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

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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.

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 being flushed with filter-sterilized O_2-free N_2-CO_2 (80:20, vol/vol) with a Hungate apparatus. The flasks were then sealed and incubated at 25°C in the dark for 2 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 the 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

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without ethanol was centrifuged, and the supernatant was anaerobically filter sterilized (0.22 μm; Millipore Co., Bedford, Mass.) and then used to resuspend the ethanol-treated inoculum. Oxygen contamination of the resuspended inoculum did not occur, as indicated by the resazurin test.

For the combined treatment, the inoculum was first treated with ethanol and 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 headspace, the bottles 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 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 a 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 cysteine 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 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 September were serially transferred, and the transferred inocula were treated each time.

All treatments were done 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 manner described above except that the vessel was a 10-ml serum bottle, the time of exposure to ethanol was 1 h, the centrifugation was performed at 8,000 × g for 10 min, and the heating temperature was 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 added to each bottle containing 29.7 ml of RAMM to make a 1% transfer.

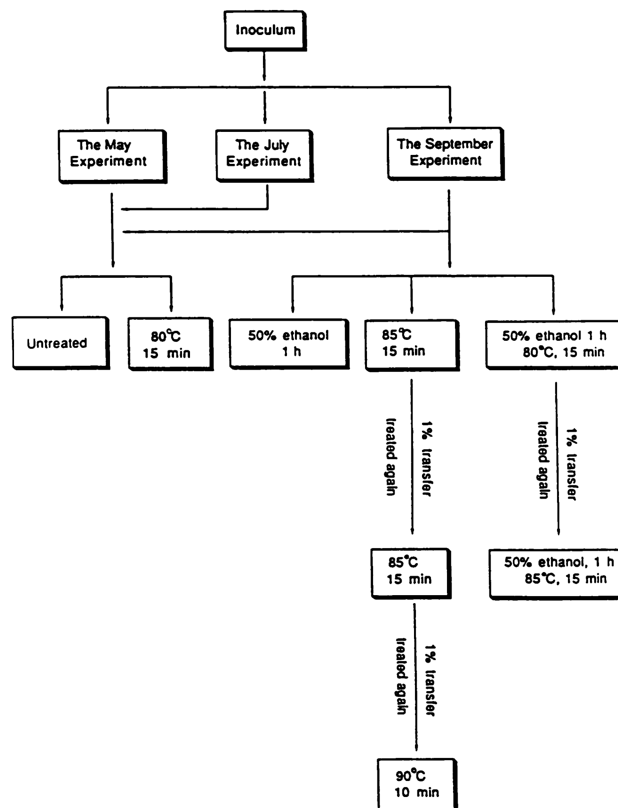

FIG. 1. Brief flow diagram of the experimental design.

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 showed a common dechlorination pattern, as illustrated in Fig. 3B. As dechlorination proceeded, the levels of most of the 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 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

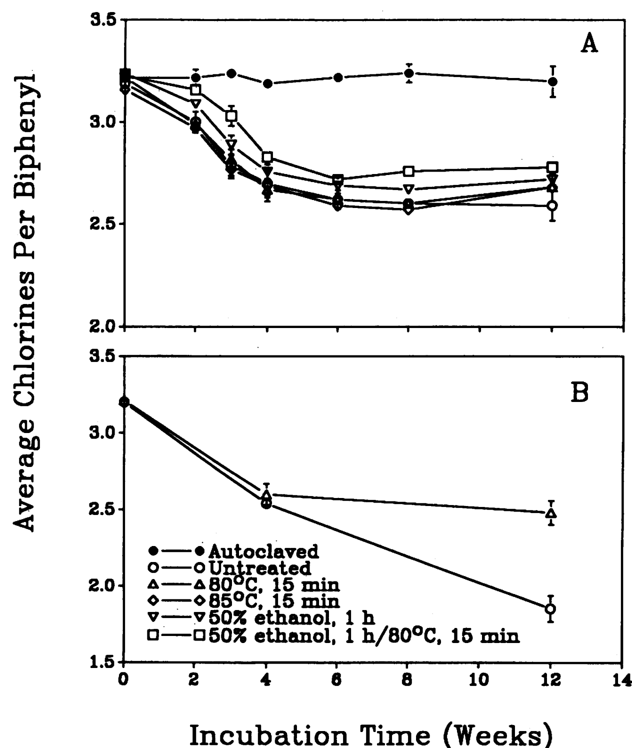

FIG. 2. Dechlorination of Aroclor 1242 by heat- and/or ethanol-treated microorganisms eluted from Hudson River sediments. Experiments were performed in (A) September and (B) May. Error bars are the standard deviation (SD) of triplicate (A) or duplicate (B) samples; where not shown, the error bars are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

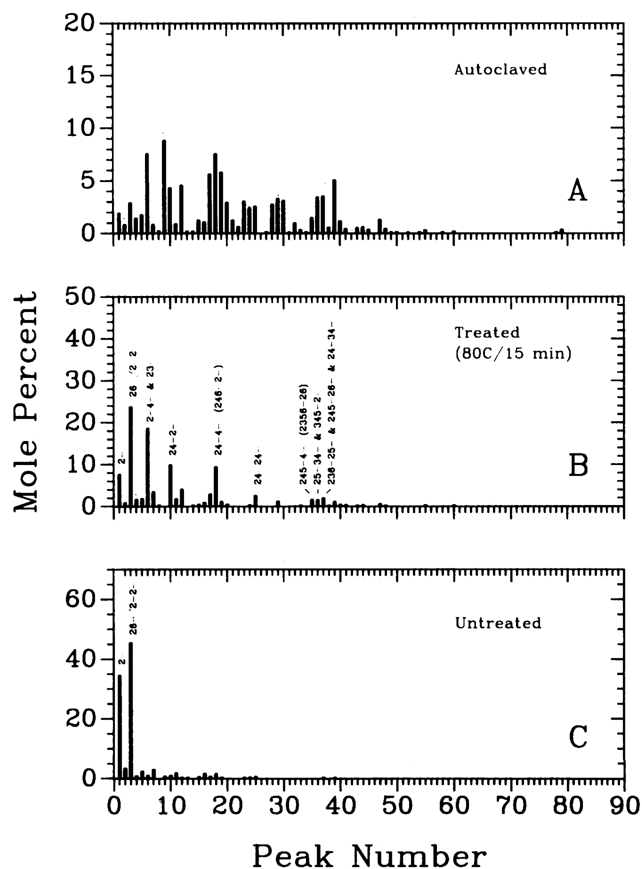

FIG. 3. Mole percentage of Aroclor 1242 represented by each chromatographic peak after 12 weeks of incubation in the May experiment with (A) autoclaved, (B) treated (80°C, 15 min), and (C) untreated microorganisms eluted from Hudson River sediments. For a complete list of the PCB congeners associated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

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 with 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 of 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, and 2,4-4-CB) tended to accumulate.

Comparison of dechlorination patterns between treated and untreated cultures. The common dechlorination pattern shown 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 a *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 differences in the heights of peaks 35, 36, and 37 (the responses of the 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 of 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

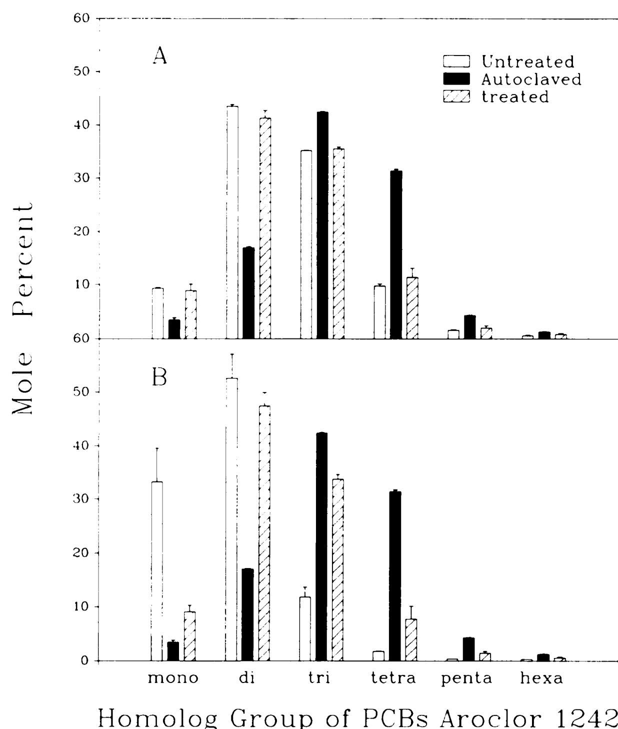

FIG. 4. Homolog distribution of Aroclor 1242 after (A) 4 and (B) 12 weeks of incubation with treated (80°C, 15 min) and untreated microorganisms (May experiment) eluted from Hudson River sediments. The hepta-, octa-, nona-, and decachlorinated homologs are not plotted because their mole percentages in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

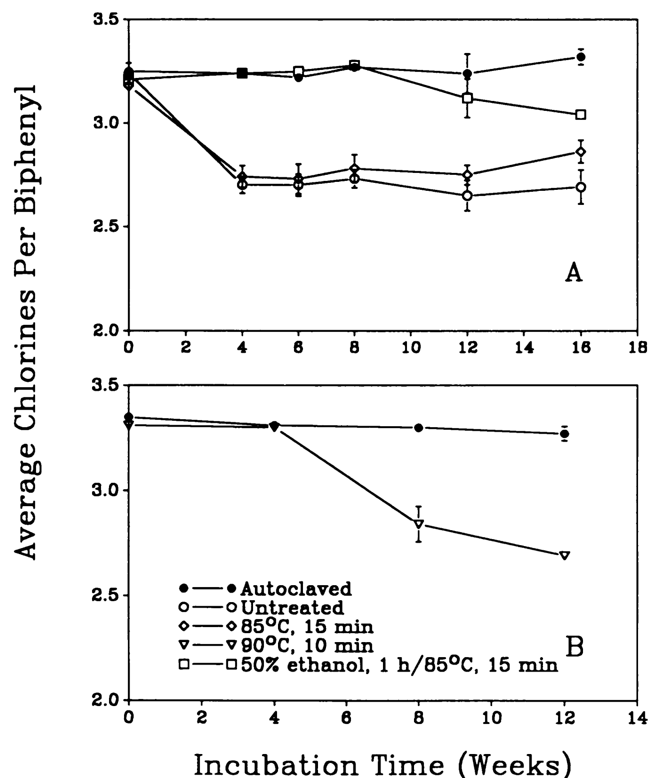

FIG. 5. Dechlorination of Aroclor 1242 by the cultures transferred from the September experiment (1% transfer): (A) second serial culture (85°C, 15 min); (B) third serial culture (90°C, 10 min). Error bars are the SD of triplicate (A) or duplicate (B) samples; where not shown, the error bars are smaller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

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), indicating that the microorganisms responsible for dechlorination grew in the culture medium. Two treated cultures from the September experiment were transferred to the second serial culture at a 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 those of 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 methanogenesis is not required for PCB dechlorination.

Gottschalk et al. (7) pointed out that heating at 70 or 80°C for 10 min is generally regarded as sufficient for elimination of nonsporeformers, including most thermophilic nonsporeformers. Koransky et al. (11) and Johnston et al. (10) reported that treatment with 50% ethanol for 1 h 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 that 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 ability 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 very similar 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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