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EFFECT OF CONTAMINANT AND ORGANIC MATTER BIOAVAILABILITY ON THE MICROBIAL DEHALOGENATION OF SEDIMENT-BOUND CHLOROBENZENES

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Abstract—The extent of reductive dechlorination occurring in contaminated, estuarine sediments was investigated. Contaminant and organic matter bioavailability and their effect on the reductive dechlorination of sediment-bound chlorobenzenes was the main focus of the work presented here. Sediment and water samples were collected from a contaminated estuarine system with more than 40 yr of contamination history. Hexachlorobenzene and other chlorinated benzene congeners were the predominant chlorinated compounds encountered. Anaerobic batch assays revealed that the sediment natural organic matter is recalcitrant and unable to support active microbial growth. However, sediment nutrients (e.g. N, P) were available in sufficient quantities to support an accelerated microbial growth. Static microcosms were constructed with sediment and water from the study site. The sediment microbial consortia were able to reductively dechlorinate the sediment-bound polychlorinated benzene congeners, primarily to dichlorobenzene isomers. The first-order rate constant for hexachlorobenzene depletion (k_{obs}) ranged from 2.2×10^{-3} to $2.9 \times 10^{-3}\ d^{-1}$, the higher value being achieved in microcosms supplemented with dextrin. These rates were about two orders of magnitude lower than those observed with the same sediment-derived, mixed dechlorinating microbial consortium in liquid cultures supplemented with freshly added hexachlorobenzene. The extent of hexachlorobenzene removal over a long incubation time (more than 480 d) was only 43%, most of which occurred during the first 200 d of incubation. Both the recalcitrant nature of the sediment organic matter and the strong partitioning of the chlorinated compounds were responsible for the low rate and extent of contaminant transformation. When frequent additions of a degradable organic carbon source and ground sediment were used, an increased rate and extent of reductive dechlorination of the sediment-bound contaminants were observed (e.g. 95% hexachlorobenzene biotransformation within 205 d of incubation; $k_{obs} = 2.1 \times 10^{-2}\ d^{-1}$). Copyright © 1996 Elsevier Science Ltd

Key words—bioavailability, chlorobenzenes, estuary, reductive dechlorination, sediments

INTRODUCTION

As a result of past unregulated industrial wastewater discharges into natural water bodies, anthropogenic, nonpolar hydrophobic and recalcitrant organic contaminants have accumulated in aquatic sediments. Chlorinated organic compounds (e.g. polychlorinated byphenyls, dioxins, chlorophenols, chlorobenzenes, DDT, and other chlorinated pesticides) predominate in contaminated sediments (Eisenreich *et al.*, 1989; El-Gendy *et al.*, 1991; Picer and Picer, 1993). Because of extensive industrial and agricultural use of chlorinated benzenes over several decades, these compounds are now ubiquitous in aquatic environments (Beurskens *et al.*, 1994). Highly chlorinated benzene congeners have low water solubility and volatility, as well as a high octanol–water partition coefficient. Because of their hydrophobic character, these compounds accumulate in aquatic sediments.

Reductive dehalogenation of chlorinated organic compounds takes place in reduced environments such as deep soils and sediments and is mediated by native microbial consortia acclimated to these contaminants (Sims *et al.*, 1990; Mohn and Tiedje, 1992). An increase in the ratio of di- and trichlorobenzenes to hexa- and pentachlorobenzene with increasing sediment depth in the Great Lakes has been taken as indirect evidence of the reductive dechlorination of highly chlorinated benzenes (Oliver and Nicol, 1982). Therefore, as a result of reductive dechlorination, less chlorinated benzene congeners—which are more soluble and volatile than the polychlorinated benzenes, and thus more mobile—are produced. Thus, a renewed source of low-level, long-term contamination to the overlying water column may exist due to these biotransformation processes.

Microbial reductive dechlorination of hexachlorobenzene (HCB) and other polychlorinated

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benzene congeners has been well documented (Tsuchiya and Yamaha, 1983; Bosma *et al.*, 1988; Fathepure *et al.*, 1988; Mousa and Rogers, 1990; Fathepure and Vogel, 1991; Liang and Grbić-Galić, 1991; Holliger *et al.*, 1992; Ramanand *et al.*, 1993; Beurskens *et al.*, 1994). Also, the less chlorinated benzene congeners (e.g. dichlorobenzenes) are amenable to aerobic degradation (Reineke and Knackmuss, 1984; deBont *et al.*, 1986; Schraa *et al.*, 1986; Spain and Nishino, 1987). However, there is a lack of information on the factors which control both the rate and extent of transformation of chlorobenzenes sorbed to natural contaminated sediments. In order to address this need, we have undertaken an investigation into the contaminant fate processes, in particular microbial reductive dechlorination taking place in the sediments of Bayou d'Inde, a tributary of the Calcasieu River near Lake Charles, Louisiana. The major chlorinated contaminants in these sediments are HCB and other less chlorinated benzene congeners.

The Bayou d'Inde tributary has received unregulated industrial waste discharges from chemical manufacturing and petroleum industries for over 40 yr. Many classes of contaminants including petroleum hydrocarbons, polycyclic aromatic hydrocarbons, and many polychlorinated aromatic and aliphatic organic compounds have been detected in the sediments, as well as the overlying water column and the native biota of the tributary and downstream regions of the estuary (Cunningham *et al.*, 1990; Pereira *et al.*, 1988; Murray *et al.*, 1992). Due to the toxicity and carcinogenicity of these contaminants, seafood consumption and primary contact recreation advisories have been issued (Mathison, 1987; LaDEQ, 1989).

The objective of this paper is to report on the extent of reductive dechlorination taking place in the contaminated estuarine sediments. Contaminant and organic matter bioavailability and their effect on the reductive dechlorination of the sediment-bound chlorobenzenes was the main focus of the work presented here.

MATERIALS AND METHODS

Sample collection and preparation

The study site is at the intersection of an industrial canal and Bayou d'Inde (Pereira *et al.*, 1988). The canal was used by a chemical plant manufacturing trichloroethylene and perchloroethylene for the discharge of effluents into Bayou d'Inde. Water quality parameters were measured *in situ* with a Hydrolab Surveyer 2 equipped with a SVR2-SU multiprobe unit. Surface water samples were collected and stored and surface sediment samples collected with a stainless steel Wildco Petite Ponar grab sampler and added to plastic carboys with minimum headspace. Water and sediment samples were transported to the laboratory and kept under refrigeration (4°C). The sediment samples were first sieved wet through a stainless steel screen (0.25 in. square openings), and then through a US Standard sieve No. 14 (1.4 mm). Subsets of the sieved and well homogenized sediment samples were added to wide-mouth glass jars and sealed with Teflon-lined screw caps providing minimal headspace. All sediment and water samples were stored at 4°C in the dark.

Nutrient availability assay

To investigate whether or not sediment nutrient availability may be limiting *in situ* microbial growth (and hence contaminant biotransformation), a nutrient bioavailability assay was conducted by monitoring microbial methanogenic activity in four series of serum bottles. Series I and II were incubated at 23°C and series III and IV at 35°C. An external electron donor was added only to series II and IV. Each of these four series was subdivided to include four separate nutrient conditions: 1) no amendment; 2) nitrogen; 3) phosphorus; and 4) methanogenic media. The methanogenic media contained (in g/l): K_2HPO_4, 0.87; KH_2PO_4, 0.54; NH_4Cl, 0.50; $CaCl_2 \cdot 2H_2O$, 0.06; $MgSO_4$, 0.05; $4MgCO_3 \cdot Mg(OH)_2 \cdot nH_2O$, 0.05; $NaHCO_3$, 1.20; and $Na_2S \cdot 9H_2O$, 0.50. Also, 1 ml/l each of vitamin (Wolin *et al.*, 1963) and trace metal (Mah and Smith, 1981) stock solutions were added. A sediment slurry with a total chemical oxygen demand (COD) concentration of 3 g/l was prepared with deionized water and flushed with helium. Aliquots of 20 ml of this slurry were added anaerobically to helium-flushed, 60 ml serum bottles and then diluted to 40 ml as described below. The final total COD concentration was 1.5 g/l. Excess alkalinity was provided by adding 4.2 g $NaHCO_3$/l (final concentration). Nitrogen and phosphorus amendments were achieved by adding 0.465 g NH_4Cl/l or 0.107 g K_2HPO_4/l (final concentration; nutrient conditions 2 and 3, respectively). In those bottles amended with external electron donor (series II and IV), acetate and lactate were added at a final total COD concentration of 1 g/l (1:1 acetate to lactate COD ratio). The balance of the 40 ml total slurry volume in each bottle was made up by adding deionized water (nutrient conditions 1–3) or methanogenic media (nutrient condition 4). The serum bottles were sealed with rubber stoppers and aluminum crimps. Five replicates of each of the 16 sample variations were prepared. Incubation took place at either 23°C (series I and II) or 35°C (series III and IV) in the dark. Periodically, excess gas production and gas composition were measured and the bottles were manually shaken.

Biotransformation assays

Biotransformation of HCB and its dechlorination products was studied under both simulated *in situ* conditions and conditions intended to enhance native microbial activity and/or increase the bioavailability of the sediment contaminants for biotransformation. Chlorinated benzenes were not added to the microcosms in any of the biotransformation assays, but rather the field contaminated sediment samples were the source of the contaminants. To perform these studies, two assays were carried out in 2 l glass bottles (1800 ml liquid volume). Incubation was carried out in the dark at 23°C. Periodically, the bottles were manually shaken. Continuous mixing was avoided to prevent breaking up of the sediment particles.

Three microcosms were prepared for the first biotransformation assay. Sediment slurries were prepared by diluting sediment samples to a final solids concentration of ca. 10% (w/w) with water obtained from the study site. No other amendments were made to the first microcosm (microcosm I). The second microcosm (microcosm II) was amended with dextrin as a supplemental electron donor at a final concentration of 400 mg/l. Dextrin requires hydrolysis and was therefore added to serve as a slowly available electron donor. Sodium azide was added at a final concentration of 2 g/l to inhibit any microbial activity in the third microcosm (microcosm III). To determine the biodegradability of the sediment organic matter, three sets of four replicate, 60 ml serum bottles (40 ml liquid volume) were also set up identically to the above described three

microcosms and incubated at 23°C in the dark. Periodically, total gas production and composition were measured, and at the end of the incubation, total COD destruction was also determined.

Four microcosms were prepared for the second biotransformation assay. Two different sediment slurries, each at a final solids concentration of ca. 10% (w/w) were used. Microcosms A and B were prepared by diluting wet sediment samples with methanogenic media (composition given above), whereas microcosms C and D were prepared with sediment that had been air dried, and then ground in a ceramic mortar until all particles passed a 500 μm sieve. The ground sediment was reconstituted in methanogenic media. Previous research (Prytula and Pavlostathis, 1996) has indicated that sediment drying and grinding improved the contaminant extraction efficiency. It was also shown that most of the contamination in the surfacial Bayou d'Inde sediments resides in the sediment fraction with a particle size range of 106–1400 μm. In light of these observations, it was postulated that the bioavailability of the sediment-bound contaminants might be enhanced by sediment grinding. To restore microbial activity to microcosm C, 180 ml (10% of the final microcosm volume) of an anaerobic, HCB-dechlorinating liquid culture derived from the wet sediment was added. To establish similar initial conditions in both microcosms A and C, 180 ml of the same inoculum was also added to microcosm A. Microcosms A and C were supplemented about every 7 to 14 days with a readily available electron donor and nutrients. Each feeding supplied 0.4 g each of acetate and lactate, and 0.04 g of yeast extract dissolved in 4 ml of deionized water. Microcosms B and D were amended with sodium azide at a final concentration of 2 g/l to inhibit any microbial activity.

For both biotransformation assays, the glass bottles were initially flushed with helium, filled with sediment slurry and corresponding amendment solutions transferred anaerobically, and then capped with Teflon-lined septa and aluminum crimps. Slurry samples were periodically withdrawn while the microcosm contents were vigorously mixed with a magnetic stirrer. Triplicate 25 ml slurry samples were transferred into 50 ml Teflon tubes and centrifuged for 25 min at 12,000 rpm. The supernatant was withdrawn and liquid/liquid extracted using iso-octane. The pellets were frozen and stored overnight, prior to Soxhlet extraction. The headspace of each microcosm was monitored for volatile organic compounds by withdrawal of duplicate 10 or 20 ml gas samples and extraction with 0.5 or 1.0 ml iso-octane, respectively.

Analytical methods

Oxidation–reduction potential (ORP), pH, and COD were measured by following procedures outlined in *Standard Methods* (APHA, 1989). A platinum electrode with an Ag/AgCl reference in a 3.5 M KCl gel (Sensorex, Stanton, CA) was used for the ORP measurements. The sediment total organic carbon was measured using a modified Mebius dichromate reflux procedure (Nelson and Sommers, 1982). The sediment specific surface area was measured by the triple-point Brunauer–Emmett–Teller (BET) method with a Nova-1000 Gas Sorption Analyzer (Quantachrome Corporation, Syosset, NY) using N_2. Sediment samples were prepared by oxidizing the organic carbon with 10% H_2O_2. The samples were sequentially washed with hydrogen peroxide solution until gas evolution ceased, washed three times with deionized water, dried at 50°C, and then gently homogenized in a small mortar.

Five methods were tested for the extraction of sediment-bound chlorinated compounds, as follows: Soxhlet extraction, sonication and solvent extraction, sequential solvent extraction, saponification and solvent extraction, and supercritical fluid extraction (Prytula and Pavlostathis, 1996). However, 20 h Soxhlet extractions using 1:1 acetone/hexane (US EPA, 1986; Method 3540) resulted in better contaminant extraction efficiency and reproducibility, and this method was adopted for the present study. After transferring the solid sample to the extraction thimble, 1 ml of a 1,3,5-tribromobenzene (TBB) stock solution in hexane was added to the thimble and served as a surrogate standard. Recoveries of TBB were consistently between 95 and 105%. Liquid samples were extracted in serum tubes with TBB-containing iso-octane at a sample to solvent volume ratio of 10:1. Phase separation was accomplished by centrifuging at 3000 rpm for 20 min. Solvent extracts from both the Soxhlet and liquid/liquid extractions were transferred to GC autosampler vials and sealed with Teflon-lined septa and aluminum crimps. Quantification of the chlorinated compounds was performed using an HP 5890 Series II gas chromatography unit (Hewlett Packard, Palo Alto, CA) equipped with an electron capture detector (ECD) and a 60 m DB-624 magabore column (J&W Scientific, Folsom, CA). The following temperature program was used: 100°C for 4 min, increase by 2°C/min to 210°C and hold for 6 min. Nitrogen was used as the carrier gas at a flow rate of 10 ml/min. Calibration curves were prepared using pure chemicals (Aldrich Chemical Co., Milwaukee, WI) dissolved in iso-octane. TBB was used as the internal standard. The method detection limit of monochlorobenzene (MCB) was 10 g/kg and 10 mg/l for the Soxhlet and liquid/liquid extraction method, respectively. All other chlorinated benzene cogeners had a detection limit of 0.2 mg/kg and 0.2 μg/l for the same two methods, respectively.

Gas composition (methane, carbon dioxide, and hydrogen sulfide) was determined using an HP 5890 Series II gas chromatography unit (Hewlett Packard, Palo Alto, CA) equipped with a thermal conductivity detector. Methane was separated using a 10 m Molsieve 5 Å megabore column and CO_2 and H_2S were separated on a 25 m PoraPlot Q megabore column (Chrompack, Raritan, NJ). Both columns were operated isothermally at 30°C with helium carrier gas. Total gas production was measured by displacement of water and collection of the excess gas in a buret. For the determination of sediment total sulfide content, sediment samples were mixed with 6 N H_2SO_4 in the ratio of 5:1 (v/v) in serum bottles flushed with helium. The samples were then incubated for 1 h at 35°C and gas samples were analyzed for H_2S by gas chromatography as described above. A calibration curve was prepared by using a standard Na_2S solution which was taken through the same acidification and gas analysis procedure. The concentration of Na_2S was determined by the iodometric method (APHA, 1989).

RESULTS AND DISCUSSION

Water and sediment analyses

The results of *in-situ* measurement of several water quality parameters at a depth of 1 m below the air–water interface during April 1993 are as follows: temperature, 29°C; pH, 7.3; DO, 5.7 mg/l; conductivity, 4630 μmho/cm; salinity, 2.5‰. Repeated *in situ* measurements, as a function of water depth all the way to the sediment–water interface (4.5 m) during March 1994, revealed little parameter variability. The water column at the study site is therefore well aerated and mixed. The water conductivity and salinity were much lower than previously reported values of 9000–14,000 μmho/cm and 5–9.6‰, respectively, for the Bayou d'Inde tributary (Demcheck *et al.*, 1990; Pereira *et al.*, 1988). The lower salinity values reported here are due to the

Table 1. Sediment and interstitial water concentrations of chlorinated organic compounds

Compound	Abbreviation	Sediment (mg/kg)	Interstitial water (μg/l)
Hexachlorobutadiene	HCBD	17.2 ± 1.0[a]	15.6 ± 0.4
Hexachlorobenzene	HCB	168.8 ± 41.1	5.5 ± 0.6
Pentachlorobenzene	PeCB	21.6 ± 3.3	2.9 ± 0.3
1,2,3,5-Tetrachlorobenzene	1,2,3,5-TeCB	6.0 ± 0.4[b]	8.0 ± 0.3[b]
1,2,4,5-Tetrachlorobenzene	1,2,4,5-TeCB	—[b]	—[b]
1,2,3,4-Tetrachlorobenzene	1,2,3,4-TeCB	1.1 ± 0.2	ND
1,2,4-Trichlorobenzene	1,2,4-TrCB	5.1 ± 0.5	13.4 ± 0.2
1,3,5-Trichlorobenzene	1,3,5-TrCB	13.0 ± 1.1	67.2 ± 1.0
1,3-Dichlorobenzene	1,3-DCB	38.1 ± 5.2	149.4 ± 6.5
1,4-Dichlorobenzene	1,4-DCB	9.5 ± 0.5	67.1 ± 4.4

[a]Mean ± standard deviation ($n = 3$).
[b]Sum of 1,2,3,5- and 1,2,4,5-TeCB.
ND = not detected.

fact that these measurements were conducted a few days after a heavy rainy season.

Water samples collected at the intersection of the industrial canal and Bayou d'Inde were analyzed in the laboratory and yielded the following results (mean ± standard deviation; $n = 3$): pH, 7.6 ± 0.1; alkalinity, 50 ± 3 mg/l as $CaCO_3$; total dissolved solids, 2555 ± 13 mg/l; volatile dissolved solids, 361 ± 26 mg/l; chloride, 1220 ± 5 mg/l; sulfate, 76 ± 5 mg/l; COD, 107 ± 10 mg/l; organic carbon, 13 ± 1 mg/l. Wet sieving of sediment samples resulted in the following particle size classification (dry weight basis): medium sand (850–1400 μm), 1.9%; fine sand (75–850 μm), 19.7%; and silt plus clay (<75 μm), 78.4%. Whole-sediment analyses gave the following results (mean ± standard deviation; $n = 3$): pH, 7 ± 0.2; alkalinity, 1760 ± 10 mg/l as $CaCO_3$; ORP, −410 ± 10 mV; total organic carbon, 4.8 ± 0.3% (dry weight basis); volatile solids, 10.3 ± 0.04% (dry weight basis); total sulfide, 1.47 ± 0.06 mg/g dry mass; COD, 30 ± 1 mg/g wet mass; specific surface area, 31.3 ± 0.5 m^2/g. The results of Soxhlet (sediment samples) and liquid/liquid (interstitial water) extractions are shown in Table 1. HCB was the predominant chlorinated organic compound in the sediment. Significant hexachloro-1,3-butadiene (HCBD) concentrations were also detected.

Nutrient bioavailability

Growth of the native microbial consortium was observed under various nutrient and temperature conditions. Total gas and methane production after 307 d of incubation are shown in Table 2. Very low microbial activity was observed at both 23 and 35°C in the unamended sediment samples. Likewise, when the sediment was supplemented with nitrogen, phosphorus, or even methanogenic media without any addition of electron donor, increase in microbial activity was not observed. At both incubation temperatures, the difference in total gas production at the four nutrient conditions was not statistically significant ($\alpha = 0.05$). The higher incubation temperature resulted in higher total gas and methane production. These results indicate that growth derived from the degradation of the sediment natural organic matter is not nutrient-limited since increased nutrient levels did not enhance the extent or the rate of organic matter degradation. The capacity of the sediment nutrients to support growth was further tested by the addition of acetate and lactate as readily utilizable organic substrates. Microbial activity in the presence of these carbon sources increased much more rapidly, as methane production was observed at both incubation temperatures after only 15 d. Again, methane production was faster at 35 than at 23°C, but there was no increase due to the addition of nitrogen, phosphorus, or methanogenic media in either case. At 35°C, methane production from the readily available carbon sources was complete by day 50, whereas it took 98 d at 23°C (Fig. 1). These results indicate that the sediment contains ample supplies of nutrients to support a level of microbial growth and activity substantially higher than that observed as a result of natural organic matter degradation.

Organic matter bioavailability

The biodegradability of the sediment organic matter was assessed by measuring total gas and gas

Table 2. Total gas and methane production during the nutrient availability assay[a]

	Series I (23°C)		Series II (23°C)		Series III (35°C)		Series IV (35°C)	
Amendment[b]	Total gas	CH_4	Total gas	CH_4	Total gas	CH_4	Total gas	CH_4
None	0.15 ± 0.07	0.07 ± 0.001	14.3 ± 0.2	9.6 ± 0.4	1.8 ± 0.5	0.66 ± 0.003	16.9 ± 0.2	12.6 ± 0.2
N	0.13 ± 0.18	0.03 ± 0.001	13.4 ± 1.8	9.6 ± 0.4	2.0 ± 0.7	0.58 ± 0.004	17.0 ± 0.9	13.7 ± 0.2
P	0.09 ± 0.13	0.12 ± 0.001	14.7 ± 0.2	10.1 ± 0.1	1.5 ± 0.5	0.59 ± 0.003	16.7 ± 0.5	13.0 ± 0.1
Media	0.28 ± 0.09	0.05 ± 0.001	13.9 ± 0.3	9.4 ± 0.1	1.8 ± 0.2	0.43 ± 0.002	17.5 ± 0.5	12.6 ± 0.1

[a]In ml at STP after 307 d of incubation (mean ± standard deviation; $n = 5$).
[b]External electron donor added only to series II and IV.

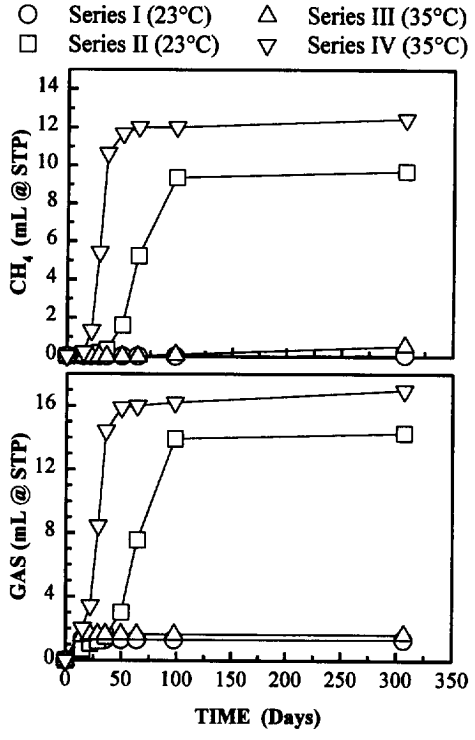

Fig. 1. Cumulative gas and methane produced during the sediment nutrient bioavailability assay (mean of all four nutrient conditions within each culture series).

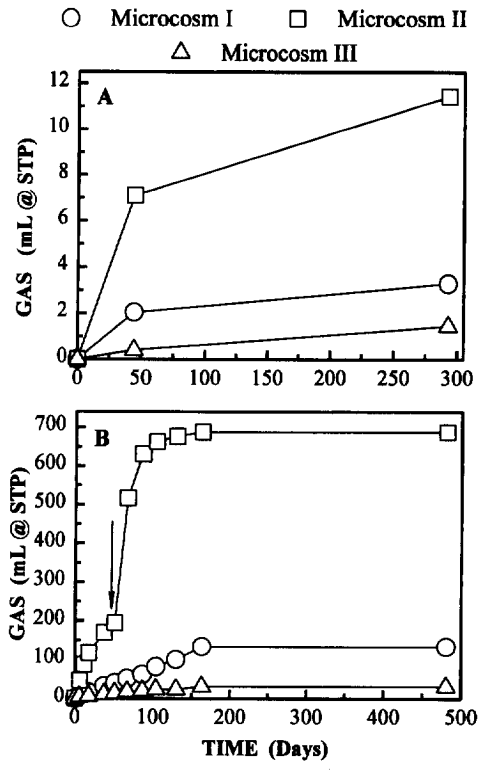

Fig. 2. Cumulative gas as a function of incubation time during the first chlorobenzene biotransformation assay. (A) Serum bottles; (B) microcosms (arrow indicates second dextrin addition to microcosm II).

composition, as well as COD destruction in both serum bottles and 2 l microcosms prepared with unamended, dextrin-amended, and azide-amended sediment samples. The incubation of the serum bottles and microcosms lasted for 293 and 481 d, respectively. Cumulative total gas profiles are shown in Fig. 2. The dextrin-amended systems produced significantly higher volumes of gas as compared to the systems which did not receive dextrin. When the rate of gas production decreased in the dextrin-amended microcosm (microcosm II) after 52 d of incubation, dextrin was added again in order to observe the effect of maintaining high microbial growth and activity on the extent of biotransformation of the sediment-bound contaminants. After the second additon of dextrin, a very rapid gas production rate was observed which practically leveled off by day 87 (Fig. 2). The initially dextrin-amended serum bottles did not receive a second dextrin addition. Significant quantities of CO_2 and CH_4 and only traces of H_2S were detected in the unamended and dextrin-amended systems. CH_4 and H_2S were not detected in the azide-amended systems.

The overall COD destruction increased from 4.8 to 17.3% and from 9.7 to 22.9% in the dextrin-amended serum bottles and microcosms, respectively (Table 3). This increase is due to the degradation of dextrin but also to the enhanced degradation of the sediment organic matter. If the dextrin contribution is taken into account, the sediment COD destruction amounts to 14.5 and 13.8% for the serum bottle and microcosms, respectively. Very low COD destruction was observed in the azide-amended systems, which is within the COD experimental error. The specific methane production (SMP; ml CH_4 produced per g COD destroyed) in this bioassay varied from 54 to 132. These values are significantly lower than the theoretical SMP value of 350 ml CH_4/g COD destroyed at standard temperature and pressure conditions (STP; 0°C and 1 atm). Therefore, alternate electron-consuming processes (e.g. iron, manganese, and sulfate reduction) may be responsible for the observed low SMP values. However, based on a stoichiometric ratio of 0.67 mg COD consumed/mg SO_4^{2-} reduced and the measured sulfate levels in the Bayou d'Inde water which was used to prepare the microcosms, sulfate reduction could account for only

Table 3. Overall COD destruction in sediment slurries

System	Amendment	COD destruction (%)[a] in: Serum bottles[b]	Microcosms[c]
I	None	4.8 ± 6.9	9.7 ± 6.7
II	Dextrin	17.3 ± 4.4	22.9 ± 5.3
III	Sodium azide	2.3 ± 3.7	5.0 ± 2.9

[a]Mean ± standard deviation.
[b]After 293 d of incubation at 23°C (n = 4).
[c]After 481 d of incubation at 23°C (n = 3).

4 and 1.7% of the total COD destroyed in microcosms I and II, respectively.

These experimental results indicate that although the carbon content of the sediment is high (4.8% on a dry weight basis), the sediment organic matter is highly recalcitrant and cannot support a high level of microbial activity. The sediment nutrients, however, appear to be sufficiently available to allow levels of growth substantially above that observed with the *in situ* carbon sources. The activity of the sediment microorganisms is thus limited by the rate at which the natural organic matter can be hydrolyzed and converted into bioavailable forms. Microbial reductive dehalogenation requires an active primary metabolic process (e.g. methanogenesis) for the continuous supply of electrons used for this reductive process (Pavlostathis and Zhuang, 1991; 1993; Zhuang and Pavlostathis, 1994). Therefore, the biotransformation rates of the sediment-bound chlorinated benzenes via microbial reductive dehalogenation would also be expected to be slow.

Biotransformation of chlorobenzenes

Biotransformation assay I. The first biotransformation assay was performed to determine the extent of reductive dechlorination of the sediment-bound chlorinated benzenes as mediated by the natural microbial consortia. Incubation lasted for a total of 481 d. Within the first 50 d of incubation, the ORP decreased from about −50 mV to −150 mV for the azide-amended microcosm and to −250 and −270 mV for the unamended and dextrin-amended microcosms, respectively. The dextrin-amended microcosm demonstrated the fastest rate of ORP decline. The pH in all three microcosms remained between 6.5 and 7 for most of the incubation period.

The solid-, liquid-, and gas-phase contaminant concentrations were periodically measured over the incubation period. The liquid- and solid-phase concentrations of HCB and its dechlorination products over the incubation period are shown in Figs 3 and 4, respectively. Contaminant mass balances were performed periodically and results are shown in Table 4. Sequential reductive dechlorination of the highly chlorinated benzene congeners occurred and DCB isomers (predominantly 1,3-DCB and 1,4-DCB) were the most abundant products. An increase in the solid- and liquid-phase concentrations of DCB in the two microbially active systems (microcosms I and II) accompanied by a drop in liquid-phase concentrations of the more chlorinated benzenes to levels at or near 1 μg/l were observed (Fig. 3). DCB accumulation and removal of the other chlorinated benzene congeners occurred most rapidly in the dextrin amended microcosm. A similar extent of biotransformation, although at a slower overall rate, was observed in the unamended microcosm. DCB did not accumulate in the azide-amended microcosm and the liquid-phase concentrations of the other contaminants increased over time as a result of desorption (Fig. 3).

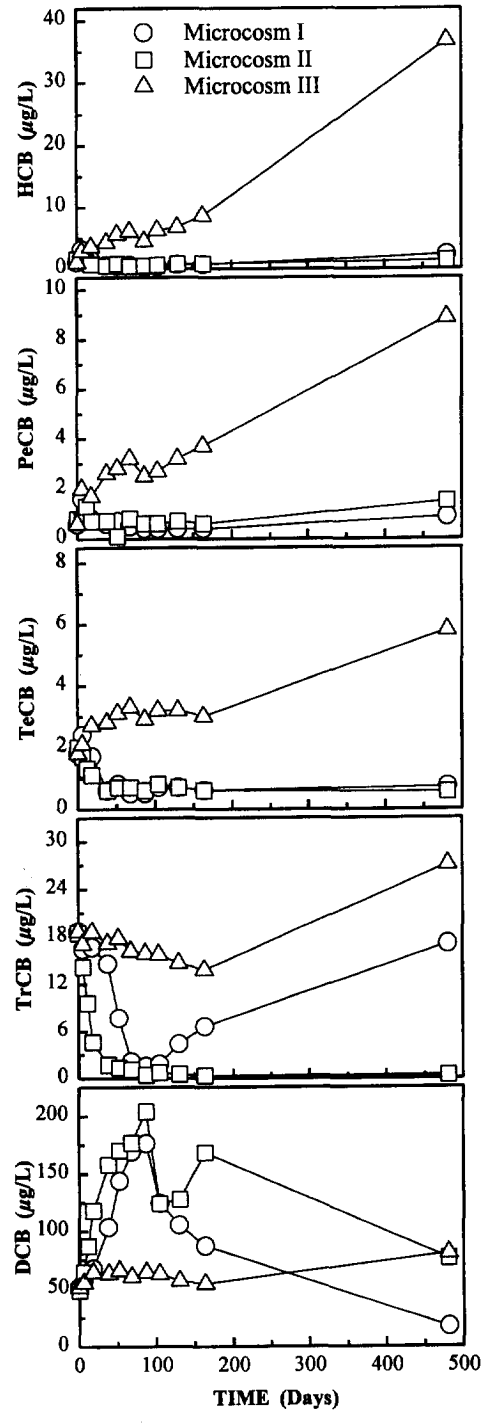

Fig. 3. Liquid-phase concentrations of the chlorinated benzene congeners during the first biotransformation assay.

Over the 481 d incubation period, the solid-phase HCB concentrations decreased by 43, 54, and 33% in unamended, dextrin-amended, and azide-amended microcosms, respectively. However, most of the observed HCB decrease took place in less than 200 d of incubation. Little change in the HCB solid-phase

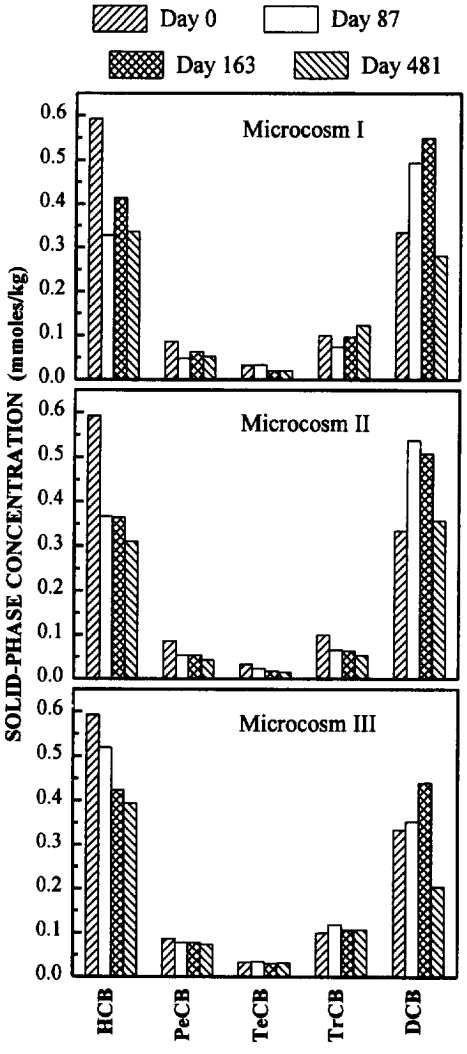

Fig. 4. Solid-phase concentrations of the chlorinated benzene congeners during the first biotransformation assay.

concentration was observed beyond this incubation time. As a result of microbial activity, the higher HCB removals observed in microcosms I and II were accompanied by higher increases in DCB as compared to the azide-amended microcosm (Fig. 4). Significant accumulation of the less chlorinated benzene congeners did not take place in the azide-amended microcosm. Desorption of sediment-bound HCB, as well as losses due to HCB sorption on to the microcosm glass may have contributed to the decline of the HCB solid-phase concentration in the azide-amended microcosm. A build-up of DCB in the solid, liquid, and gas phases of microcosms I and II was observed. Over time, a general decrease in DCB concentrations in all three reactors was observed. This could be due to the fact that the rate of accumulation due to biotransformation was exceeded by losses, possibly resulting from volatilization and diffusion of DCB through the Teflon-lined septum over the long incubation period.

Table 4. Mass balance of chlorobenzene congeners during the first biotransformation assay as a function of incubation time[a]

Incubation time (d)	Microcosm I	Microcosm II	Microcosm III
0	100	100	100
87	86.0	95.2	89.0
163	93.4	93.9	89.3
481	83.8	87.2	81.9

[a]Total (i.e. sum of solid, liquid, and gas phase) contaminant mass balance relative to the initially measured contaminant mass.

Because of very slow desorption, only about 4% of the initial total moles of the contaminants were found in the liquid and gas phases of the azide-amended reactor at 87 d of incubation. In contrast, for the same incubation period, 15 and 19% of the initial contaminant mass was released—and accumulated in both the liquid and gas phase as the less chlorinated benzenes—in the unamended and electron donor-amended microcosms, respectively. Therefore, microbial activity not only results in contaminant transformation, but can enhance the release of contaminants from the sediment. As a result of microbial reductive dechlorination, less chlorinated and hydrophobic compounds are produced, which in turn are more soluble and volatile, and hence more mobile. It is noteworthy that a larger mass of contaminants was released in the biologically active microcosms, which is probably due to maintaining a high driving force for contaminant desorption as the aqueous concentrations of the polychlorinated contaminants were kept at very low levels in these systems.

Extremely low desorption rates and extent of the sediment-bound chlorinated benzenes were previously observed for sediments collected from the Bayou d'Inde tributary (Gess, 1994). In addition, due to salinity effects, elevated octanol–water partition coefficients of chlorinated organic compounds encountered in the Bayou d'Inde have been reported (Pereira *et al.*, 1988). Therefore, salinity and the long contaminant residence time may be responsible for the observed limited contaminant bioavailability.

Biotransformation assay II. A second biotransformation assay was carried out to investigate the effects of organic carbon and contaminant bioavailability on the rate and extent of biotransformation of the sediment-bound contaminants. Figure 5 shows the cumulative gas production profiles for all four microcosms during the 205 d incubation period. Both pH and ORP remained fairly constant due to the presence of the pre-reduced and buffered methanogenic media (pH ranged from 7.3 to 7.7; ORP ranged from −225 mV to −265 mV). The two microbially active microcosms (A and C) achieved the lowest ORP values. Methane was only produced in microcosms A and C, and amounted to 3.49 and 3.44 l at STP, respectively, after 205 d of incubation. Carbon dioxide was measured in all four microcosms (0.71, 0.02, 0.76, and 0.02 l at STP after 205 d of incubation in microcosms A to D, respectively). It is important to note that the total gas, carbon dioxide,

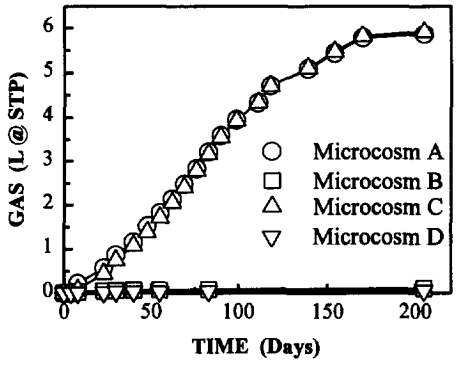

Fig. 5. Cumulative gas as a function of incubation time during the second chlorobenzene biotransformation assay.

and methane production resulting from microbial activity are almost identical in microcosms A and C. This indicates that the biological activity initiated in microcosm C by the addition of the sediment-derived liquid culture inoculum was equivalent to that in the wet sediment microcosm which, in addition to the inoculum amendment, contained native sediment microorganisms. Therefore, a comparison of the biotransformation activity between these two microcosms and its effect on contaminant bioavailability could be made.

The dechlorination pattern during the second biotransformation assay was analogous to that observed during the first biotransformation assay. The more highly chlorinated benzene congeners accumulated in the azide-amended microcosms, whereas the concentrations of DCB isomers increased in the microbially active microcosms (Fig. 6). A large production of TrCB and conversion to DCB was observed in microcosm C. The fact that the liquid-phase DCB concentration remained relatively stable for the remainder of the incubation period indicates that further DCB transformation to MCB in this system either did not take place or was very slow. The predominant DCB isomer was 1,3-DCB. The biotransformation of HCB was most rapid in microcosm C (ground sediment and electron donor-amended microcosm). After 83 d of incubation, 84% of the original HCB had been transformed in this system and by day 205, 95% of the original HCB had been converted (Fig. 7). In contrast, after 83 d of incubation, about 33% of the initial HCB was transformed in microcosm A and did not change for the remainder of the incubation period (205 d). The solid-phase concentrations of the highly chlorinated benzene congeners remained constant in the azide-amended microcosms, with some loss of DCB occurring as discussed above. Mass balances of chlorinated benzenes in all four microcosms (A to D) at the end of the incubation accounted for between 61.4 and 89.1% of the initially measured contaminant mass (Table 5).

The results of this study indicate that the *in situ* biotransformation of sediment-bound chlorobenzenes is slow due to both the lack of degradable carbon sources to promote and sustain high microbial growth and activity, as well as poor bioavailability of the strongly sorbed contaminants. The addition of a degradable organic carbon source and sediment grinding resulted in a high rate and

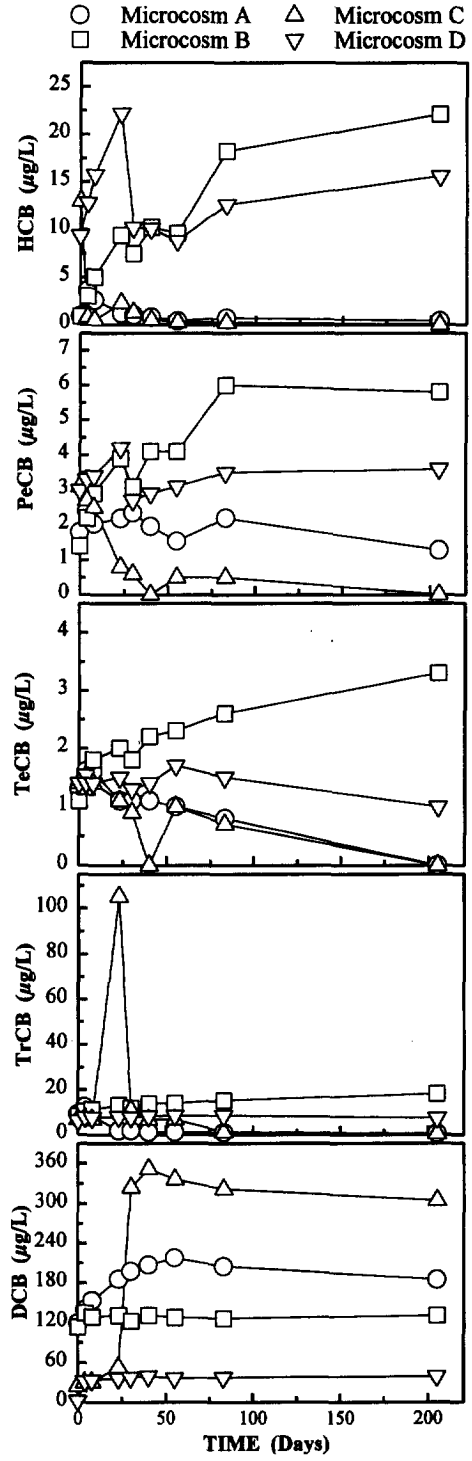

Fig. 6. Liquid-phase concentrations of the chlorinated benzene congeners during the second biotransformation assay.

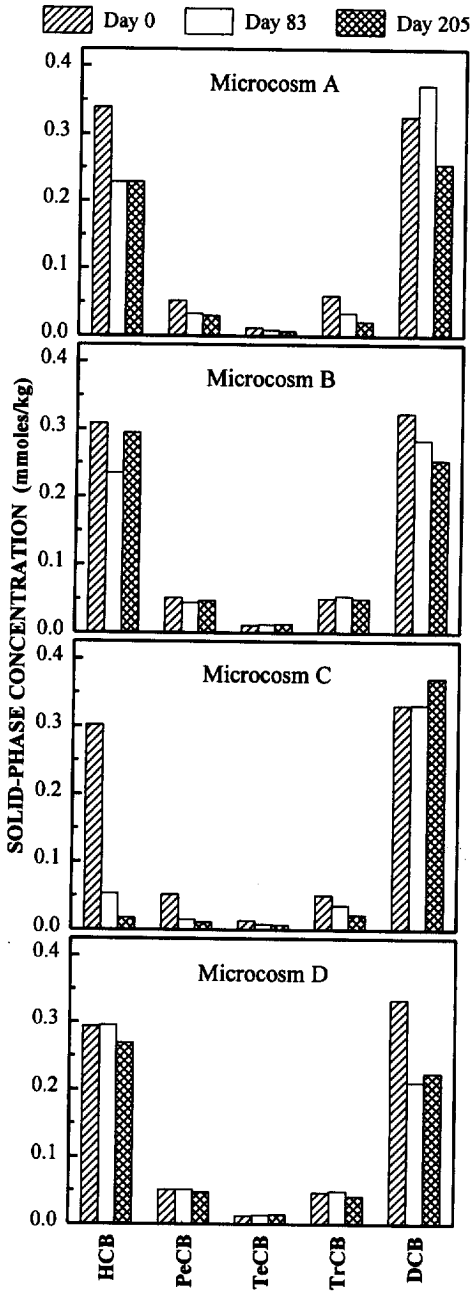

Fig. 7. Comparison of the solid-phase concentrations of the chlorinated benzene congeners between time zero and two incubation periods (biotransformation assay II).

extent of HCB dechlorination in microcosm C. The fact that limited HCB biotransformation was observed in microcosm A, even though its methanogenic activity was identical to that of microcosm C, indicates that the contaminants in the intact sediment were not biologically available. It is not clear, however, how the contaminant is "shielded" from the organisms in the natural sediment. The increased bioavailability afforded by drying and grinding the sediment may be a result of shorter diffusive path lengths, or simply increased sediment surface area. In the latter case, it may not be necessary for solid-phase chlorobenzenes to dissolve prior to biotransformation. The sediment organisms may have limited access to the HCB in the natural sediment, as some of the contaminant may have diffused and sorbed within the sediment particles. Once the sediment has been ground, more of this contamination will be at or near a surface, where microorganisms might be able to attach and initiate contaminant biotransformation. This argument also holds for contaminant dissolution, since shorter path lengths and increased surface area could lead to faster dissolution/diffusion, and hence higher bioavailability.

HCB dechlorination rates. The observed, first-order rate constants (k_{obs}, d^{-1}) for the depletion of HCB in all four microbially active microcosms are given in Table 6. Based on these data, the effect of electron donor and contaminant availability on the reductive dechlorination rate is evident. Frequent additions of electron donor and therefore maintenance of microbial activity resulted in a faster HCB dechlorination rate in the lactate/acetate-amended microcosm (A) as compared with the unamended microcosm (I). In addition, preparation of the sediment by drying and grinding resulted in about one order of magnitude higher HCB dechlorination rate (microcosm A vs microcosm C). In contrast to these biotransformation rates of sediment-bound HCB, enriched methanogenic, liquid cultures derived from the Bayou d'Inde sediment, when amended with freshly added HCB, achieved HCB dechlorination rates with k_{obs} values ranging from 3.2×10^{-1} to $3.8 \times 10^{-1}\,d^{-1}$ at 23°C (Pavlostathis *et al.*, 1995). Based on data reported by Ramanand *et al.* (1993), a first-order rate constant of $2.2 \times 10^{-2}\,d^{-1}$ was calculated for the depletion of freshly added HCB in a soil slurry inoculated with a culture previously acclimated to a mixture of di-, tri-, and tetrachlorobenzenes. Dechlorination of HCB began after a 2 month lag phase. Beurskens *et al.* (1994) reported k_{obs} values ranging from 7.2×10^{-1} to $1.07\,d^{-1}$ for the dechlorination of freshly added HCB to a mixed liquid culture derived from a freshwater sediment contaminated with chlorobenzenes. Based on these rates, it appears that the reductive dechlorination rate of the sediment-bound HCB is severely limited by slow desorption/diffusion and low biodegradability of the sediment organic matter.

In the first biotransformation assay, where sulfate at a concentration of 80 mg/l was present—contributed by the Bayou d'Inde water—less than 4% of the total COD destruction was attributed to sulfate reduction. Therefore, sulfate reduction played a minor role in terms of carbon and electron flow. Although reductive dehalogenation of chlorinated hydrocarbons has been successfully carried out under sulfate-reducing conditions (see, for example, Fathepure *et al.*, 1987; Egli *et al.*, 1987; Bagley and Gossett, 1990; Pavlostathis and Zhuang, 1991),

Table 5. Distribution of chlorobenzene congeners and mass balance during the second biotransformation assay as a function of incubation time

	Chlorobenzene (%)[a]					Contaminant mass balance[b] (%)
	HCB	PeCB	TeCB	TrCB	DCB	
Microcosm A						
Day 0	42.6	6.5	1.5	7.6	41.8	100
Day 83	33.0	4.8	1.3	4.9	56.0	87.0
Day 205	40.9	5.3	1.2	3.7	48.9	70.1
Microcosm B						
Day 0	41.0	6.8	1.5	6.7	44.0	100
Day 83	36.8	7.0	1.9	8.6	45.7	85.0
Day 205	44.1	7.0	2.0	7.6	39.3	89.1
Microcosm C						
Day 0	40.2	6.9	1.7	6.9	44.3	100
Day 83	11.3	3.2	1.6	7.7	76.2	62.4
Day 205	3.9	2.4	1.4	4.9	87.4	61.4
Microcosm D						
Day 0	39.7	6.9	1.6	6.4	45.4	100
Day 83	47.5	8.1	2.1	8.0	34.3	84.2
Day 205	44.9	7.9	2.3	7.1	37.8	81.4

[a]Based on total mass (i.e. sum of solid, liquid, and gas phase) of chlorobenzenes measured at each time point.
[b]Total contaminant mass balance relative to the initially measured contaminant mass.

several researchers have reported an adverse impact of sulfate on the reductive dechlorination of haloaromatic compounds (Gibson and Suflita, 1986; Kohring *et al.*, 1989; Kuhn *et al.*, 1990). Preliminary experiments with freshly added HCB to liquid mixed cultures derived from the Bayou d'Inde sediments and with a relatively high sulfate concentration (550 mg/l as SO_4^{2-}) resulted in a first-order HCB dechlorination rate constant of $1.0 \times 10^{-1}\ d^{-1}$. This rate compares favorably with the HCB dechlorination rates achieved by liquid cultures in the absence of sulfate (see above). Therefore, the relatively low sulfate concentration in the microcosms of this study has an insignificant effect on the observed reductive dechlorination rates of HCB.

Biotransformation pathway. HCB was the primary contaminant released into the Bayou d'Inde tributary. The distribution of less chlorinated benzenes in the sediment should therefore reflect the transformation pathway that has resulted in their production and hence accumulation. All isomers and congeners of chlorinated benzenes were detected in the sediment except 1,2,3-TrCB, 1,2-DCB, and MCB. The most abundant chlorobenzene congener besides HCB was 1,3-DCB, indicating that this compound is the main product of the *in situ* microbial transformations of HCB. The presence of penta- as well as tetra- and trichlorobenzene isomers in the sediment is in line with the previously reported sequential reductive dechlorination of HCB (Fathepure *et al.*, 1988; Mousa and Rogers, 1990; Fathepure and Vogel, 1991; Liang and Grbić-Galić, 1991; Edwards *et al.*, 1992; Holliger *et al.*, 1992; Mohn and Tiedje, 1992; Beurskens *et al.*, 1994).

Table 6. Observed HCB dechlorination rate constants

Assay	Microcosm	Microcosm description	k_{obs} (d^{-1})
I	I	Native sediment	2.2×10^{-3}
	II	Native sediment with two dextrin additions	2.9×10^{-3}
II	A	Native sediment with frequent lactate/acetate additions	4.8×10^{-3}
	C	Air dried/ground sediment with frequent lactate/acetate additions	2.1×10^{-2}

The data from the biotransformation assays are in line with the distribution of chlorobenzene congeners in the Bayou d'Inde sediments. HCB was transformed in the microbially active microcosms and accumulated predominantly as 1,3-DCB with some accumulation of 1,4-DCB as well. Although there is evidence of anaerobic dechlorination of DCB, these transformations occur very slowly (Tsuchiya and Yamaha, 1983; Bosma *et al.*, 1988; Mousa and Rogers, 1990; Liang and Grbić-Galić, 1991; Edwards *et al.*, 1992; Ramanand *et al.*, 1993). In addition, lack of further dechlorination of DCB in methanogenic liquid cultures derived from the Bayou d'Inde sediments (Pavlostathis *et al.*, 1995) suggests that further dechlorination of DCB is not significant in these sediments. In the simulated microcosms, the transformation pathway appears to be as follows: HCB→PeCB→1,2,3,5- and 1,2,4,5-TeCB→1,3,5- and 1,2,4-TrCB→1,3- and 1,4-DCB. A frequent observation in previous research has been the production of 1,3,5-TrCB as a dominant and stable product from microbially mediated HCB reduction, with further transformations occurring much more slowly. Although the reduction of 1,3,5-TrCB to 1,3-DCB is the least energetically favorable reaction of all the possible dechlorination reactions in the chlorobenzenes series (Beurskens *et al.*, 1994), and while some of the 1,3-DCB could have been formed by the reduction of 1,2,4-TrCB, the former reaction appears to have been responsible for the accumulation of 1,3-DCB in the sediment under study. The accumulation of TrCB in the liquid phase of microcosm C at day 23 was almost 95% 1,3,5-TrCB. The subsequent

removal of 1,3,5-TrCB and concomitant accumulation of 1,3-DCB in this microcosm by day 30 indicates that there is substantial potential for this microbial transformation to occur rapidly.

Electron balance. Electron balance calculations were made by assigning 8 electron equivalents (eeq) per mole of methane produced and 2 eeq per chlorine substituent removed. Relative to methanogenesis, at 87 d of incubation, only 0.2% of the available electrons were diverted to the dechlorination reactions in the dextrin-amended microcosm during the first biotransformation assay. Similarly, in the second biotransformation assay, only 0.04% of the total eeq were used for the dechlorination process in the most microbially active microcosm C. The regular supply of a degradable electron donor resulted in much higher methane production in the second biotransformation assay compared to that in the first assay, thus leading to an even smaller fraction of all available electrons used for the dechlorination process. It has been previously observed that in reductive dechlorination transformations mediated by mixed cultures, only a small fraction (less than 1%) of the total reducing power (i.e. total electroequivalents) transferred through metabolic reactions are diverted and used for the reduction of the chlorinated contaminants (Bagley and Gossett, 1990; Pavlostathis and Zhuang, 1991, 1993). This observation, in conjunction with the requirement for a continuous supply of electron donor(s) to maintain microbial activity, and thus the reductive dechlorination process, have far-reaching implications—in terms of nutrient requirements and cost—for the *in situ* bioremediation of subsurface environments.

CONCLUSIONS

Naturally occurring microorganisms mediate the reductive dechlorination of the sediment-bound chlorobenzenes. However, because of the refractory nature of the sediment natural organic matter, as well as strong contaminant adsorption, a low rate and extent of biotransformation were attained. The dechlorination rate of sediment-bound HCB was two orders of magnitude lower than that of freshly added, and therefore bioavailable, HCB. An increase in contaminant hydrophobocity due to the effect of salinity, as well as the relatively high contaminant residence time, may have contributed to the observed reduced contaminant bioavailability. On the other hand, microbial reductive dechlorination of the sediment-bound contaminants leads to the long-term release of less chlorinated and more mobile chlorinated benzenes. Sediment nutrients (such as nitrogen and phosphorus) did not limit the biodegradation of the natural organic matter. The low electron transfer efficiency towards reductive dechlorination, as well as the low extent of biotransformation of the sediment contaminants, need further investigation. A better understanding of the factors involved in the bioavailability and transformation of the sediment-bound contaminants will lead to a more accurate estimation of the impact of contaminant release on the aquatic environment, as well as to the development of means to either accelerate or control the dechlorination process for remediation purposes.

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