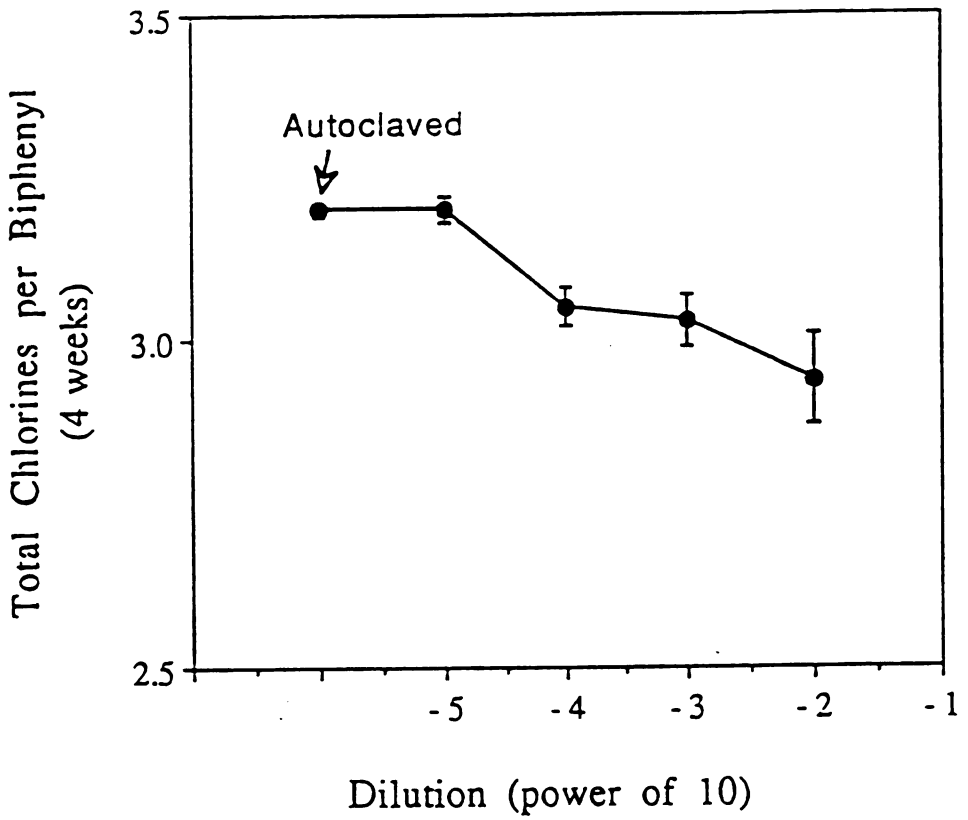

Fig. 4. Results of dilution experiment after 4 weeks of incubation. Error bars are the standard derivation (SD) of triplicate samples; where not shown, the error bars are smaller than the symbols.

Table 1. Results of the Dilution Experiment (8 weeks)
(total chlorines per biphenyl)

	Power of 10						
# of triplicates	-2	-3	-4	-5	-6	-7	-8
1	2.81	2.87	2.94	2.99	3.21	3.24	3.24
2	2.98	3.03	2.88	3.23	3.26	3.28	3.27
3	3.00	2.98	2.93	3.23	2.93*	3.23	3.23

Table 2. Results of the Dilution Experiment (6 months)
(total chlorines per biphenyl)

	Power of 10			
# of triplicates	-5	-6	-7	-8
1	2.90	3.18	3.20	3.20
2	3.11	3.19	3.18	3.18
3	3.16	2.75*	3.21	3.21

* Dechlorination ocurred

negative cultures, the major metabolites were butyrate and acetate. There was no significant difference in the amount of the metabolites between the positive and negative cultures.

In preparation of this experiment, 3 ml inoculum was mixed with 3 ml of 0.9% NaCl containing 1 mM $Na_2S.9H_2O$ and 0.0001% of resazurin. After re-pasteurization, the inoculum was serially diluted 10-fold and 0.6 ml of the diluted inoculum was introduced into each 60-ml serum bottle containing 29.4 ml RAMM to make an 1% transfer.

The number of the microorganisms in the pasteurized inoculum was counted by the Most-Probable-Number (MPN) method with a mixed medium of RAMM and AC medium (10); and the ratio of RAMM to AC was 4:1. Each tube was inoculated with 0.1 ml inoculum. The MPN result was as follows (12 weeks):

Transfer (power of 10)	-2	-3	-4	-5	-6	-7	-8	-9	-10	-11
Dilution (power of 10)	0	1	2	3	4	5	6	7	8	9
positive:	5	5	5	5	5	4	2	0	0	0

From the data, a population of 1.32×10^{-8} organisms was calculated for the original inoculum, i.e. 10^{-6}, theoretically 132 microorganisms were present. Since only 1 of 3 of the cultures was positive, this is rear the end point of dechlorination active members. These numbers only give a roughly idea, because some microorganisms might not grow in this medium. The reason to replace sediments with AC medium is to

facilitate determination of growth. These results also imply that the dominant strains are not the active dechlorinators, and therefore this approach will not easily yield a pure strain capable of PCB dechlorination.

II. Attempts to Stimulate PCB Dechlorination by Spore Germinants

Effects of spore germinants on dechlorination was investigated. L-alanine and a variety of alanine analogues are common germinants, while D-alanine is the inhibitor of L-alanine (4). In this experiment, both L-alanine and D-alanine were introduced at concentration of 2 mM. D-alanine was used either as a negative control (amendment of D-alanine only), or as a competitive inhibitor (amendment of L-alanine plus D-alanine). Another group was the heat-treated group (which was heated at 80°C for 15 min); was used as a positive control since heat stimulates spore germination.

No difference in the lag time among different groups was observed (data not shown), indicating that most spore-forming bacteria in the inoculum may have already been present as vegetative cells instead of bacterial spores. This conjecture is supported by the fact that in previous experiments a difference in lag time between the heated and untreated cultures was observed (see Chapter 5). However, no difference in these two groups in lag time was seen this time. This may have been due to the fact that two weeks before preparing this experimental batch, some inoculum was taken for another experiment. At this time some RAMM was added to the stored sediments. It is known that fresh medium stimulates spore germination (3, 4).

To investigate the effects of a germinant, addition of the germinant directly to the sediments is recommended. However, whether the presence of sediment may alter the

effects of the germinants is unclear. Another alternative approach way is to introduce sporulant to the inoculum and wait for some time to allow sporulation occur before the inoculum is used.

III. Isolation and Testing Sporeformers

Microorganisms from the pasteurized cultures were isolated on different media. All these media contained RAMM and sediment extract (either pre-incubated (8) or not) with different modifications. The modifications included addition of the following media, carbon or energy sources, or growth factors at different low concentrations: AC medium, reinforced clostridium medium, VL medium, cooked meat medium, blood agar base, trypticase, yeast extract, malt extract, lactate, H_2. all media were from Difco (9). These were added either separately or as a combination of two or three items in different proportions. For some cultures, 500 ppm of Aroclor 1242 was also incorporated.

The inocula used were from the following cultures: Those pasteurized at 90°C for 20 min (Section I.1), the positive culture of the 10^{-6} dilution (Section I.3), and the culture pasteurized at 100°C for 10 min (Section I.2). Roll tubes, anaerobic agar plates, and deep agar tubes were employed.

After the colonies appeared, colonies of different morphologies were picked. Microorganisms from similar morphological colonies were examined under microscope, and those colonies containing different cell morphologies were also picked. About 140 isolates from different experimental batches were tested for the dechlorination activity. For this, the isolates were inoculated into 60-ml serum bottles containing 10 g preincubated PCB-free Hudson River sediment, 30 ml RAMM, 0.15 ml cysteine (final

Table 3. Results of the Isolates Experiment

Samples	Number of Isolates	Isolation medium	Average Cl^- per biphenyl
Autoclaved			3.23
Mix-A	5	RAMM, SE*, H_2, AC	2.79
Mix-B	4	RAMM, SE, H_2, RC**	2.73
MIx-C	6	RAMM, SE, H_2, RC	2.79
Mix-D	5	RAMM, SE, H_2, PCB	2.72

* Pre-incubated sediment extract.
** Reinforced *Clostridium* medium.

concentration was 330 mg/liter liquid), and 60 μl 10% Aroclor 1242 in acetone. No pure isolate showed dechlorination activity, however, some cultures with combined isolates retained the dechlorination capacity. Results are listed in Table 3. The results listed in table 3 are actually only one of many batches of the isolation experiments, positive results were obtained only in this batch.

The inoculum for this batch was from the 10^{-6} transfer of the dilution experiment.

Further isolation and purification are needed to finally isolate and identify either pure strain(s) or a minimum syntrophic consortium capable of anaerobic PCB dechlorination.

IV. Evaluation of PCB bioavailability

PCBs are nonionic organic compounds (NOCs). In soil and sediment they distribute mainly between soil solution and soil organic matter. It is generally believed that only the NOCs in soil solution would be accessible to microorganisms, those partitioned into soil organic matter would be unavailable because they are separated from the microorganisms by the organic bulk phase. Additionally, as time goes by, the NOCs in soil can diffuse to remote sites in the soil matrix, and become entrapped in micropores in soil aggregates, also influencing their bioavailability. The availability of NOCs to microorganisms is, therefore, controlled by both desorption and diffusion rates of NOCs, which is determined by both soil conditions and the nature of NOCs, that is, the partition coefficient and mobility. When either one of these rates is slower then the dechlorination rate, bioavailability will sooner or later be a limiting factor.

To investigate the bioavailability to microorganisms of PCBs in historically

contaminated soils, a bioavailability experiment was conducted. The investigated soils were a Glens Falls, New York, drag strip soil (GFDS soil) and Saginaw, Michigan, soil site 8RC 10-20 cm. Both soils are historically contaminated by PCBs; the GFDS soil is contaminated by Aroclor 1242.

The experiment was performed in two batches. One batch was set up with GFDS soil, the control PCB-free soil was from New Bedford Harbor, Mass., while the other batch was set up with Saginaw 8RC soil, and PCB-free Hudson River sediments served as control.

Since different soils have different physical and chemical conditions such as structure, PH, nutrients, sulfuroxy/nitroxy anions, heavy metals, et al., and all of these may effect dechlorination. Therefore, any difference in dechlorination rate and extent between the contaminated soil and the PCB-free soil can not be simply attributed to the bioavailability. In order to account for the effects from any factor other than bioavailability, both contaminated and the PCB-free soils were spiked with the same concentration of late eluting congeners, 2,3,4-3,4-CB and 2,3,4,5,6-CB (15 μg/g dry soil) as internal standards. Dechlorination of both historically contaminated and freshly added PCBs were first normalized by the standards (calculated as the ratio to the standards), and then the normalized rates were comparable.

There were two amendment groups for the historically contaminated soils. One was only spiked with the PCB congener standards (group B), while the other group (group A) received both the standards and Aroclor 1242 (300 μg/g dry soil, which is about the same concentration of PCBs as found for the historically contaminated PCBs).

The experimental results did demonstrate that the aged PCBs were less available

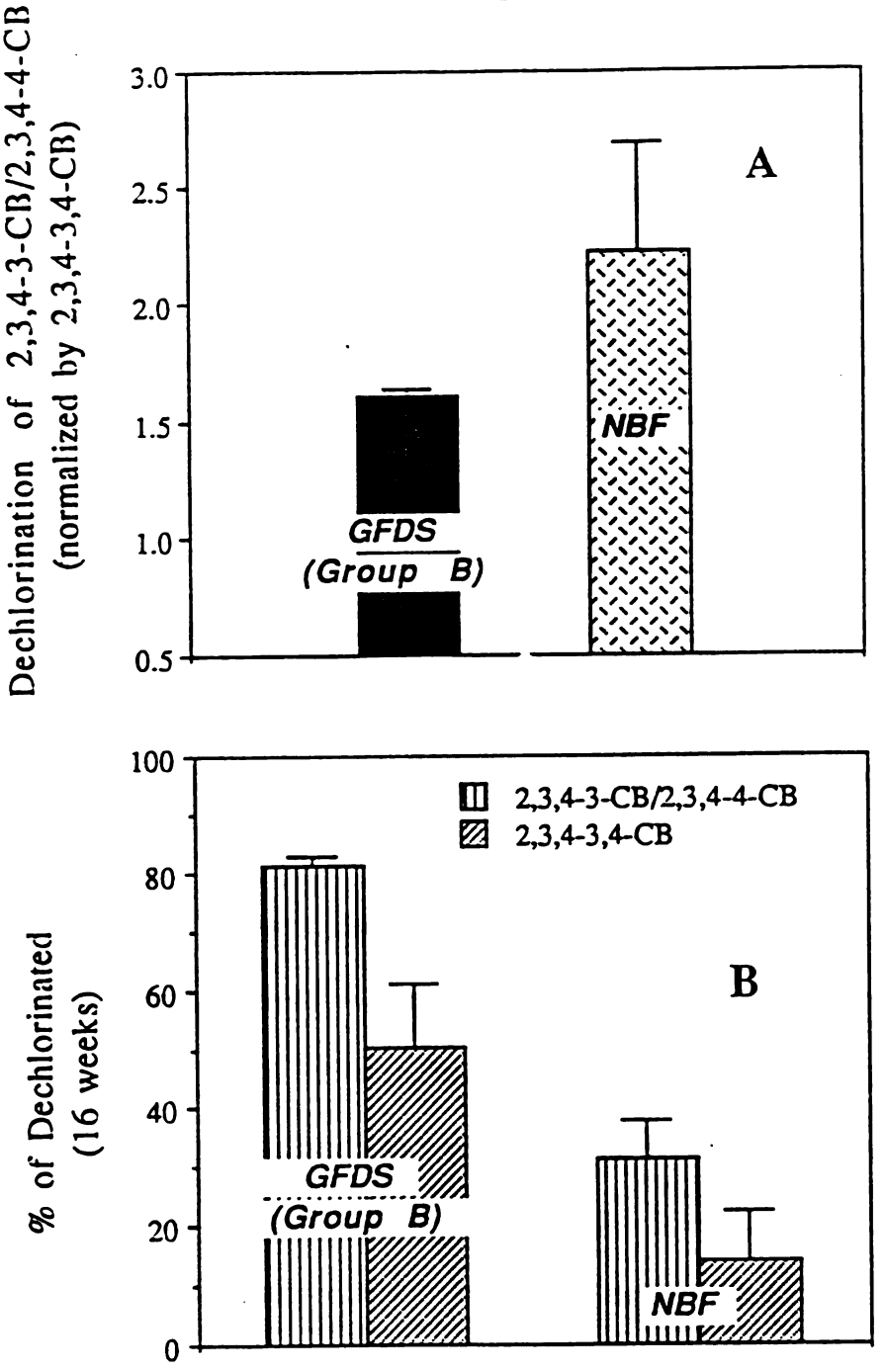

Fig. 5. Dechlorination of aged (GFDS soil, experimental group B) and freshly spiked (NBF soil) 2,3,4-3-CB and 2,3,4-4-CB by microorganisms from Hudson River sediments after 16 weeks of incubation. (A) normalized by 2,3,4-3,4-CB; (B) percentage dechlorinated. Error bars are the standard derivation (SD) of duplicate samples; where not shown, the error bars are smaller than the symbols.

than the freshly spiked ones. Figure 5A shows PCB dechlorination in both freshly spiked (NBF soil) and the historically contaminated (group B, GFDS soil) 2,3-3,4-CB/2,3,4-4-CB (coeluting congeners) normalized by 2,3,4-3,4-CB, as an example. Comparison between group A (with addition of fresh Aroclor 1242) and group B (without introducing fresh PCBs) in GFDS soil also showed that the amount of dechlorinated 2,3-3,4-CB/2,3,4-4-CB in group A is 1.56 time that in group B (data not shown).

On the other hand, despite of less availability of the aged PCBs, the overall dechlorination of the aged PCBs in GFDS soil was much greater than that of the fresh ones in NBF soil (Fig. 5B), suggesting that soil conditions are more important than bioavailability in determining dechlorination rate and extent. Additionally, comparison between group A and group B of the GFDS soil showed that the dechlorination amount of 2,3,4-3,4-CB (standard) in group A (with addition of fresh Aroclor 1242) was only about half that in group B (data not shown), despite the fact that the initial amount of 2,3,4-3,4-CB in both groups was almost equal since the residual 2,3,4-3,4-CB in GFDS soil is negligible (0.5% of the spiked one) and the amount of 2,3,4-3,4-CB in the introduced Aroclor 1242 was also negligible. This result indicated that the increase in PCB concentration inhibited dechlorination of 2,3,4-3,4-CB. The inhibitory effect was obviously not due to the possible toxic effect of the introduced PCBs since the overall dechlorination was much greater after introducing the PCBs (data not shown). Instead, the inhibitory effect was most probably because the addition of extra PCBs increased the concentration of other congeners, and consequently increased the competitors competing for the dechlorination capacity (dechlorinating enzymes/microorganisms). This indicated that the dechlorination conditions in GFDS were mainly capacity-limiting.

From the bioavailability results It can be concluded that bioavailability, soil conditions, as well as dechlorinating capacity (dechlorinating enzymes/microorganisms) are all factors in determining the dechlorination rates and extent. However, according to the experimental results, it appeared that bioavailability is less important than the other two factors. At least, as determined by this experiment, substantial dechlorination of environmental PCB residues can occur.

V. Influences of Some Carbon and Energy Amendments on PCB Dechlorination by Microorganisms from Hudson River Sediments

To enhance dechlorination and to enrich microorganisms able to dechlorinate PCBs, influences of some carbon and energy sources as well as other nutrients such as yeast extract on anaerobic dechlorination were investigated. Results are as follows:

1) Effects of acetate, acetone, yeast extract, and sediment extract on the on-set dechlorination

This experimental batch was prepared in 160-ml serum bottles. Each bottle contained 25 g of pre-incubated PCB-free Hudson River Sediment, 75 ml RAMM containing the eluted microorganisms, and 50 μl of 10% Aroclor 1242 in acetone. The final concentration of PCBs was 200 μg/g dry sediment. The cultures were first incubated at 25°C for 4 weeks until dechlorination activity was confirmed by sample analysis. Amendments were made on the fifth week. Acetate, acetone, and yeast extract amendments consisted of 1.5 ml of 10% solution of each. Sediment extract consisted of 10 ml extract prepared by autoclaving PCB-free Hudson River sediment with deionized water (1:1, wt:vol) for 1 hour. The mixture was then shaken for another hour,

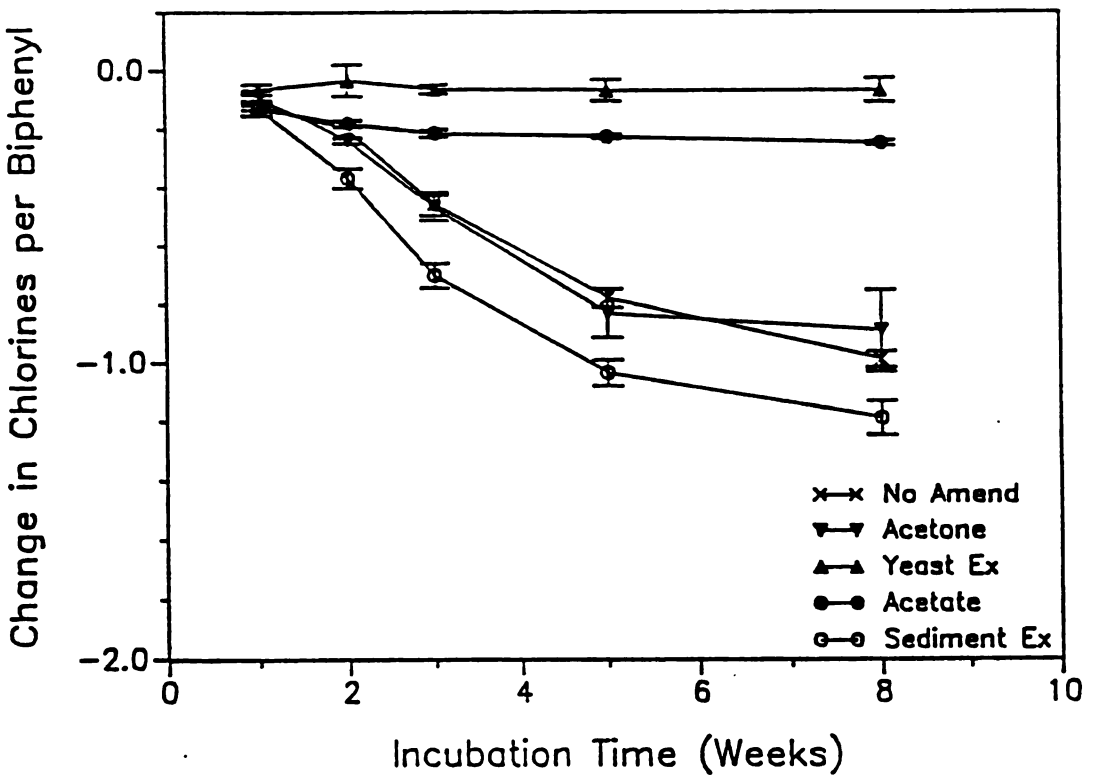

Fig. 6. Effects of different amendments on on-set dechlorination of Aroclor 1242 by microorganisms from Hudson River sediments. Error bars are the standard derivation (SD) of triplicate samples; where not shown, the error bars are smaller than the symbols.

centrifuged and filtered. All amendment solutions were bubbled with N_2-CO_2 and were autoclaved in serum bottles sealed with Teflon-coated stoppers and aluminum crimp seals.

No continued dechlorination was observed in any group including the non-amendment group which served as the control. This was probably because the more readily dechlorinated congeners had already been dechlorinated to a level below the threshold for a activity. Therefore, an additional 100 μl of 10% Aroclor 1242 in acetone was added, and that time was designated as 0 time. The cultures were incubated for additional 8 weeks.

Both acetate and yeast extract completely inhibited dechlorination. In contrast, sediment extract enhanced dechlorination (Fig. 6). Acetone at this concentration had no effect on dechlorination.

2) **Effects of butyrate and formate on the on-set dechlorination**

Butyrate and formate were both added at 0 time and at 4 weeks at a final concentration of 10 mM and 100 μM, respectively. No significant effect on dechlorination was observed.

3) **Influences of some amendments on dechlorination by the pasteurized cultures**

The amendments for this experiment were cellulose, glucose, lactate, methanol, H_2, yeast extract, peptone, and lactate plus sulfate along with three nails (5) per bottle. The concentration of sulfate was 2 mM (final concentration), H_2 was introduced with a gas manifold; cellulose was added as shredded filter paper; the concentrations of all amendments, except sulfate and H_2, were 0.5 g/liter liquid. All amendments were

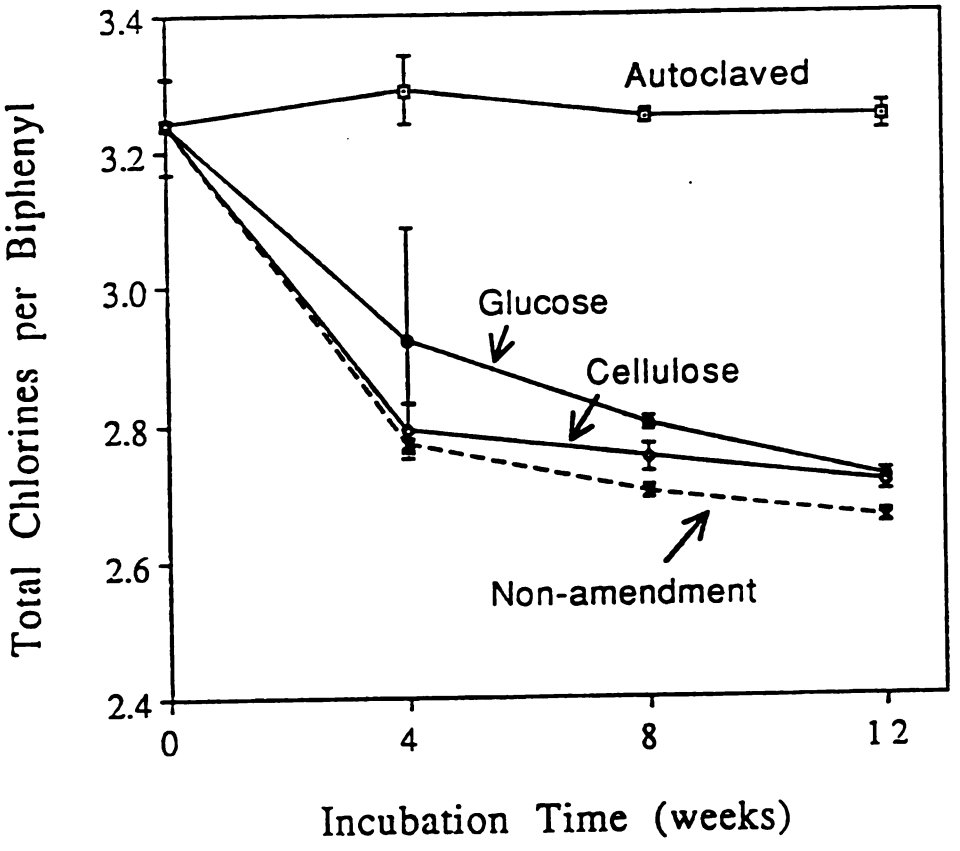

Fig. 7. Influences of cellulose and glucose on dechlorination of Aroclor 1242 by the pasteurized microorganisms from Hudson River sediments. The cellulose and glucose were added at 0 time and 4 weeks at a final concentration of 0.5 g/liter liquid. Error bars are the standard derivation (SD) of duplicate samples; where not shown, the error bars are smaller than the symbols.

autoclaved except H_2 which was filter sterilized, and the nails which were flamed.

The cultures were prepared in 60-ml serum bottles containing 10 g preincubated PCB-free Hudson River sediment, 29.4 ml RAMM, 0.3 ml inoculum, 0.15 ml cysteine (final concentration was 330 mg/liter liquid, 0.15 ml of stock amendments solution, and 60 μl 10% Aroclor 1242 in acetone.

The inoculum was taken from the 2nd serial pasteurized culture (Section I.1), therefore, the culture of this experiment was actually the 3rd serial culture (re-pasteurized, 1% transfer). The amendments were added at 0 time and at 4 weeks except sulfate which was only added at 0 time.

Both cellulose and glucose showed slight but discernible inhibitory effects after 4 weeks of incubation (Fig. 7), other amendments had no effects on dechlorination (data not shown). In the experiments described in Chapter 4, sulfate inhibited dechlorination by the pasteurized cultures at 2 mM. However, in this experiment, with addition of lactate, no inhibitory effect by sulfate was observed (data not shown), indicating that the sulfate effect was balanced by lactate.

Since no amendments enhanced dechlorination, it seemed that with presence of PCB-free Hudson River sediments, carbon and electron donor were not limiting factors for PCB dechlorination by the pasteurized cultures.

4) Investigation of solid supports for PCB culture

Ashed PCB-free Hudson river sediments and sloppy agar were used as supporting material to replace non-ashed sediments in order to have an organic matter free solid support. Lactate, pyruvate, acetate, formate, ethanol, and methanol were supplemented as amendments each at two experimental concentrations: 0.5 g/liter liquid, and 5 g/liter

liquid. H_2-CO_2 (80:20) was also among the investigated substrates and was introduced to 0 (equal to the atmospheric pressure) and 0.5 atm, respectively. For the 0.5 atm amendment the gas was introduced by needle attached to a gas manifold. The needle hole was then immediately sealed with silicon glue. In the sloppy agar experiment, 0.125% agar was added, while in the ashed sediment experiment, the ashed sediment was 10 g/60-ml serum bottle. The liquid portion consisted of 97.5% RAMM and 2.5% of sediment extract containing 0.5 g/liter of yeast extract and 0.4 g/liter of cysteine.

The inoculum was taken from the 5th serial transfer of the pasteurized cultures (Section I.1) The inoculum was re-pasteurized at 80°C for 10 min, and the inoculum was 5%.

The RAMM turned pink after being introduced into the ashed sediment, despite the fact that the ashed sediment was evacuated three times, filed with H_2-CO_2, and stored in an anaerobic chamber for 2 days. This may have been because, after being heated and ashed, the metals associated with the sediments were highly oxidized, and thus consumed the reducing power. The RAMM then had to be changed couple times, the medium then turned colorless before inoculation.

No dechlorination was observed in any culture, suggesting that either the supporting materia failed to effectively disperse PCBs which are highly hydrophobic, or no experimental amendment supported growth of the dechlorinating microorganisms.

5) Effects of preincubation on PCB dechlorination

In previous experiments, the preincubation procedure (8) was always followed for preparation of the experimental batches. The reason for the preincubation is to allow the facultative microorganisms to consume any residual oxygen. Strictly anaerobic

conditions can also be ensured by detection of methane formation since methanogens only grow under strictly anaerobic conditions.

Preincubation should also consume some sediment organic matter and nutrients, and produce some metabolites. All of these might influence dechlorination. To investigate the possible effects of the preincubation procedure on dechlorination, an experiment was set up with the following amendments:

a. autoclaved control

b. preincubated serum bottles

c. non-preincubated serum bottles

d. preincubated serum bottles washed with RAMM four times (the *pre/wash* amendment group, described below)

e. non-preincubated serum bottle with addition of 0.1% methanol

The purpose of amendment "e" was to investigate if introducing 1% methanol has any effect on dechlorination, since in the pre-incubation procedure, 0.1% of methanol is added as electron donor to scavenge the residual oxygen.

The preincubated cultures were incubated at 37°C for 10 days and methane production was confirmed by gas analysis. After preincubation, triplicate bottles were taken for the pre/wash group. The liquid portion (10 ml) was withdrawn, the preincubated sediment (10 g, dry weight) in the serum bottles was then washed four times with 30 ml of RAMM and shaken for 10 min each time. To avoid washing out sediment-associated nutrients, the RAMM had been added into other sediments, autoclaved along with the bottles to be washed, and then had been shaken for 10 min after autoclaving. After the washing process, fresh RAMM and inoculum were

introduced. Therefore, the pre/wash group was the same as the preincubated group except that the RAMM was fresh RAMM, therefore, no nutrient in the RAMM was consumed, and it did not contain metabolites from preincubation. This group was also the same as the non-preincubated group except that the sediment was preincubated and thus contained less organic matter due to the preincubation. Differences among the three amendments (amendment "e" is not included) are listed in Table 4.

Results showed that addition of 0.1% methanol had no effect on dechlorination (Fig. 8A). Compared with the non-preincubated cultures, preincubation inhibited dechlorination (Fig. 9). The preincubated culture showed dechlorination pattern M, while the non-preincubated culture exhibited pattern C (Fig. 10). The differences in the dechlorination pattern between the preincubated and the non-preincubated cultures are shown by the bottom panel in Figure 10.

Consuming sediment organic matter appears was not to be the reason that the preincubation had side effects on dechlorination, as evidenced by the fact that the dechlorination by the pre/wash group was not effected, even though this group also contained less sediment organic matter. Both accumulation of metabolites and consumption of nutrients may contribute to the inhibition effect. The nutrients consumed were most probably from RAMM instead of sediment, since nutrients from the sediment in the pre/wash group were equally consumed as those of the preincubated group. Metabolites proved to be unlikely cause of the effect, according to the methanol experiment (described below). Therefore, this result suggested that the effect of preincubation was probably due to deficiency of nutrients from RAMM that resulted in the inhibition effect. This result is also consistent with the previous observation that the

Table 4. Differences Among the Four Amendment Groups*

	Sediment organic matter		Nutrients in RAMM		Metabolites	
Amendments	More	Less	More	Less	Yes	No
Preincubated		–		–	+	
Pre/wash		–	+			–
Non-preincubated	+		+			–

* Preincubation should consume some sediment organic matter and some nutrient, and prodce some metabolites.

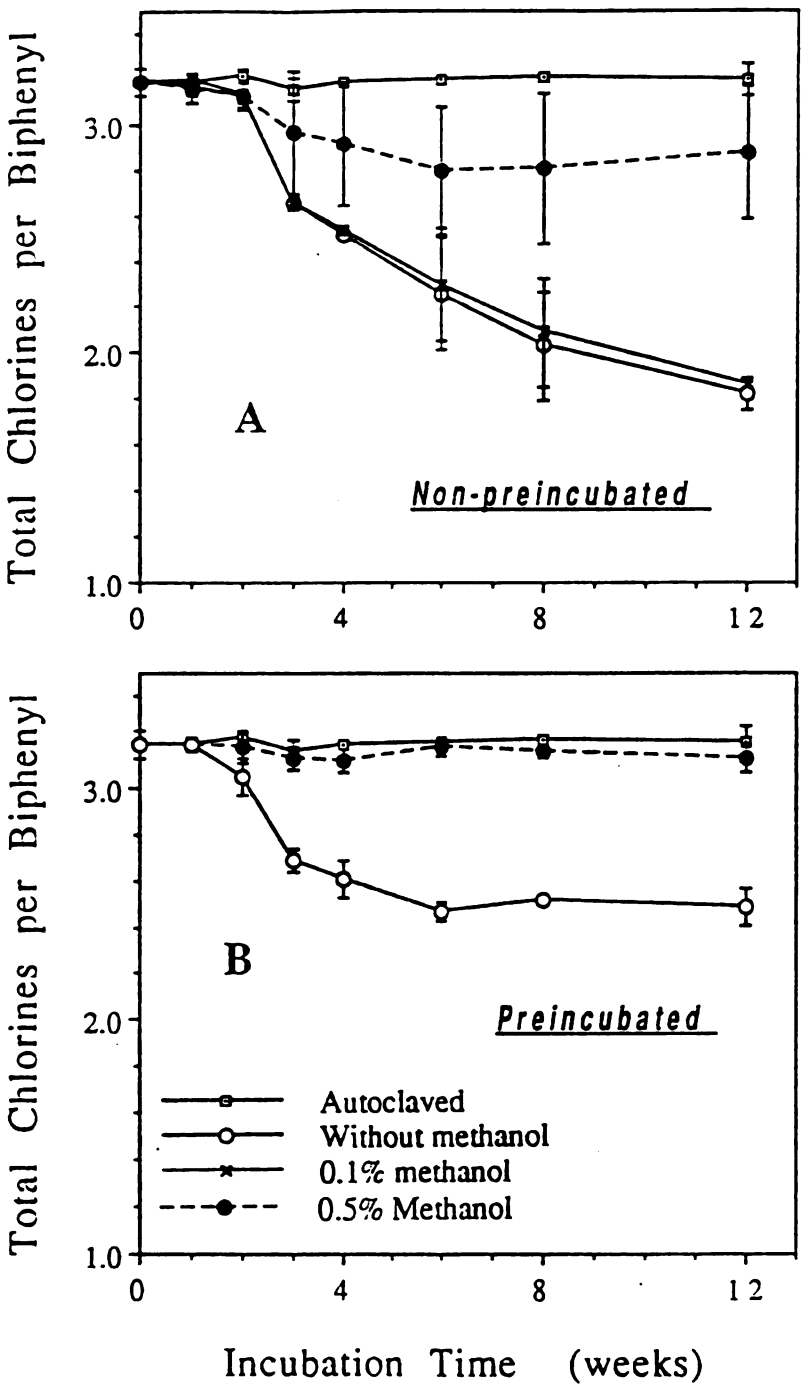

Fig. 8. Effects of methanol on dechlorination of Aroclor 1242 by (A) non-preincubated and (B) preincubated cultures. The 0.5% amendments received methanol at 0 time and 4 weeks, while the 0.1% amendment only received methanol at 0 time. Error bars are the standard derivation (SD) of triplicate samples; where not shown, the error bars are smaller than the symbols.

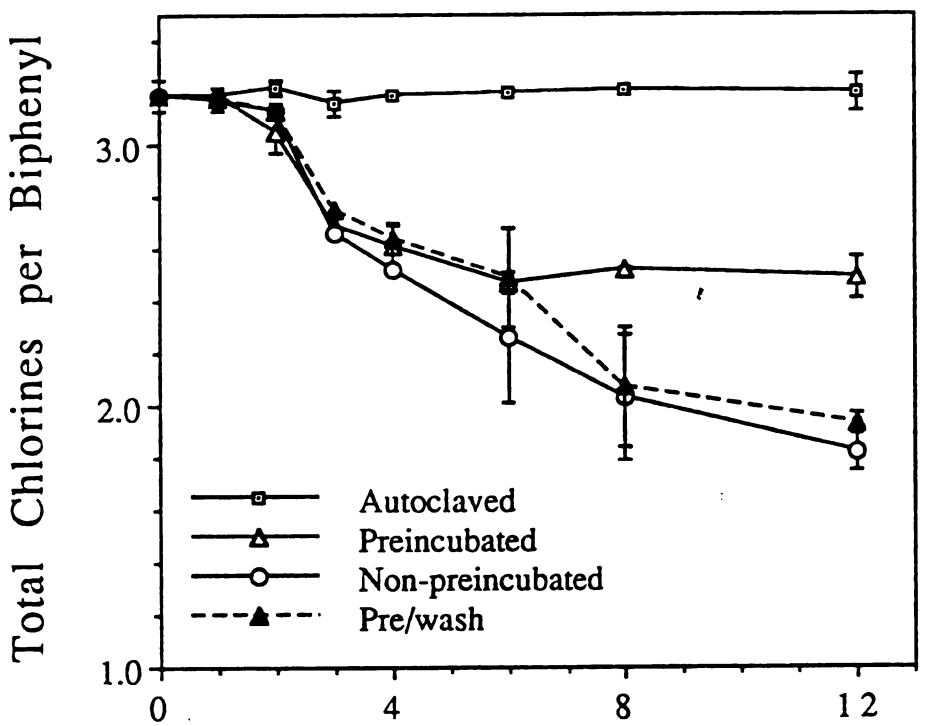

Fig. 9. Dechlorination of Aroclor 1242 by different experimental groups of the preincubation experiment. Error bars are the standard derivation (SD) of triplicate samples; where not shown, the error bars are smaller than the symbols.

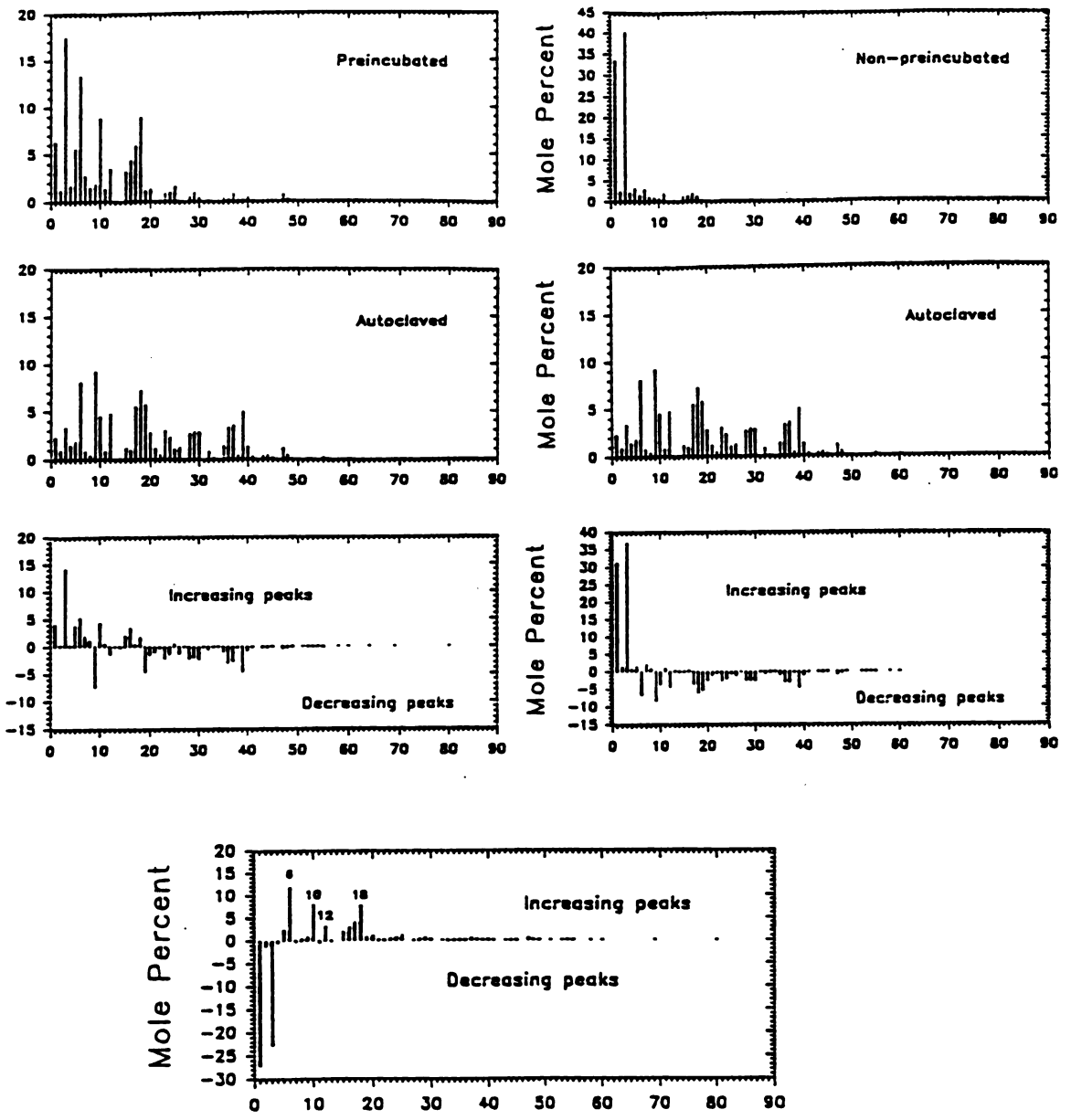

Fig. 10. Mole percentage of Aroclor 1242 peaks after 12 weeks of incubation with the preincubated and non-preincubated cultures. The bottom panel shows differences in mole percentage between preincubated and non-preincubated cultures after 12 weeks of incubation. For peak assignments, please see reference 6.

activity that was lost from the preincubated group was also lost from aged inoculum, and that the lose could be overcome by transferring the old culture to fresh RAMM (refer to Chapter 5). All these results suggested that the activity loss was unstable and was nutrient dependent.

However, whether pattern M or pattern C is expressed appears to depend not only on the preincubation time, but also on inoculum size and the activity of the inoculum. For example, in previous experiments, despite the standard preincubation treatment, pattern C was often expressed. It appears that all factors affecting nutrients in the culture may affect the expressed patterns.

6) **Effects of methanol on both preincubated and non-preincubated cultures**

As mentioned above, 1% methanol had no effect on dechlorination. However, as shown in Fig. 8, addition of methanol at a concentration of 5 g/liter liquid (at 0 time and 4 weeks) completely inhibited dechlorination by the preincubated cultures and partially inhibited dechlorination by the non-preincubated cultures. This result provided additional evidence that the culture was nutrient-limiting. As listed in Table 2, the differences between the preincubated and non-preincubated cultures were that the preincubated cultures contained less sediment organic matter, less nutrient, and metabolites. Less sediment organic matter and metabolites in the preincubated cultures do not likely enhance the inhibition by methanol. However, addition of methanol should accelerate consumption of nutrients. If the inhibitory effects of methanol on dechlorination is to accelerate consumption of nutrients, then the less nutrients present in the culture, the stronger the inhibitory effects. Results of the methanol experiment support this postulation, since methanol has stronger inhibitory effects on dechlorination by the

preincubated cultures, and the preincubated cultures contained less nutrient due to the preincubation procedure.

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