

Effects of Polychlorinated Biphenyl Congener Concentration and Sediment Supplementation on Rates of Methanogenesis and 2,3,6-Trichlorobiphenyl Dechlorination in an Anaerobic Enrichment

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We have employed a method of enrichment that allows us to significantly increase the rate of reductive polychlorinated biphenyl (PCB) dechlorination. This method shortens the time required to investigate the effects that culture conditions have on dechlorination and provides an estimate of the potential activity of the PCB-dechlorinating anaerobes. The periodic supplementation of sterile sediment and PCB produced an enhanced, measurable, and sustained rate of dechlorination. We observed volumetric rates of the dechlorination of 2,3,6-trichlorobiphenyl (2,3,6-CB) to 2,6-dichlorobiphenyl (2,6-CB) of more than 300 $\mu\text{mol liter}^{-1} \text{day}^{-1}$ when the cultures were supplemented daily. A calculation of this activity that is based on an estimate of the number of dechlorinating anaerobes present indicates that 1.13 pmol of 2,3,6-CB was dechlorinated to 2,6-CB $\text{day}^{-1} \text{bacterial cell}^{-1}$. This rate is similar to that of the reductive dechlorination of 3-chlorobenzoate by *Desulfomonile tiedjei*. Methanogenesis declined from 585.3 to 125.9 $\mu\text{mol of CH}_4 \text{ liter}^{-1} \text{day}^{-1}$, while dechlorination increased from 8.2 to 346.0 $\mu\text{mol of 2,3,6-CB dechlorinated to 2,6-CB liter}^{-1} \text{day}^{-1}$.

The release of polychlorinated biphenyls (PCBs) into the environment has caused public concern for several decades because of the purported recalcitrance and potential carcinogenicity of PCBs. The debate over the danger that PCBs pose to human health, wildlife, or the environment continues (18, 21). In the United States, PCBs were released as Aroclors which are mixtures of PCB congeners that possess various degrees of chlorination. PCBs are now known to be susceptible to biodegradation, and recent studies have shown PCBs to be extensively dechlorinated in the Hudson River (1, 2). The rates of these dechlorinations in anaerobic environments have been slow, with major changes occurring only over a period of months or years.

In the laboratory, many PCB congeners can be reductively dechlorinated under anaerobic conditions (14-17, 23). These dechlorinations transpire within several weeks. From a bioremediation perspective, understanding how these dechlorinations can be stimulated is of value. Our laboratory investigations of anaerobic PCB dechlorination involve defining specific bacterial activities involved in the process. Determining rates of dechlorination and how to increase them is complicated by the presence of several PCB congeners which may all have different rates of dechlorination, depending on the positions and substitutions of the chlorines. Different bacterial populations may also confound the issue if the populations have different specificities for different PCB congeners.

Increasing the dechlorination rates of individual congeners would allow quicker analysis of culture conditions. Our objective was to test the effects of PCB concentration and sediment supplementation upon the rate of PCB dechlorina-

tion and methanogenesis. To accomplish this objective, we employed a technique involving resupplementing cultures with slurries of 2,3,6-trichlorobiphenyl (2,3,6-CB), sterile sediment, and an anaerobic mineral medium referred to as RAMM (19). There are three advantages to this approach. (i) Analysis of PCB dechlorination is simplified. (ii) An enhanced rate of dechlorination allows for the rapid testing of culture variables. (iii) The single dechlorinating reaction may provide a selection method for the enrichment and eventual isolation of the dechlorinating organism(s). From this work, we have been able to measure rates of *meta* dechlorination in sediment-supplemented cultures in the laboratory, how fast this may occur per bacterial cell, and how the rate of methanogenesis is affected when dechlorination increases.

MATERIALS AND METHODS

Aroclor 1242 enrichment. An initial enrichment was prepared with Hudson River H7 sediment (inoculum) (15% [vol/vol]), the sterile reduced anaerobic mineral medium RAMM (19) (60% [vol/vol]), air-dried Spier Falls sediment (33% [wt/vol]), Aroclor 1242 (400 mg/liter), 2,3,6-CB (100 μM), 2,4,6-CB (100 μM), 2,2',6,6'-CB (70 μM), cysteine-HCl (0.1% [wt/vol]), and $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ (0.025% [wt/vol]). Before inoculation, slurries were sterilized by exposure to 121°C for 3 h. Sterile reducing agents (cysteine-HCl and $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$) were added shortly before inoculation. After 15 weeks of incubation, the initial Aroclor 1242 enrichment was transferred (15% [wt/vol]) to identical medium. The transfer was performed under a stream of filter-sterilized, O_2 -free N_2 by the Hungate technique (3). After inoculation, sterile controls were autoclaved for an additional 3 h. The PCB congeners and Aroclors were obtained from Accustandard Inc. (New Haven, Conn.), prepared in acetone, and added at the time of inoculation. The PCB-acetone solutions were added to each transfer culture while the culture was flushed with filter-sterilized oxygen-free N_2 . The final concentration of acetone in the cultures was 0.01% (vol/vol).

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The Aroclor 1242 enrichments and transfers of the enrichments were incubated under N_2 at $30^\circ C$ in the dark in 50-ml serum bottles capped with Teflon-coated butyl rubber stoppers and aluminum seals. The Hudson River H7 sediment and Hudson River Spier Falls sediment were supplied by General Electric Company (Schenectady, N.Y.) and were collected at river miles 193.5 and 205, respectively, of the Hudson River. In later work, Raritan River sediment (Hunterdon County, N.J.) was used in place of Spier Falls sediment. Analysis of the Raritan River and Spier Falls sediments showed that these sediments were PCB free, and we observed no difference in dechlorinating activity when Raritan River sediment was used in place of Spier Falls sediment.

Incubation with 2,3,6-CB. After 15 weeks of incubation, the Aroclor 1242 enrichment was transferred (15% [vol/vol]) into identical medium, except $100 \mu M$ 2,3,6-CB replaced the Aroclor 1242 and congener mixture of PCBs. This culture was initially sampled monthly, then every 2 weeks, and then weekly over a 4-month period. The 2,3,6-CB culture was then transferred (15% [vol/vol]) into identical medium and incubated for 3 months. This transfer culture was then resupplemented weekly with $2.34 \mu mol$ of 2,3,6-CB and 4 ml of a fresh, sterile slurry of RAMM and Raritan River sediment (1 g of dry sediment per 2 ml of RAMM). This culture was then given the designation C-146. C-146 has been maintained with 2,3,6-CB for more than 1 year at 28 ml by sampling (2 ml) before and after supplementation with 4 ml of sediment and RAMM. C-146 was used as the source of inoculum for the rate experiments reported here.

Culture sampling and PCB analysis. All culture sampling and transfer was done under O_2 -free N_2 by the methods of Quensen et al. (16), using sterile 5-ml pipettes in the reverse orientation. Quantitation of 2,3,6-CB was based upon the known amount of 2,3,6-CB added, the measured moles percent of 2,3,6-CB and 2,6-CB in the samples, and the dilution of congener caused by sampling and supplementation. PCBs were extracted from the sediment by shaking 2-ml samples overnight in 10-ml portions of ethyl acetate in 15-ml glass vials that were sealed with Teflon-lined caps. The samples were then passed over a Florisil-copper powder column which was prepared by the method of Quensen et al. (16). The PCB congeners were analyzed by passage of 1- μl samples through a gas chromatograph (Tracor 9000 Series; Tracor, Austin, Tex.) equipped with a DB-1 capillary column (30 m by 0.25 mm; J&W Scientific, Folsom, Calif.) and an electron capture detector, as described by May et al. (10).

Methane determination. Analysis of CH_4 was made with a Tracor 9000 Series gas chromatograph equipped with a Carbosieve S-II column (1/4-in. [0.64-cm] outer diameter, 2-mm inner diameter, 6 ft [182.9 cm] long, 80/100 mesh) and a thermal conductivity detector. The column was operated with an initial column temperature of $165^\circ C$ and the temperature was increased to $225^\circ C$ at a rate of $15^\circ C$ per min. After determination of headspace volume, 100- μl gas samples were taken from the cultures and analyzed. All punctured Teflon-coated stoppers were replaced after sampling in order to prevent adsorption of the PCBs to the stoppers.

Determination of the most probable number of dechlorinating microorganisms. Duplicate cultures of C-146 were serially diluted (1:10) into aluminum-seal-type anaerobic culture tubes (Bellco, Vineland, N.J.) containing 9-ml samples of the sediment-RAMM slurry described above with $70 \mu M$ 2,3,6-CB as the only PCB. The dilution tubes had been autoclaved before inoculation for 3 h at $121^\circ C$. Two sets of dilutions were made for each of the duplicate enrichments,

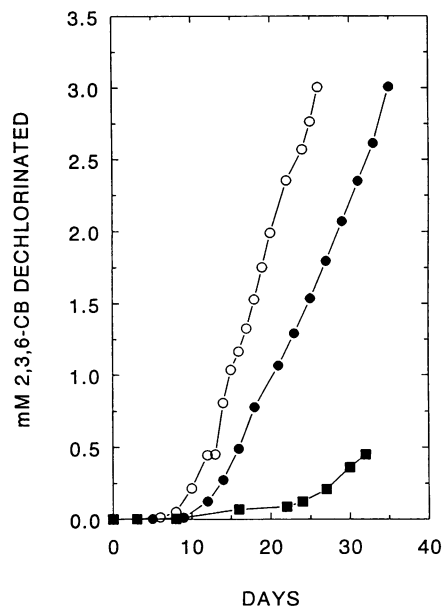


FIG. 1. Cumulative dechlorination of 2,3,6-CB by C-146 on river sediment. Each point represents the average dechlorination of two cultures. Symbols: ■, cultures that had 30 to $85 \mu M$ 2,3,6-CB available from days 0 to 16 and 115 to $183 \mu M$ 2,3,6-CB available from days 16 to 32; ●, cultures that had 168 to $312 \mu M$ 2,3,6-CB from days 0 to 9 and 406 to $643 \mu M$ 2,3,6-CB available from days 9 to 35; ○, cultures that had 84 to $287 \mu M$ 2,3,6-CB available from days 0 to 8 and 289 to $999 \mu M$ 2,3,6-CB available from days 8 to 26.

and the dilutions ranged from $1:10^1$ to $1:10^{12}$. After 10 weeks of incubation, 2-ml samples were removed from the dilution tubes for PCB extraction and analysis.

RESULTS

Aroclor 1242 enrichment. An anaerobic culture capable of removing chlorines from the *meta* positions of PCBs was acquired by enrichment of Hudson River H7 sediment. The initial enrichment was grown in the presence of Aroclor 1242 and select congeners including 2,3,6-CB. After 15 weeks of incubation, significant dechlorination was observed. Removal of both *meta* and *para* chlorines occurred after 15 weeks (10). This type of activity has been reported by several investigators (14, 15, 17, 24). Transfers were made from this enrichment into sediment slurries containing a single congener, 2,3,6-CB. Initially, only nanomole amounts of congener were dechlorinated per day in 28-ml cultures. These cultures were not resupplemented with sediment nor was the amount of 2,3,6-CB increased.

Sustained rate of dechlorination. The 2,3,6-CB (*meta*)-dechlorinating culture C-146 was obtained by transfer from the Aroclor 1242 enrichment. C-146 was periodically supplemented with sterile sediment and 2,3,6-CB. Figure 1 shows the net dechlorination of 2,3,6-CB by C-146 over time. Volumetric rates were sustained at levels ranging from 71.4 to $182.1 \mu mol$ of 2,3,6-CB dechlorinated liter $^{-1}$ day $^{-1}$. These cultures were maintained at 33% (wt/vol [dry wt]) of sediment, so the activity per gram of soil ranged from 0.21 to $0.55 \mu mol$ of 2,3,6-CB dechlorinated to 2,6-CB day $^{-1}$. The rate of activity with more 2,3,6-CB and an estimate of this activity in relation to the number of dechlorinating microorganisms are given below. In general, dechlorination in-

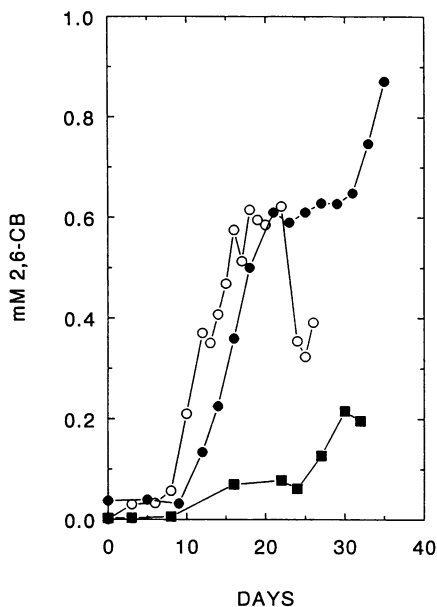


FIG. 2. Accumulation of 2,6-CB in cultures of C-146. These data are from the cultures shown in Fig. 1. For explanation of symbols, see the legend to Fig. 1.

creased with corresponding increases in the amount of PCB and/or frequency of supplementation. Dechlorination could be maintained indefinitely as long as PCB and sediment were supplied in this semicontinuous fashion.

Production of 2,6-CB. Since the levels of 2,3,6-CB and 2,6-CB are determined by the mole percentage of the known amount of congener added to the culture, the amount of 2,6-CB produced is equivalent to the 2,3,6-CB consumed. However, the amount of 2,6-CB that accumulated in the cultures was not equivalent to the amount produced as a result of sampling, resupplementation of the cultures, and transfer from the cultures. The 2,6-CB that accumulated in the cultures in Fig. 1 is shown in Fig. 2. The concentration of 2,6-CB in many of these cultures exceeded 500 μM and concentrations greater than 1 mM have been observed in active cultures (data not shown). These levels of 2,6-CB do not decrease activity, which suggests that the product of the reductive dechlorination does not inhibit the reaction at these PCB concentrations.

Effect of PCB concentration and nutrient (sediment) resupplementation. When C-146 was incubated with a low level of 2,3,6-CB (83.6 μM) and was not resupplemented, the volumetric rate of dechlorination was 2.1 μmol of congener dechlorinated $\text{liter}^{-1} \text{day}^{-1}$ (data not shown). Dechlorination could be increased more than 100-fold by increasing the level of PCB and by resupplementing congener and sediment daily. The effect that PCB concentration and sediment supplementation have on dechlorination is demonstrated in Fig. 3. The data show that the 1-day resupplementation gave the fastest rate of activity; therefore, the cultures become limited for something other than PCB with longer periods of incubation. The data also show that dechlorination was enhanced by increases in the level of 2,3,6-CB available. Volumetric rates of dechlorination of more than 300 μmol of 2,3,6-CB dechlorinated $\text{liter}^{-1} \text{day}^{-1}$ were obtained when the cultures were supplemented daily and supplied with between 1.0 and 2.0 mM 2,3,6-CB.

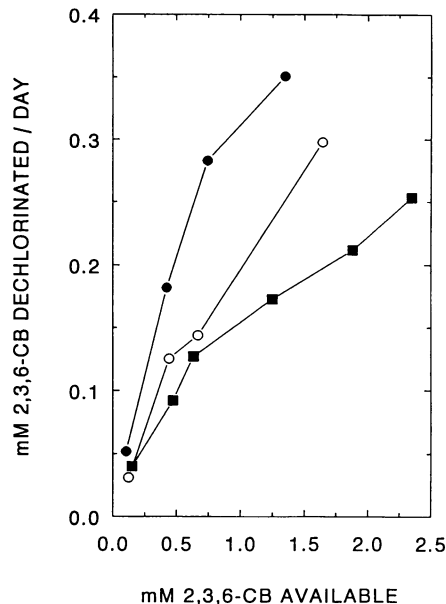


FIG. 3. Daily dechlorination of 2,3,6-CB versus 2,3,6-CB available to C-146 cultures that were sampled and resupplemented daily (\bullet), once every 2 days (\circ), and once every 3 days (\blacksquare).

Estimation of rate per bacterial cell. A duplicate set of C-146 was maintained at a high rate of activity for nearly 2 months. These cultures were used as a stock for fresh transfers and for the determination of dechlorination rates. After 42 days of incubation, duplicate transfers were prepared from these cultures in order to determine the number of dechlorinating organisms present at the time of sampling. These transfers took place in the midst of several 1-day feedings while the activity was high. The transferred slurries were diluted in a modified most probable number experiment, and after 10 weeks of incubation, the transfers were assayed for dechlorination of 2,3,6-CB. Approximately 3×10^5 dechlorinating cells per ml of culture were present in the examined cultures. The sampled cultures had dechlorinated 339.3 μmol of 2,3,6-CB to 2,6-CB $\text{liter}^{-1} \text{day}^{-1}$ (1.13 pmol of 2,3,6-CB dechlorinated to 2,6-CB $\text{day}^{-1} \text{bacterial cell}^{-1}$) during the 24 h before sampling and dilution.

Rates of dechlorination and methanogenesis. Table 1 summarizes the dechlorination and methanogenesis of C-146 in the presence and absence of 2,3,6-CB. Low levels of PCB

TABLE 1. Methanogenesis and dechlorination of 2,3,6-CB by C-146 in the presence and absence of 2,3,6-CB

Amt (μM) of 2,3,6-CB available ^a	Supplement schedule ^b	Amt (μmol) of CH_4 produced $\text{liter}^{-1} \text{day}^{-1}$	Amt (μmol) of 2,3,6-CB dechlorinated $\text{liter}^{-1} \text{day}^{-1}$
1,062.9 \pm 228.4 (4)	Daily	125.9 \pm 64.1	346.0 \pm 60.4
84.3 (2)	Weekly	585.3	8.2
0.0 (8)	Daily	627.7 \pm 100.9	

^a The numbers in parentheses represent the number of samples assayed. When there were more than three samples, a standard deviation for the entire population of numbers was calculated.

^b Cultures were resupplemented with sterile sediment, RAMM, and 2,3,6-CB.

with weekly supplementation resulted in low levels of dechlorination and high rates of methanogenesis. This rate of methanogenesis was similar to the methanogenic rate of C-146 that was supplemented daily with fresh sediment and RAMM without PCB. High levels of 2,3,6-CB (near 1 mM) and daily supplementation resulted in an elevated rate of dechlorination and a fivefold reduction in methanogenesis.

DISCUSSION

Investigation with Aroclor mixtures is a slow process (14–16, 24), and the quantification of dechlorination to specific congeners is complicated by the presence of many different congeners. The presence of congeners makes measurement of activity that may be specific to a biological system complicated. Other investigators have studied the dechlorination of individual PCB congeners (23, 24) in order to clarify the activities associated with different PCB congeners. In order to quantitate dechlorination more quickly, we used higher concentrations of the congeners and frequent supplementation of the PCBs and sediment. This method increased PCB reductive dechlorination more than 100-fold in our cultures. Extrapolating from the data of Van Dort and Bedard (23), we calculated that their anaerobic cultures dechlorinated 2,3,5,6-CB to 2,3,6-CB and 2,5-CB at rates between 9 and 10 $\mu\text{mol liter}^{-1} \text{day}^{-1}$. These cultures dechlorinated approximately 4 μmol of 2,3,6-CB to 2,6-CB $\text{liter}^{-1} \text{day}^{-1}$. The data of Williams (24) show that 25 to 30 μmol of 3,4,5-CB $\text{liter}^{-1} \text{day}^{-1}$ could be dechlorinated in anaerobic cultures. These investigations were concerned with the type of dechlorination (*meta*, *para*, and *ortho*), so attempts were not made to resupplement the cultures and increase the rate of activity. Our results show that such resupplementation increases the rate of PCB dechlorination 10- to 50-fold over that of the previous studies.

The ability to sustain a more rapid dechlorination of PCBs will be useful for analyzing changes in culture conditions. In addition, the continued supplementation of a specific congener at higher levels may assist in the enrichment of organisms capable of specific dechlorinations. We have already demonstrated that such a technique is useful in the comparison of methanogenesis and PCB dechlorination and in the comparison of the reductive dechlorination of PCBs and other chlorinated compounds. The addition of nutrients to Aroclor-contaminated sediments has not resulted in significant increases in the rate of dechlorination (14, 15). However, we have used our technique to further investigate the effect of supplements other than sediment and PCBs on the anaerobic dechlorination of PCBs (unpublished data).

Although dechlorination of an aryl chlorine has been shown to occur under many anaerobic conditions (13), only two microorganisms capable of reductively dehalogenating a chlorinated aromatic compound have been isolated (9a, 20). The first to be isolated, *Desulfomonile tiedjei* formerly (DCB-1) (4), is capable of dehalogenating chlorinated aliphatic compounds (9), chlorophenols (11), and halobenzoates (8). Its rate of dechlorination of 3-chlorobenzoate has been measured at 0.1 $\text{pmol day}^{-1} \text{bacterial cell}^{-1}$ (22). Our estimate of the rate of removal of a *meta* chlorine from 2,3,6-CB (1.13 $\text{pmol day}^{-1} \text{bacterial cell}^{-1}$) compares favorably with this rate. Our estimate establishes a point of reference for future investigation.

Previous studies have shown that methane is produced in anaerobic PCB-dechlorinating cultures (12, 17); however, the role of methanogens, if any, is unknown. Dingyi et al. (5) have recently shown that pasteurized cultures of anaerobes

capable of PCB dechlorination were not methanogenic. In another investigation, we enriched a PCB-dechlorinating anaerobic culture (not C-146) that is no longer methanogenic after transfer on a solid medium (10). From these studies, it can be concluded that methanogenesis is not required for the biologically mediated reductive dechlorination of all PCBs. We did not establish whether methanogenesis is required for the dechlorination of 2,3,6-CB by C-146. We did observe, however, that methanogenesis decreased when dechlorination increased. Although we do not know the mechanism responsible for this decrease in methanogenesis, it is of interest that this pattern of decreasing methanogenesis with increasing dechlorination has been observed in the biologically mediated reductive dechlorination of other halogenated compounds (6, 7).

The rate of PCB dechlorination can be significantly increased in the laboratory. Studying PCB dechlorination at an increased rate with mixed or individual congeners will enhance the researchers' ability to characterize the dechlorinating organisms. This enrichment method can also be used as a tool in the investigation of required culture conditions.

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