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$FeSO_4$ Amendments Stimulate Extensive Anaerobic PCB Dechlorination

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Anaerobic microbial reductive dechlorination of PCBs is important because it removes the chlorine substituents that block aerobic metabolism and it reduces PCB toxicity. Although this process occurs widely in nature, its extent is often limited to dechlorination of some of the chlorines in the meta positions of biphenyl. In this report we demonstrate the ability to achieve nearly complete meta plus para dechlorination of Aroclor 1242. This involves the additions of $FeSO_4$ to PCB contaminated sediments and results in ∼90 mol % of the total PCBs being converted to aerobically degradable ortho-substituted mono- and dichlorinated congeners. We propose that iron sulfate provides two mutually beneficial effects leading to its stimulation of anaerobic PCB dechlorination. Sulfate stimulates growth of sulfate reducing organisms responsible for PCB dechlorination, while Fe^{2+} reduces sulfide bioavailability and hence toxicity by forming the insoluble precipitate FeS. Ferrous sulfate is an inexpensive, innocuous compound which could be utilized to overcome factors limiting both the extent of in situ dechlorination as well as the implementation of sequential anaerobic/aerobic biotreatment systems. In addition it is expected that the toxicities of Aroclors, and hence the risk they pose, will be substantially reduced at sites where PCBs have been extensively dechlorinated.

Introduction

It is estimated that ∼600 million kilograms of polychlorinated biphenyls (PCBs) have been produced worldwide and that several million kilograms have been released into the environment (*1*). Commercial PCBs were manufactured and used as complex mixtures of chlorine substituted biphenyl molecules, typically consisting of 60−90 of a possible 209 PCB congeners. Several commercial PCB mixtures (e.g. Aroclors) exist, each with a specific chlorine content and congener profile. These mixtures are distributed throughout the global ecosystem at relatively low concentrations but can be found at much higher concentrations at specific locations, often in sediments. PCBs are generally considered persistent environmental contaminants primarily because chlorine substituents prevent common microbial oxygenase enzymes from attacking the aromatic rings of biphenyl. The reductive dechlorination of PCBs by anaerobic bacteria has recently been established as an important environmental fate of these otherwise recalcitrant compounds (*2*−*4*). This process replaces chlorines on the biphenyl ring with hydrogen, reducing the average number of chlorines per biphenyl in the resulting product. Reductive dechlorination of PCBs is important because the dechlorinated products are more susceptible to aerobic metabolism including ring opening and mineralization. Furthermore, reductive dechlorination generally reduces the toxicity of PCBs. It has recently been established that the inhibitory effects of PCBs on mouse gamete fertilization and their Ah receptor mediated activity ("dioxin-like" toxicity) are reduced or eliminated by anaerobic microbial dechlorination (*5*, *6*).

In situ reductive dechlorination has been documented in anaerobic sediments at numerous locations including the Hudson River (NY), Silver Lake (MA), Sheboygan River (WI), Waukegon Harbor (IL), New Bedford Harbor (MA), Hoosic River (MA), River Raisin (MI), and the Housatonic River (MA) (*7*). Although the intrinsic anaerobic reductive dechlorination of PCBs is well documented, the extent of dechlorination varies considerably among sites, ranging from <10 to >90% removal of meta plus para chlorines; removal of ortho chlorines is not generally observed. Based on chromatographic profiles of dechlorinated product mixtures, several dechlorination processes have been described (*7*). They may occur singularly or in combination in the environment. Apparently these processes result from differences in congener specificities of the distinct species or strains of dechlorinating microorganisms active at each site (*8*, *9*). The singular processes M and Q are the most extensive meta and para dechlorination processes, respectively. This is because neither requires that chlorine be adjacent to the position dechlorinated. All other described meta and para dechlorination processes have this requirement, hence resulting in a lesser extent of dechlorination.

Process M removes chlorines from the meta (3,3′,5,5′) positions of biphenyl and appears to be the most widely distributed in anaerobic sediments. This is also the process most commonly exhibited by the unamended Hudson River (HR) inoculum used in our present investigation. The resulting dechlorinated PCB product mixture consists of an accumulation of numerous ortho and para chlorinated congeners. Two enrichment cultures obtained from the same parent microbial consortium (i.e. HR) provide some information on the organisms responsible for process M. Ye et al. (*9*, *10*) observed process M dechlorination by pasturized cultures and concluded that the organisms responsible for this activity were sulfate reducing spore formers. A second enrichment culture capable of of process M dechlorination was established by large additions of the single congener 2,3,6-CB (*11*). Using antibiotics as specific inhibitors it was concluded that these organisms were most likely gram-positive.

Process Q removes para (4,4′) chlorines from the biphenyl ring, is rarely observed in situ, and is difficult to obtain in laboratory incubations. This activity has only been reported for organisms originating from PCB contaminated HR sediments (*7*). Recently, Williams (*11*) developed the only known enrichment culture exclusively displaying process Q activity. From this culture he concluded that, like process M activity, nonmethanogenic gram-positive organisms were essential for process Q dechlorination.

The most extensive overall dechlorination activity, designated process C, is process M and Q acting in conjunction. This results in PCB congeners substituted solely in the ortho positions. These congeners are less toxic (*5*, *6*), have lower bioaccumulation factors (*12*), and are readily susceptible to

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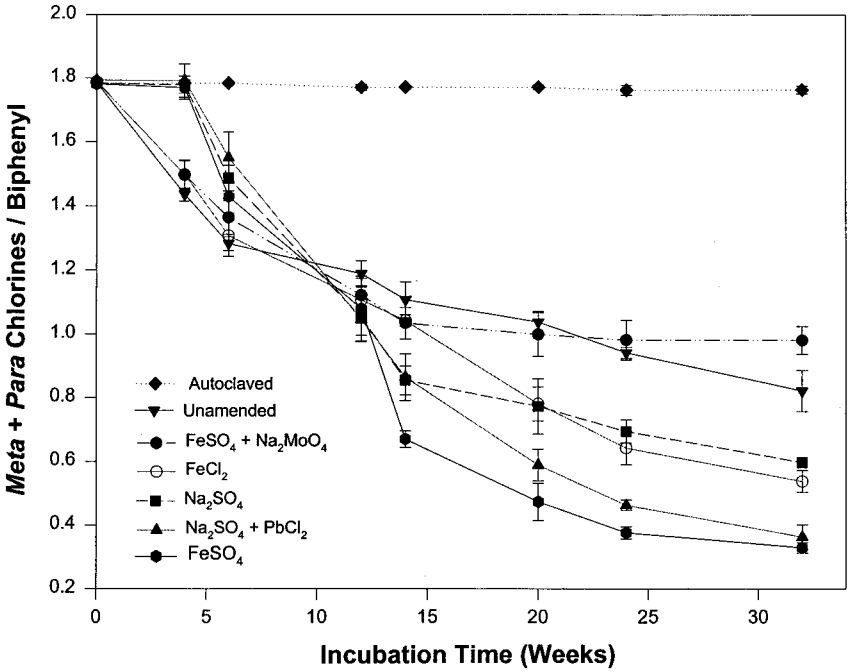

FIGURE 1. Effects of $FeSO_4$ amendments on anaerobic microbial dechlorination of Aroclor 1242. Rates and extents of dechlorination were determined by comparing changes in the average number of meta + para chlorines per biphenyl (no ortho dechlorination was observed). Error bars indicate standard error of triplicate samples. Unamended samples served as positive controls to establish indigenous dechlorination activity; autoclaved samples served as negative biological controls.

rapid aerobic mineralization (*13*). Unfortunately, the full dechlorination potential of process C is often unrealized in PCB contaminated sediments. In fact, this activity has only been documented in situ in PCB contaminated sediments of the Hudson River (*2*, *14*).

Reliable achievement of process C is desirable for both the development of sequential anaerobic/aerobic PCB biotreatment technologies as well as minimal-input in situ bioremediation of PCB contaminated sites (*13*, *15*, *16*). In recent experiments investigating the efficacy of adding various reagents to alleviate inhibition of the PCB dechlorination by heavy metals, we discovered that process C dechlorination of Aroclor 1242 could be achieved by amending HR sediment slurries with $FeSO_4$. The objective of this study was to document the stimulation of anaerobic PCB dechlorination by $FeSO_4$ and to elucidate the underlying mechanisms involved.

Methods and Materials

Sediment Collection. Sediment was sampled from two different sites on the upper Hudson River (HR) near Hudson Falls, NY. Non-PCB contaminated ("clean") sediments used in the dechlorination assays were collected just upstream from the origin of the PCB contamination at river mile 205. Sediment contaminated with Aroclor 1242 was obtained downstream at river mile 193.5. The PCB dechlorinating microbial consortium utilized herein was eluted from these sediments. Sediments were collected via a post-hole digger to a depth of approximately 25 cm and transported to the laboratory in completely filled and tightly sealed Teflon lined paint cans to minimize exposure to oxygen.

Dechlorination Assays. Laboratory assays similar to those used previously were designed to simulate the anaerobic sediment environment (*3*, *4*). Anaerobic sediment slurries consisted of 2 g of air-dried clean upstream Hudson River sediment and 3 mL of reduced anaerobic minimal media (RAMM) (*17*). Slurries were contained within O_2 free 15 × 150 mm glass Balch tubes sealed with Teflon coated butyl rubber stoppers (The West Co. Phoenixville, PA). A preincubation procedure was used to ensure anaerobic conditions prior to initiation of the actual dechlorination assay. For this, each tube received 1 mL of inoculum eluted from clean upstream HR sediments and was then monitored for methane production. When methane was detected in the headspace (~10 days), the tubes were autoclaved at 121 °C for 2 h on 2 consecutive days. Various amendments (described below) were added to each preincubated tube via sterile anaerobic technique. After 24 h each tube was inoculated with 2 mL of a microbial consortium eluted from PCB contaminated HR sediment obtained as previously described (*4*). A 10% solution of Aroclor 1242 (Monsanto Co., St. Louis MO) in acetone was then added to each tube to give a final PCB concentration of 250 μg per g air dried sediment. During this procedure the tubes were flushed with filter-sterilized O_2-free N_2/CO_2 (80:20, vol/vol) using a Hungate apparatus. The assays tubes were crimp sealed with sterile Teflon coated butyl rubber stoppers, vigorously vortexed, and then incubated statically in the dark at 22 °C.

Treatments. Various amendments were added to dechlorination assays in order to elucidate the mechanistic basis for the stimulatory effect of $FeSO_4$ on PCB dechlorination. These include an unamended control, an autoclaved plus $FeSO_4$ (10 mM) control, and the following treatments: $FeSO_4$ (10 and 20 mM), Na_2SO_4 (10 mM), $FeCl_2$ (10 mM), $FeSO_4$ (10 mM) plus Na_2MoO_4 (3.7 mM), and Na_2SO_4 (10 mM) plus $PbCl_2$ (10 mM). The treatments were preformed in triplicate. The amendments (1 mL) were added as sterilized, degassed solutions.

Headspace Methane Content. Prior to PCB extraction the headspace gas of each assay was analyzed for methane content utilizing a gas chromatograph coupled to a thermal conductivity detector (Carle Instruments Inc.).

Sample Extraction and PCB Analysis. Triplicate samples of each treatment were sacrificed at predetermined time intervals. The entire contents were solvent extracted, purified, and analyzed for congener specific PCB content as previously described (*4*).

Sulfate and Sulfide Analysis. Additional triplicate samples of each treatment were sacrificed at predetermined time intervals (except weeks 6 and 12 where duplicate samples were sacrificed). Assay vessels were centrifuged and transferred to an anaerobic glovebox where the supernatant was removed and filtered (45 μm filter). A 1 mL portion of the supernatant was transferred to a sample vial and analyzed for sulfate content via. ion exchange chromatography (Dionex, model 2000i); 4 mL were processed for colorometric analysis of sulfide as described by Cline (*18*).

Results and Discussion

The dechlorination of Aroclor 1242 by HR microorganisms was stimulated by the addition of $FeSO_4$. Figure 1 depicts this graphically by plotting change in the average number of meta plus para chlorines per biphenyl over time. In the $FeSO_4$ amended sediments (10 or 20 mM) the average number of meta plus para chlorines per biphenyl was reduced from 1.78 ± 0.02 in the parent Aroclor to 0.30 ± 0.01 in the dechlorinated product mixture. In the unamended controls a more limited dechlorination occurred resulting in 0.80 ± 0.04 meta plus para chlorines per biphenyl. As generally observed for the dechlorination of Aroclors, there was no evidence for the removal of ortho chlorines. Dechlorination did not occur in autoclaved biological controls amended with $FeSO_4$.

The impact of $FeSO_4$ amendment on PCB dechlorination can be better understood in the context of the different dechlorination processes. Microbial dechlorination of individual congeners may vary greatly, even within the same sediment, depending on the PCB mixture, time of incubation, environmental conditions, and microbial populations. Process M is most commonly exhibited by the Hudson River (HR) inoculum used in our investigation and is characterized by the removal of both flanked and unflanked meta chlorine (*9*). As expected, process M occurred in our unamended positive controls resulting in the accumulation of numerous ortho and para chlorinated congeners (Figure 2, histogram B), namely 2-4 CB (peak 7), 24-2 CB (peak 11), 24-4 CB/246-2 CB (peak 19), and 25-25 CB (peak 24). The loss of virtually all para chlorines (process Q) in addition to the loss of meta chlorines (process M) occurred when $FeSO_4$ was used in conjunction with Hudson River inoculum (Figure 2, histogram D). The combination of these two activities (process C) resulted in ~90 mol % of the total PCBs being converted to ortho-substituted mono- and dichlorinated congeners, i.e., 2 CB (peak 1) and 2-2 CB/26 CB (peak 4). Thus, the greater extent of dechlorination observed in the $FeSO_4$ amended treatments occurred because processes M and Q were both active, but only M occurred in the unamended treatment. Quensen and Bedard (*7*) described pattern C as process M and Q occurring in succession, each likely due to the activity of an individual bacterial species or strain.

Pattern C is distinguished by the accumulation of 2 CB and 2-2 CB/26 CB and was originally described in 1987 for PCBs extracted from sediment samples taken from the upper Hudson River (*2*). We also observed this when sediments containing Aroclor 1242 were incubated in the laboratory with organisms freshly eluted from the same location (*4*). However, all our subsequent attempts to obtain process C activity using organisms eluted from the same sediment after cold storage or from fresh samples have been unsuccessful. Additionally, more limited types of dechlorination are more commonly observed in situ and in laboratory experiments using organisms from various locations including New Bedford Harbor, Hudson River, Woods Pond, Silver Lake, and the River Raisin (*7*). It was not until the current study that process C activity could be reproducibly obtained through the use of $FeSO_4$ additions.

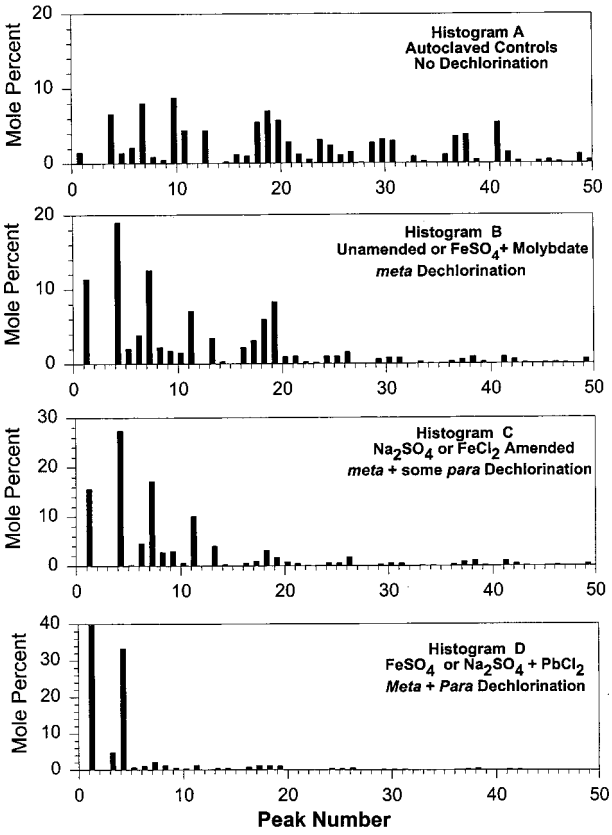

FIGURE 2. Changes in PCB congener profiles resulting from dechlorination (at 32 weeks) can be seen through histogram representations of GC chromatograms. In general peak numbers correlate to chlorine content, with lower numbered peaks representing lesser chlorinated congeners; for complete peak number assignments see Mousa et al. (*5*). Histogram A represents unaltered Aroclor 1242. Histogram B represents end products for the unamended positive controls. This is a typical pattern M congener profile resulting from the accumulations of the congeners 2-4 chlorobiphenyl (CB) (peak 7), 24-2 CB (peak 11), 24-4 CB/246-2 CB (peak 19), and 25-25 CB (peak 24). Histogram C represents congener profiles obtained from slurries amended with Na_2SO_4 or $FeCl_2$. These treatments resulted in more extensive meta dechlorination and a minor amount of para dechlorination. Additions of $FeSO_4$ or Na_2SO_4 + $PbCl_2$ (Histogram D) result in extensive meta and para dechlorination (process C). The result is ~90 mol % of the total PCBs converted to the primarily ortho-substituted mono- or dichlorinated congeners, i.e. 2 CB (peak 1) and 2-2 CB/26 CB (peak 4).

Other researchers attempting to maximize PCB dechlorination have had some success in stimulating more limited para dechlorination processes (P and LP) but not in the presence of process M. Bedard et al. (*19*) demonstrated the priming of para dechlorination of PCBs in Housatonic River and Wood's Pond sediment by the addition of single PCB or polybrominated biphenyl (BB) congeners (e.g. 25-34 CB, 24-34 CB, 245 CB, 25 BB, 26 BB, 25-3 BB). Similarly they have shown that addition of the single congener 23456-CB resulted in both partial meta and para dechlorination, process N and LP, respectively (*20*). Unfortunately, both of these enhancements require the additional introduction of high concentrations of PCB or PBB congeners (~750 ppm) so their practical utility is questionable. Furthermore, to obtain the maximum extent of dechlorination it is critical that both process M and Q are operative. The significance of our observation is that we have identified an innocuous compound that can be used at a reasonable concentration (10 mM or ca. 10.6 lbs $FeSO_4$/ton sediment) to enhance the overall extent of dechlorination by activating the most extensive para dechlorination process (process Q) without inhibiting

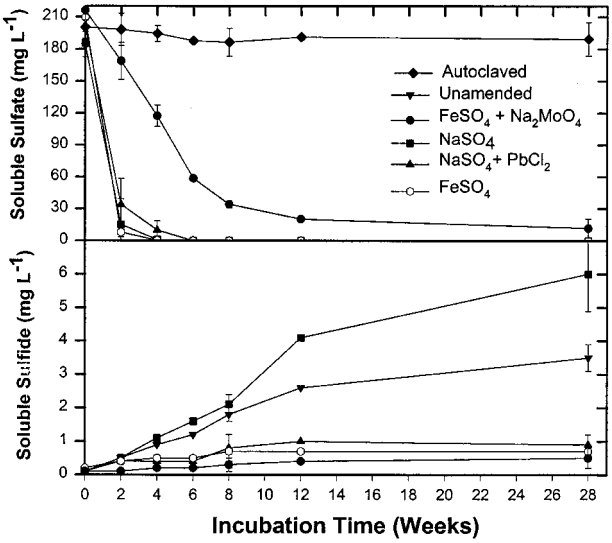

FIGURE 3. Soluble sulfate and sulfide concentrations in assay vessels over time. Data plotted are averages of triplicate samples except those of 6 and 12 weeks, which consisted of duplicates (error bars omitted). Sulfate and/or sulfide data is not shown for treatments in which their respective concentrations remained below 2 and 1 ppm over the course of the experiment. (Data omitted: sulfate and sulfide for $FeCl_2$ treated assays, sulfide in autoclaved controls, and sulfate in untreated Hudson River assays).

process M. No other feasible approaches for enhancing microbial PCB dechlorination have been described.

We propose that $FeSO_4$ provides two mutually beneficial effects. First, it provides sulfate as an electron acceptor, which stimulates the growth of sulfate-reducing bacteria, which are responsible for the para dechlorination activity (process Q). Second, Fe^{2+} removes sulfide formed during sulfate reduction by forming the insoluble precipitate FeS, reducing sulfide bioavailability and hence toxicity. Once sulfate is consumed, an increased number of sulfate reducers utilize PCBs as an alternate electron acceptor, leading to extensive meta and para dechlorination.

Desulfomonile tiedjei, a sulfate reducer that is able to dechlorinate chlorobenzoates, provides a good model for conceptualizing the results reported here. Sulfoxy ions stimulate growth of this organism but inhibit its dechlorination of 3-chlorobenzoate (*21*, *22*). However if the sulfoxy ions become limited, the organism can reductively dechlorinate chlorinated benzoates (*23*, *24*). It seems plausible that the microorganisms responsible for para-dechlorination of PCBs described here, and *Desulfomonile tiedjei*, are both sulfate reducers, whose growth is stimulated by sulfate additions. Then, following depletion of sulfate, they utilize chloroaromatic compounds as electron acceptors resulting in dechlorination. Numerous researchers have tried unsuccessfully to stimulate dechlorination by adding various electron acceptors (SO_4^{2-}, NO_3^-, CO_2, and ferric oxyhydroxide) (*8*, *25*, *26*). However, if the primary electron acceptor must be limiting before dechlorination will occur, as for *Desulfomonile tiedjei*, then large or repeated additions of electron acceptors should inhibit dechlorination, as has been the case in previous studies (*8*). Also, accumulation of reduced substrates such as sulfide can be toxic to these sulfate reducing organisms (*27*). *Desulfomonile tiedjei* is known to be particularly sensitive to sulfide toxicity (*28*).

A series of treatments were designed to separate the effects of Fe^{2+}, sulfate, and sulfide and to test our hypothesis regarding the stimulatory effect of $FeSO_4$. Experimental controls included no amendment (deionized H_2O), and $FeSO_4$ amended sterile and nonsterile controls. A treatment of $FeSO_4$ plus Na_2MoO_4 was used to provide solution concentrations of $FeSO_4$ while simultaneously blocking sulfate reduction. This was designed to establish the involvement of sulfate reducers in the stimulation. An amendment of Na_2SO_4 provided an equal amount of sulfate as the $FeSO_4$ amendment but did not provide Fe^{2+} as a means to bind sulfide; a $FeCl_2$ treatment provided Fe^{2+} but not sulfate. A treatment consisting of Na_2SO_4 and $PbCl_2$ provided sulfate as an electron acceptor as well as an alternate metal (Pb^{2+} rather than Fe^{2+}) to bind sulfide but not provide excess Fe^{2+}.

There was no evidence of para dechlorination in the unamended controls but rather only partial meta dechlorination (Figure 1). Additions of $FeSO_4$ or Na_2SO_4 plus $PbCl_2$ resulted in the activation of para dechlorination to nearly identical extents and patterns of dechlorination, greatest among all treatments (Figure 1). The form of bivalent metal made no difference in the stimulatory effect observed in these

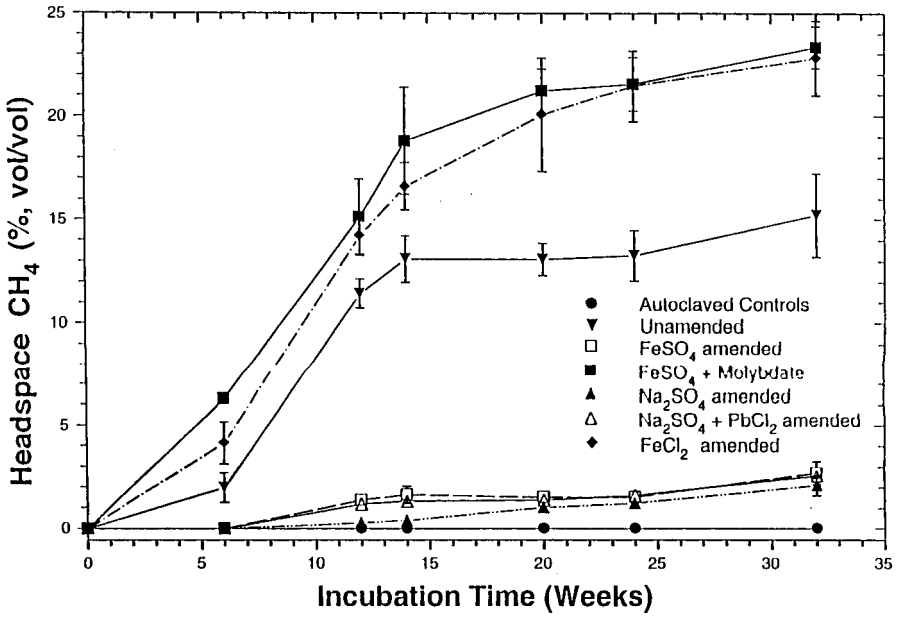

FIGURE 4. Methane content of assay vessel headspace. No methane was detected in the headspace of autoclaved controls. Error bars indicate the standard error of triplicate samples.

two treatments. Both Pb^{2+} and Fe^{2+} will form insoluble metal sulfides due to their extremely low solubility products ($K_{sp} = 1 \times 10^{-19}$ and 7×10^{-29} for FeS and PbS) (*29*). The removal of sulfide was observed visually in the $FeSO_4$ and $PbCl_2/Na_2SO_4$ treatments which produced a black precipitate commencing at week 6, but not in the other two $NaSO_4$ treatments. Measurements of soluble sulfide showed considerably lower sulfide concentrations when sulfate was added with Fe^{2+} or Pb^{2+} as compared to its addition as Na_2SO_4 alone (Figure 3). The addition of Fe^{2+} (as $FeCl_2$) or sulfate (as Na_2SO_4) alone did not manifest the extensive dechlorination observed in the $FeSO_4$ or $PbCl_2/Na_2SO_4$ treatments. These results are consistent with the concept of sulfate additions stimulating growth of the dechlorinating bacteria and Fe^{2+} (or Pb^{2+}) reducing sulfide toxicity.

We propose that the stimulatory effect of $FeSO_4$ on para dechlorination resulted from an increase in the population of sulfate reducing bacteria. The $FeSO_4$ plus Na_2MoO_4 treatment resulted in an extent of dechlorination similar to the unamended controls. Thus, when sulfate was not added (unamended control), para dechlorination did not occur, nor did it occur when a sulfate reduction inhibitor (Na_2MoO_4) was added in conjunction with an otherwise stimulatory sulfate source (i.e. $FeSO_4$). While the available sulfate provided in each of these treatments is not a specific inhibitor of any physiological group, the addition of sulfate usually stimulates sulfate-reducing bacteria and concomitantly inhibits methanogenic bacteria due to bioenergetic advantages (*30*). Here the shift in the terminal electron acceptor to sulfate is evidenced by the lack of methane production in treatments where sulfate is added and the production of methane in the $FeSO_4$ plus Na_2MoO_4 treatment where sulfate reduction is blocked (Figure 4). Furthermore, when sulfate is added in the absence of Na_2MoO_4 (i.e. as Na_2SO_4, $FeSO_4$ or $Na_2SO_4/PbCl_2$) it is depleted rapidly during the first 2−4 weeks of incubation (Figure 3). Each of these observations is consistent with our proposal that sulfate additions stimulated the growth of the dechlorinating bacteria.

Numerous studies have reported that the presence of available sulfate inhibits PCB dechlorination (*8*, *21*, *22*, *25*, *26*, *31*). Here, in treatments where microcosms were supplied with sulfate ($FeSO_4$ or $Na_2SO_4/PbCl_2$), dechlorination was initially inhibited (through week 4) as compared to the no amendment control (Figure 1). Dechlorination in these treatments commenced between weeks 4 and 6, corresponding exactly to the depletion of sulfate (Figure 3). We suspect that the initial inhibition of dechlorination was due to a shift in the electron acceptor from PCBs to sulfate; the higher free energy available from sulfate reduction initially stimulated the growth of the dechlorinating microorganisms while simultaneously suspending PCB dechlorination. Consistent with this is the observation that after the initial inhibition the dechlorination rate in the $FeSO_4$ and $Na_2SO_4/PbCl_2$ treatments was significantly higher than in the unamended controls. This would not be expected in $FeCl_2$ or $FeSO_4$ plus Na_2MoO_4 amended treatments and was not observed. These results suggest that sulfate initially stimulates growth of the dechlorinating population but inhibits dechlorination, which commences once sulfate is depleted. Last, microbial community analysis via denaturing gradient gel electrophoresis (DGGE) of 16S rDNA revealed similar microbial community genotypic makeup in these two treatments which differed from each of the other treatments (unpublished data).

Our interpretation of the underlying mechanisms is also consistent with other observations regarding the microbial physiology of microorganisms able to dechlorinate PCBs. In the anaerobic environment two of the main metabolic pathways are sulfate reduction and methanogenesis. Both meta and para dechlorinating activities obtained from HR sediment occurred in the absence of measurable methanogenic activity, suggesting that the responsible microorganisms were not methanogens (*10*, *31*). In addition May et al. (*31*) have had some success subculturing HR organisms on solid media with the ability to para dechlorinate PCBs. Consistent with our observations, these subcultures were based on a primary enrichment for sulfate reducers. In addition these cultures did not express dechlorination activity until sulfate was depleted.

The data presented herein demonstrates that $FeSO_4$ addition to sediment slurries containing PCB dechlorinating bacteria stimulates the para dechlorination of PCBs. This, in conjunction with the more stable and widely distributed meta dechlorination activity of the unamended controls, resulted in nearly complete removal of meta and para chlorines from Aroclor 1242 and the accumulation of 2 CB and 2-2 CB/26 CB as terminal products. $FeSO_4$ appears to be an effective, inexpensive, and innocuous amendment for stimulating extensive PCB dechlorination. This greatly improves the potential to utilize PCB dehalogenation as a practical and effective remediation method both in situ and ex situ.

Acknowledgments

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