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ORIGINAL PAPER

Influence of sulfate reduction on the microbial dechlorination of pentachloroaniline in a mixed anaerobic culture

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Abstract The reductive dechlorination of pentachloroaniline (PCA) was investigated in the absence and presence of sulfate in batch assays using a PCA-dechlorinating mixed anaerobic culture with methanol as the external electron donor at neutral pH and 22°C. PCA at an initial concentration of 7.8 μM was sequentially dechlorinated to dichlorinated anilines in the sulfate-free culture and the culture amended with 300 mg sulfate-S/L. At an initial concentration of 890 mg sulfate-S/L, a higher sulfate reduction rate was achieved, but PCA dechlorination was not observed until the sulfate concentration dropped to about 100 mg S/L. The transient inhibition of PCA is attributed to competition between sulfate reducing and dechlorinating species for electron donor, more likely for H_2 resulting from methanol fermentation. A long-term (118 days) PCA dechlorination assay with the sulfate-amended culture, which included five feeding cycles, resulted in accumulation of both sulfide (886 mg S/L) and acetate (1,900 mg COD/L). Under these conditions, the sulfate reducers were inhibited, while the rate and pathway of PCA dechlorination were not affected. The results of this study show that the rate of sulfate reduction rather than the sulfate concentration alone dictates the outcome of the competition between sulfate reducers and either dechlorinators or methanogens. The findings of the present study have significant implications relative to the fate and transport of PCA and its dechlorination products in sulfate-laden subsurface systems.

Keywords Reductive biotransformation · Pentachloroaniline dechlorination · Sulfate reduction · Sulfide toxicity

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Introduction

Halogenated pesticides are pollutants of increasing concern because they are found in all major environmental media, including surface water, ground water, sediments, and soil. Chloroanilines are of particular environmental concern because of their persistence and toxicity (Kuhn and Suflita 1989). The fate of chlorinated anilines in the environment has been of particular interest because they are associated with widely used herbicides, which contain chloroaniline moieties and precursors (Bunce et al. 1983; Kuhn et al. 1990). Also, complete reduction of the nitro group during the anoxic transformation of chlorinated nitroaromatic compounds results in the formation of chloroanilines (Hakala et al. 2007; Klupinski et al.

2004; Okutman Tas and Pavlostathis 2005; Susarla et al. 1996; Thiele et al. 1988; Wu et al. 2006).

Soils and sediments represent a major sink for organic, hydrophobic pollutants. In anoxic environments, nitrate, sulfate, carbon dioxide, as well as metals serve as terminal electron acceptors. The abundance of sulfate in many subsurface environments is of particular importance for the degradation of organic contaminants (Mort and Dean-Ross 1994; Pavlostathis and Zhuang 1991). In marine sediments, at high sulfate concentrations (20–30 mM), sulfate reduction is the dominant process in carbon metabolism and sulfate may be the most important electron acceptor influencing the anaerobic degradation of halogenated compounds (Häggblom and Young 1995).

Provided that appropriate environmental conditions exist, chlorinated aliphatic and aromatic hydrocarbons undergo reductive dechlorination in sulfate-reducing environments with different electron donors (Alder et al. 1993; Aulenta et al. 2008; Bagley and Gossett 1990; Cabirol et al. 1998; De Best et al. 1997a, b; Häggblom and Young 1990; Kennedy et al. 2006; Ndon et al. 2000; Palekar et al. 2003; Pavlostathis and Zhuang 1991; Prytula and Pavlostathis 1996; Sonier et al. 1994). Degradation of chlorinated aromatic compounds coupled to sulfate reduction yields higher free energy than degradation coupled to methane production (Häggblom and Young 1995). However, inhibition by sulfuroxy anions has been reported for chlorinated anilines and benzoates (Kuhn et al. 1990). Inhibition of dehalogenation under sulfate-reducing conditions has been attributed to competition between sulfate and chlorinated compounds for electron donor (Alder et al. 1993). Conflicting reports exist as to the effect of sulfate reduction on microbial dechlorination, from lack of inhibition (Bagley and Gossett 1990; DeWeerd et al. 1991; Hoelen and Reinhard 2004; Pavlostathis and Zhuang 1991), to partial inhibition (Aulenta et al. 2008; Cabirol et al. 1998), or to complete inhibition (Nelson et al. 2002). Considering the significant role molecular hydrogen (H_2) plays as a terminal electron donor in microbial reductive dechlorination reactions (Aulenta et al. 2008; DiStefano et al. 1992; Fennell et al. 1997; Kassenga and Pardue 2006; Löffler et al. 1999; Smatlak et al. 1996; Yang and McCarty 1998), the outcome of competition among various microbial groups for H_2 utilization at relatively low H_2 concentrations may be explained by the fact that the H_2 threshold of dechlorinating bacteria (<0.3–2 nM) is significantly lower than that of hydrogenotrophic methanogens (5–95 nM), and similar to that of hydrogenotrophic sulfate reducers (1–4 nM) (Aulenta et al. 2008). However, in addition to H_2 threshold considerations and microbial competition, the detrimental effect of sulfate reduction on microbial dechlorination may also be attributed to direct inhibition of dehalogenases by sulfate or intermediates of sulfate reduction such as thiosulfate, sulfite, or sulfide (DeWeerd et al. 1991).

Pentachloroaniline (PCA) is of great environmental concern due to its high potential for bioaccumulation in the food chain, especially in environments where PCA dechlorination does not take place (Okutman Tas and Pavlostathis 2005). PCA is the first product of pentachloronitrobenzene (PCNB) biotransformation under reductive biotic (Okutman Tas and Pavlostathis 2005) and abiotic (Hakala et al. 2007; Klupinski et al. 2004) conditions. Due to the presence of several electron acceptors in the environment, which may enhance or inhibit contaminant biotransformation processes, the reductive biotransformation of PCA under different electron accepting conditions needs to be assessed. Susarla et al. (1997) determined the PCA transformation pathway in anaerobic sulfidogenic sediment/water systems containing approximately 20 mM sulfate. The reductive dechlorination pathway and kinetics of PCA in a mixed, methanogenic culture derived from a contaminated sediment have been reported (Okutman Tas and Pavlostathis 2005; Okutman Tas et al. 2006). Okutman Tas and Pavlostathis (2007) reported that reductive dechlorination of PCA in a mixed fermentative, methanogenic culture amended with Fe(III) EDTA was not observed until nearly all of the added Fe(III) was reduced to Fe(II), implying that competition between dechlorination and iron reduction for electron donor occurred. Okutman Tas and Pavlostathis (2008) investigated the effect of nitrate reduction on the reductive biotransformation of PCA in a mixed fermentative, methanogenic culture. PCA sequentially dechlorinated to dichlorinated anilines in a culture amended with 10 mg nitrate-N/L, but partially dechlorinated to tetrachloroanilines (TeCAs) in cultures amended with 50–200 mg nitrate-N/L. The inhibition of PCA dechlorination was attributed to the accumulation of nitric (NO) and nitrous (N_2O) oxides resulting from partial nitrate reduction.

In view of the fact that sulfate is a widespread electron acceptor in natural systems, especially in estuarine and marine sediments, its effect on the reductive dechlorination of PCA needs to be further investigated. The objective of the research reported here was to assess the influence of sulfate reduction on the sequential reductive dechlorination of PCA in a methanol-fed mixed, PCA-dechlorinating culture.

Materials and methods

Culture

The PCNB-transforming enrichment culture used in this study was developed from a contaminated sediment and maintained as previously reported (Okutman Tas and Pavlostathis 2005). The predominant PCNB biotransformation pathway was as follows: PCNB → PCA → 2,3,4,5- and 2,3,5,6-tetrachloroaniline (TeCA) → 2,4,5- and 2,3,5-trichloroaniline (TrCA) → 2,5- and 3,5-dichloroaniline (DCA) → 3-chloroaniline (CA) (low levels). Under fermentative/methanogenic conditions, the enrichment culture did not dechlorinate five out of six dichlorinated anilines and three monochlorinated aniline isomers in short dechlorination assays (Okutman Tas et al. 2006). The PCNB-transforming, enrichment culture had been maintained in our laboratory for over 4 years when the present study was conducted. The steady-state biomass concentration of the sediment-free culture was 315 ± 40 mg/L (expressed as particulate organic carbon; POC). In a previously conducted assay using the stock, enrichment culture, addition of 2-bromoethanesulfonate (BES) resulted in the complete cessation of methanogenesis, but did not have any detrimental effect on the sequential dechlorination of PCA, suggesting the existence of halorespiring species in the enrichment culture (Okutman Tas and Pavlostathis 2005). In a subsequent study, among the dechlorinating bacterial groups tested (*Dehalococcoides*, *Dehalobacter*, *Desulfuromonas*, *Geobacter*, and *Anaeromyxobacter*) by 16S rRNA gene analysis, only *Dehalococcoides* was detected in the mixed fermentative/methanogenic culture (Okutman Tas et al. 2006). The possibility of the existence of other dechlorinating organisms in the mixed culture should not be discounted.

Abiotic PCA controls

The culture media contained sulfide (67 mg S/L), Fe(II) (28.1 mg/L), and vitamin B_{12} (2 μg/L). An abiotic assay was conducted using 160 ml serum bottles because of the potential of culture media components, as well as culture products to abiotically mediate the reductive transformation of PCA. Three types of autoclaved, abiotic controls were used (all in triplicate): deionized (DI) water, culture media, and culture. The effect of sulfide alone on the transformation of PCA was assessed by setting up two control series with DI water and an initial PCA concentration of 7 μM (dissolved in methanol) and at two sulfide concentration levels, 500 and 1,000 mg/L as total S, resulting in pH of 9.7 and 11.1, respectively. Three autoclaved culture media control series were set up with an initial PCA concentration of 7 μM at three sulfide concentrations of 67, 500 and 1,000 mg/L as total S, resulting in pH of 7.5, 8.4, and 9.1, respectively. To compare the data obtained with the active culture and to assess the effect of reductant(s) formed in the culture, two autoclaved culture control series were set up with an initial PCA concentration of 7 μM and at two sulfide concentration levels, 500 and 1,000 mg/L as total S, resulting in pH of 11.2 and 11.9, respectively. All serum bottles were autoclaved twice on two consecutive days at 121°C for 30 min. All abiotic controls were incubated in a 22°C constant temperature room and manually shaken once a day.

PCA dechlorination assays

The reductive dechlorination of PCA by the enrichment culture in the presence and absence of sulfate reduction was investigated in two separate batch assays. The first short-term assay was conducted with four culture series, each series prepared using four replicate 160 ml serum bottles (120 ml liquid volume), sealed with Teflon-lined septa and preflushed with helium gas. The cultures were amended with 7 μM PCA dissolved in methanol (1,318 mg/L), yeast extract (17 mg/L) and culture media. The media had the following composition (in mg/L): K_2HPO_4, 900; KH_2PO_4, 500; NH_4Cl, 500; $CaCl_2{\cdot}2H_2O$, 100; $MgCl_2{\cdot}6H_2O$, 200; $FeCl_2{\cdot}4H_2O$, 100; $Na_2S{\cdot}9H_2O$, 500; $NaHCO_3$, 1,200; resazurin, 2; 0.2 ml/L vitamin stock solution and 1 ml/L trace metal stock solution

(Tugtas and Pavlostathis 2007). The first culture series was a sulfate-free control. The second culture series was amended with 7 μM PCA and an initial sulfate concentration of 300 mg S/L. The initial sulfate concentration was chosen based on the results of a preliminary test conducted with the stock enrichment culture using five culture series amended with 7 μM PCA and initial sulfate concentrations of 20, 50, 100, 150 and 200 mg S/L, respectively. The preliminary test resulted in relatively low sulfate reduction rates and had no effect on the rate and pathway of PCA dechlorination as compared to the sulfate-free control (data not shown). The electron donor provided in the second culture series (371 milli electron equivalents/L; meeq/L) was well in excess of the required meeq for the reduction of the added sulfate to sulfide (300 mg sulfate-S/L; 75 meeq/L). The third culture series was set up identically to the first culture series, except that BES was added at an initial concentration of 25 mM to assess the role of methanogens on the dechlorination of PCA in the mixed culture. In order to investigate the effect of BES on both the sulfate reduction and PCA dechlorination, the fourth culture series was amended with PCA, sulfate, and BES at 7 μM, 300 mg sulfate-S/L and 25 mM, respectively. Prior to the addition of methanol and PCA, the BES-amended cultures were incubated for one day to enhance the effectiveness of the inhibitor. Incubation of the four culture series took place in a 22°C constant temperature room and the serum bottles were manually shaken once a day. All liquid-phase analyses were conducted in triplicate using three out of the four replicate bottles, whereas gas-phase analyses were conducted only once using the fourth replicate bottle due to limited excess gas production at each sampling time.

Based on the results obtained from the short-term assay, another batch assay was conducted to examine the long-term effect of sulfate reduction on the dechlorination of PCA at relatively high sulfate reduction rates. Two cultures were prepared by diluting 0.6 L of the stock enrichment culture with 1.2 L mineral culture media using 2-L glass flasks preflushed with helium gas (1.8 L liquid volume). This assay included five successive feeding cycles and lasted 118 days. PCA (7.1–7.8 μM) dissolved in methanol (1,318 mg/L) and yeast extract (17 mg/L) were introduced at the beginning of each feeding cycle. One culture served as the sulfate-free control, whereas the other culture was amended with 300 mg sulfate-S/L in cycles 1 through 3. The initial sulfate concentration in the second culture was increased to 890 mg sulfate-S/L in cycles 4 and 5 to determine the effect of higher sulfate concentration, and thus higher sulfate reduction rates, on the sequential reductive dechlorination of PCA. The electron donor provided at the beginning of each feeding cycle (370 meeq/L) was well in excess of the required meeq for complete sulfate reduction to sulfide at each feeding cycle (75–223 meeq/L). BES (25 mM) was added once to the sulfate-amended culture after 17 days of incubation (beginning of third feeding cycle). Fresh culture media were supplied at the beginning of each feeding cycle and the culture pH was kept around 7 by the addition of $NaHCO_3$. Gas production was monitored throughout the incubation period as described below. Gas and liquid samples were taken periodically to monitor gas composition, pH, chlorinated compounds, methanol, chemical oxygen demand (COD), sulfate and volatile fatty acids (VFAs). At the end of the assay, both total and soluble sulfide were measured. All analyses were conducted in triplicate. The two cultures were kept in a 22°C constant temperature room and gently stirred with magnetic stirrers for 30 min daily before and during culture sampling.

Sulfide assays

In order to investigate the effect of produced and accumulated sulfide in the above-described, long-term PCA dechlorination assay, a batch, sulfide-amended culture was set up in triplicate 160 ml serum bottles sealed with Teflon-lined septa and preflushed with helium gas using the stock, enrichment culture. PCA (7.1 μM) dissolved in methanol (1,318 mg/L), yeast extract (17 mg/L) and total sulfide (700 mg S/L) were added only once.

In order to assess the effect of sulfide on the sulfate reduction activity of the above-described sulfate-amended culture during the long-term assay, another batch assay was carried out in triplicate by transferring 40 ml aliquots of the sulfate-amended culture at the end of the fifth feeding cycle and 78 ml fresh culture media in 160 ml serum bottles preflushed with helium. The cultures were then amended with 1,318 mg/L methanol, 25 mM BES and 900 mg sulfate-S/L. For both assays, incubation of the cultures took place in a 22°C constant temperature

room and the serum bottles were manually shaken once a day.

Chemicals

PCA stock solutions were prepared by dissolving a neat standard (96.1%) obtained from Supelco (Bellefonte, PA) in HPLC-grade (99.99%) methanol obtained from Fisher Scientific (Pittsburg, PA). Standards of all expected PCA dechlorination products were obtained from Sigma-Aldrich (St. Louis, MO), except 2,3,5-TrCA and 2,3,5,6-TeCA, which were obtained from Neosyn Laboratories (New Milford, CT) and VWR (Buffalo Grove, IL), respectively. Sodium sulfate and isooctane (99.7%) were purchased from Fisher Scientific (Fair Lawn, NJ). BES was obtained from Acros Organics (Morris Plains, NJ).

Analytical methods

PCA and chlorinated anilines were liquid/liquid extracted from culture samples with isooctane, which contained 0.5 mg/L of 1,3,5-tribromobenzene used as an internal standard. The extracts were analyzed by gas chromatography (electron capture detection) as reported before (Okutman Tas and Pavlostathis 2005).

Total gas production was measured by displacement of an acidified brine solution (10% NaCl w/v and 2% H_2SO_4 v/v) in a graduated burette equilibrated to atmospheric pressure. Culture headspace samples were withdrawn using a gas tight syringe and injected (100 µL injection volume) into an Agilent Technologies 6890N gas chromatograph (Agilent Technologies Inc., Palo Alto, CA) equipped with two columns and two thermal conductivity detectors. Methane was separated with a 15-m HP-Molesieve fused silica, 0.53 mm i.d., and 50-µm film thickness column (Agilent Technologies, Inc.). Separation of CO_2 and H_2S was performed with a 25-m CP-PoraPLOT Q fused silica, 0.53 mm i.d., and 20-µm film thickness column (Varian Inc., Palo Alto, CA). Helium was used as the carrier gas at a constant flow rate of 6 ml/min. The 10:1 split injector was maintained at 150°C, the oven was set at 40°C and the detector temperature was set at 150°C. Molecular hydrogen analysis used the above described GC unit and Molsieve column, except that the carrier gas was nitrogen. The minimum gas-phase detection limit for CH_4, CO_2, H_2S, and H_2 was 0.5, 0.8, 0.1, and 0.5 ml/L, respectively.

C_2–C_7 VFAs and methanol were measured using a HP 6890 Series gas chromatography (GC) unit (Hewlett Packard, Palo Alto, CA) equipped with a flame ionization detector (FID) and a 30-m Restek 11025 Stabilawax-DA, 0.53-mm i.d. column (Restek Company, Bellefonte, PA) as previously reported (Tugtas and Pavlostathis 2007). Aqueous samples were first centrifuged at 12,000 rpm for 10 min, filtered directly into 1.8 ml glass autosampler vials, and then acidified (sample/acid, 2:1 v/v) with 2.5% v/v H_3PO_4 which contained 1.5 g/L acetoin, used as an internal standard. The vials were capped with Teflon-lined septa and aluminum crimp caps and stored at 4°C until analysis. COD was measured following the spectrophotometric method outlined in *Standard Methods* (APHA 2005).

Sulfate was determined using a Dionex DX-100 ion chromatography unit (Dionex Cooperation, Sunnyvale, CA) equipped with a conductivity detector, a Dionex IonPac AG14A (4 × 50 mm) pre-column, and a Dionex IonPac AS14A (4 × 250 mm) analytical column. The eluent was a mixture of 8 mM Na_2CO_3 and 1 mM $NaHCO_3$ used at an isocratic flow rate of 1 ml/min. Autosuppression mode was used.

Total and soluble sulfide measurements in liquid samples were determined using an acid–H_2S gas measurement procedure similar to that described by Bagley and Gossett (1990) as follows. Aliquots of 1 ml of a 6 N H_2SO_4 solution were transferred to 12-ml glass vials, capped with Teflon-lined septa and aluminum crimps, and flushed with helium gas for 2 min. Total sulfide analysis was conducted by injecting a 5 ml culture sample into each vial, whereas for soluble sulfide, 10 ml filtered (0.2 µm Whatman syringe filter) culture sample was injected into each vial. The vials were then shaken vigorously for 2 min and incubated in an inverted position at 35°C for 1 h. After incubation, the headspace of each vial was analyzed for H_2S gas by injecting 100 µL of headspace gas into a GC unit (thermal conductivity detector). The hydrogen sulfide was quantified based on a conditional calibration curve prepared by plotting peak areas against total sulfide mass using sodium sulfide standards prepared in culture media and following the above-described procedure used for the culture samples.

Results and discussion

Assessment of abiotic PCA transformation

The abiotic transformation of PCA was monitored over a 60 day period. In all autoclaved controls (DI water, sulfide-amended media, and autoclaved culture), the PCA concentration did not change and less chlorinated anilines were not observed over the incubation period. Therefore, it is concluded that PCA dechlorination in active (i.e., non autoclaved) cultures reported below was biologically mediated. Similar to our results, Okutman Tas and Pavlostathis (2005) reported that over a 60 day incubation period, abiotic dechlorination of PCA was not observed in the presence of sulfide at concentration levels of 3 and 67 mg S/L in three types of autoclaved abiotic controls (DI water, culture media, and culture).

Assessment of PCA dechlorination—Short term assay

PCA dechlorination in the control (i.e., sulfate-free) culture as well as the cultures amended with either sulfate, BES, or sulfate plus BES was monitored for 7 days. The pH in all cultures ranged from 6.9 to 7.1. Table 1 presents the cumulative methane, carbon dioxide, and hydrogen sulfide volume produced, as well as the fraction of COD processed during the 7 days of incubation in the control culture and the three cultures amended with either sulfate, BES, or sulfate plus BES, respectively. The time course of PCA dechlorination in the sulfate-free, control culture is shown in Fig. 1. PCA was sequentially dechlorinated to two tetrachloroanilines (2,3,5,6-TeCA, 2,3,4,5-TeCA), three trichloroanilines (2,3,5-TrCA, 2,4,5-TrCA, 2,4,6-TrCA), followed by dichloroanilines (2,4+2,5-DCA, 3,5-DCA and 2,6-DCA; note that 2,4 and 2,5-DCA coeluted). 2,3,5-TrCA was the predominant intermediate and 2,4+2,5-DCA were the predominant end products. The same PCA dechlorination pathway was observed by Okutman Tas and Pavlostathis (2005) using the same enrichment culture. Kuhn and Suflita (1989) reported sequential transformation of 2,3,4,5-TeCA to form 2,3,5-TrCA and eventually 3,5-DCA. Susarla et al. (1997) investigated the dechlorination of PCA in an anaerobic sulfidogenic sediment/water system and reported its transformation to 2,3,4,5-TeCA, 2,3,5-TrCA, and 3,5-DCA.

PCA dechlorination proceeded at an initial rate of 6 μM/day in the sulfate-amended (300 mg sulfate-S/L) culture and the dechlorination pathway was similar to that observed in the control culture, indicating that PCA dechlorination was not affected at a relatively low sulfate reduction rate (2.3 mg sulfate-S/L-day) (Table 1). Amendment of the sulfate-free culture with BES at an initial concentration of 25 mM completely inhibited methanogenesis (Fig. 2e), but did not affect the rate and pathway of PCA dechlorination. Therefore, the methanogens were not directly involved in the sequential dechlorination of PCA. Similar results

Table 1 Gas production, COD consumption, sulfate reduction, and PCA removal in the sulfate-free (control) culture, as well as cultures amended with either sulfate, BES, or sulfate plus BES during the short-term dechlorination assay

Parameter	Control culture	Cultures amended with		
		Sulfate	BES	Sulfate + BES
Gas volume (ml)				
Total	78.0	82.5	3.3	4.5
CH_4	55.9	55.5	0.1	0.1
CO_2	20.3	20.1	2.9	2.5
H_2S	0.8	5.6	0.2	1.7
COD processed (%)[a]	100.0	99.9	0.1	14.8
Sulfate reduction rate (mg S/L-day)	NA	2.3	NA	13.1
Initial PCA removal rate (μM/day)	6.0	6.0	6.0	6.0

After 7 days of incubation; culture conditions: 22°C, pH 7

NA not applicable

[a] Normalized to the COD processed by the control (i.e., sulfate-free) culture

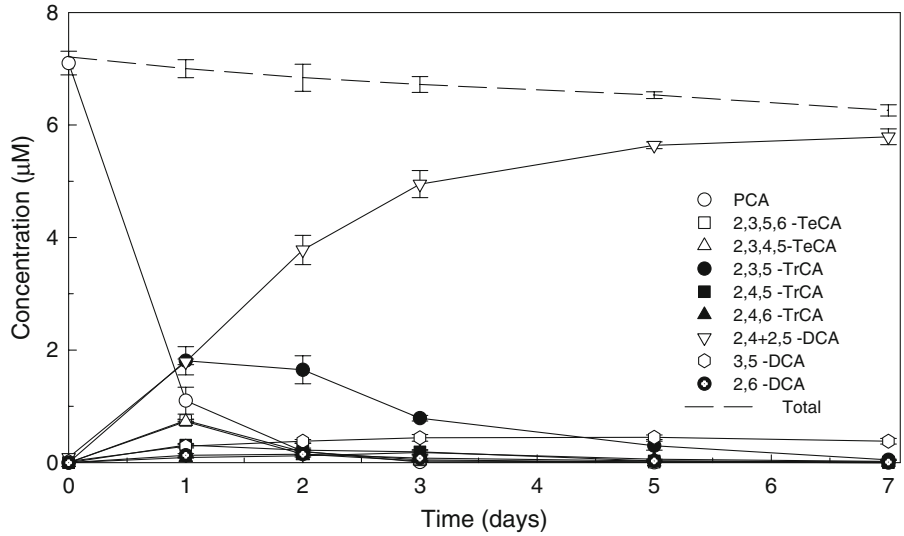

Fig. 1 Time course of PCA dechlorination in the sulfate-free, control culture during the short-term PCA dechlorination assay (*error bars* represent ± one standard deviation; $n = 3$)

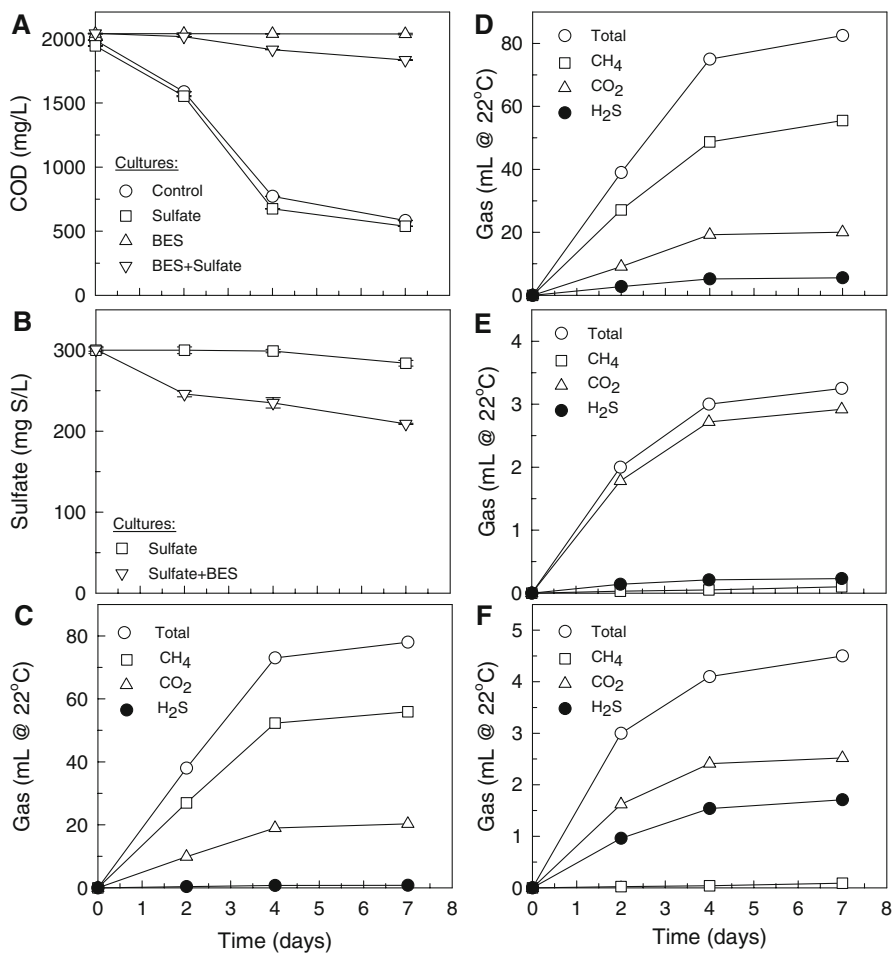

Fig. 2 Short-term PCA dechlorination assay. **a** COD consumption, **b** sulfate reduction, **c**, **d**, **e**, and **f** gas production in the control (i.e., sulfate-free), sulfate-amended, BES-amended, and sulfate- plus BES-amended culture, respectively (*error bars* for COD and sulfate data represent ± one standard deviation; $n = 3$)

were reported by Okutman Tas and Pavlostathis (2005) for the same enrichment culture. Simultaneous PCA dechlorination and sulfate reduction at a rate of 6 μM/day and 13.1 mg sulfate-S/L-day, respectively, was observed in the culture amended with BES and sulfate at initial concentrations of 25 mM and 307 mg sulfate-S/L, respectively. The relatively higher sulfate reduction rate in the BES-amended culture had no adverse effect on the PCA dechlorination process. As expected, the addition of BES to the sulfate-free culture and the sulfate-amended culture affected the total gas production and composition, as well as the COD utilization (Table 1; Fig. 2).

Electron equivalence calculations were performed at the end of the 7 day incubation period by accounting for each process, except for microbial growth (Table 2). The meeq consumed via methane production, sulfate reduction, and dechlorination were based on 8 meeq/mmole of methane produced, 8 meeq/mmole of sulfate reduced to sulfide, and 2 meeq/mmole of chlorine substituent removed. With the exception of the BES-amended culture, which had a very low microbial activity, between 87.2 and 89.5% of the electron donor-derived meeq were accounted for at the end of the 7 day incubation (Table 2). The fraction of the total meeq utilized diverted to the sequential dechlorination of PCA to dichloroanilines in the control (i.e., sulfate-free) and sulfate-amended culture was very small (ca. 0.017%), which is typical of mixed, dechlorinating cultures maintained with a very high external electron donor/chloroorganic eeq ratio (Aulenta et al. 2008; Pavlostathis et al. 2003). PCA dechlorination in the BES- and BES + sulfate-amended cultures accounted for 10 and 0.1% of the electron donor-derived meeq, respectively. The amount of consumed but unaccounted meeq (6.7–12.8%) is assumed to be related to the microbial growth. It is noteworthy that in the absence of BES, sulfate reduction consumed only 2.3% of the electron donor-derived meeq. These calculations show that the small population size of sulfate reducers could not outcompete the methanogens for electron donor utilization in the sulfate-amended culture series as discussed below. Addition of BES to the sulfate-amended culture highly affected the meeq fraction used by sulfate reducers (88.5%) and methanogens (1.0%). However, as mentioned above, both the rate and pathway of PCA dechlorination were the same in the control culture and the cultures amended with either sulfate, BES, or sulfate plus BES.

The relatively low sulfate reduction rate in the sulfate-amended culture was attributed to the low population density of sulfate reducers in the stock culture, which was enriched and maintained as a sulfate-free culture for over 4 years before the present study was conducted, as well as to competition by methanogens for electron equivalents. The sulfate reduction rate increased by more than five fold when methanogenesis was blocked in the BES- and sulfate-amended culture, further indicating the impact of competition by the methanogens in the BES-free culture for electron equivalents, in addition to the difference in relative population size between sulfate reducers and methanogens in this batch assay. For electron donors such as methanol and H_2, based on

Table 2 Electron balance for the short-term PCA dechlorination assay

Culture	Total electron equivalents used (meeq/L)[a]	Electron equivalents (meeq/L) used for[b]			Total electron equivalents accounted for (meeq/L)	Recovery (%)
		Methanogenesis	Sulfate Reduction	Dechlorination		
Control (sulfate-free)	175.8	153.3	NA	0.03	153.33	87.2
Sulfate-amended	176.0	150.0	4.0	0.03	154.03	87.5
BES-amended	0.3	0.25	NA	0.03	0.28	93.3
BES + sulfate-amended	26.0	0.25	23.0	0.03	23.28	89.5

At the end of the 7-day incubation; culture initial conditions: 7 μM PCA, 1,318 mg/L methanol (=371 meeq/L), 17 mg/L yeast extract, and 300 mg sulfate-S/L (75 meeq/L; where indicated)

NA not applicable

[a] Based on soluble COD measurements and net soluble COD destruction

[b] Ignores microbial growth

both free energy and threshold concentration considerations, sulfate reducers are expected to outcompete methanogens (Cord-Ruwisch et al. 1988; Liamleam and Annachhatre 2007; Löffler et al. 1999; McCartney and Oleszkiewicz 1993). However, in addition to thermodynamic considerations, substrate preferences as well as the concentration of the electron donor(s) relative to that of electron acceptor(s) play a major role in microbial competition. It has been reported that methanol, when used directly by sulfate reducing bacteria, results in low rates of sulfate reduction under mesophilic conditions, leading to a slow growth rate of sulfate reducers. Methanol can also be used indirectly by sulfate reducers in the presence of other anaerobic microorganisms, which convert methanol to H_2/CO_2, formate, and acetate (Liamleam and Annachhatre 2007). McCartney and Oleszkiewicz (1993) reported that the potential for methanogens to outcompete sulfate reducers exists when the electron donor:sulfate ratio is high or when a build up of sulfide occurs. In the present study, the relatively high COD concentration (1,978 mg/L) and the low sulfate concentration (300 mg S/L), along with the low population density of sulfate reducers explain why sulfate reducing bacteria competed less efficiently with methanogens for reducing equivalents in the batch assay.

Assessment of PCA dechlorination—Long term assay

PCA dechlorination and sulfate reduction

The long-term PCA dechlorination assay lasted for 118 days and included five feeding cycles (Fig. 3). All analyses were conducted in triplicate, but as the coefficient of variation for all data was less than 5%, error bars are not shown in Fig. 3. Throughout the entire incubation period, the culture pH ranged from 6.9 to 7.1. The culture head space composition was analyzed throughout the incubation period and CH_4, CO_2, and H_2S data are shown in Fig. 3. H_2 was not detected in any of the five cycles. In a recent study conducted by Aulenta et al. (2008), liquid-phase H_2 concentrations in the order of 10–20 nM and about 3 nM were measured in butyrate-fed mixed, tetrachloroethylene dechlorinating cultures in the absence and presence of sulfate reduction, respectively. Based on such reports, along with the above discussed H_2 threshold concentration values for dechlorinators (<0.3–2 nM) and hydrogenotrophic sulfate reducers (1–4 nM), the lack of H_2 detection in our study is the result of the high detection limit (500 ppmv, corresponding to a liquid-phase H_2 concentration of about 0.4 μM). The PCA dechlorination in the sulfate-free (control) culture proceeded at an initial rate of 6.5 μM/day and the dechlorination pathway was similar to that observed in the short-term assay (Fig. 1). A similar initial PCA dechlorination rate and pathway was observed in the sulfate-amended culture during the first and second feeding cycles, confirming the results obtained in the short-term assay. During the first and second feeding cycles, the initial sulfate concentration was 300 mg S/L. In both cycles, simultaneous sulfate reduction and dechlorination took place, but sulfate reduction was relatively slow (2.7 mg sulfate-S/L-day; Fig. 3b). The reasons for the observed low sulfate reduction rate (i.e., relatively low population size of sulfate reducers, slow growth rate, and competition by methanogens for electron equivalents) were discussed above.

At the beginning of the third feeding cycle, 25 mM BES was added to the sulfate-amended culture to enhance sulfate reduction by blocking methanogenesis occurring either by direct methanol utilization or indirectly via H_2/CO_2 and/or acetate produced by other methanol-using microorganisms in the mixed culture. Indeed, a relatively higher initial sulfate reduction rate (7.3 mg sulfate-S/L-day) was observed in this cycle. The increase in the sulfate reduction rate is reflected in an increased level of H_2S gas formation (Fig. 3e). The efficiency of BES in blocking methanogenesis is shown by the virtual cessation of methane production (Fig. 3d) and the accumulation of acetate (Fig. 3c). The rate of PCA dechlorination and its pathway were as those observed in the first and second feeding cycle, indicating no inhibition or any adverse effect on the sequential dechlorination of PCA by either BES or a relatively higher sulfate reduction rate.

In order to investigate the influence of a much higher sulfate reduction rate on the PCA dechlorination, the initial sulfate concentration was increased to 890 mg sulfate-S/L in the BES-amended culture at the beginning of the fourth feeding cycle. A noticeably higher sulfate reduction rate (39 mg sulfate-S/L-day) and a higher H_2S production were observed (Fig. 3b, e). However, PCA dechlorination practically ceased

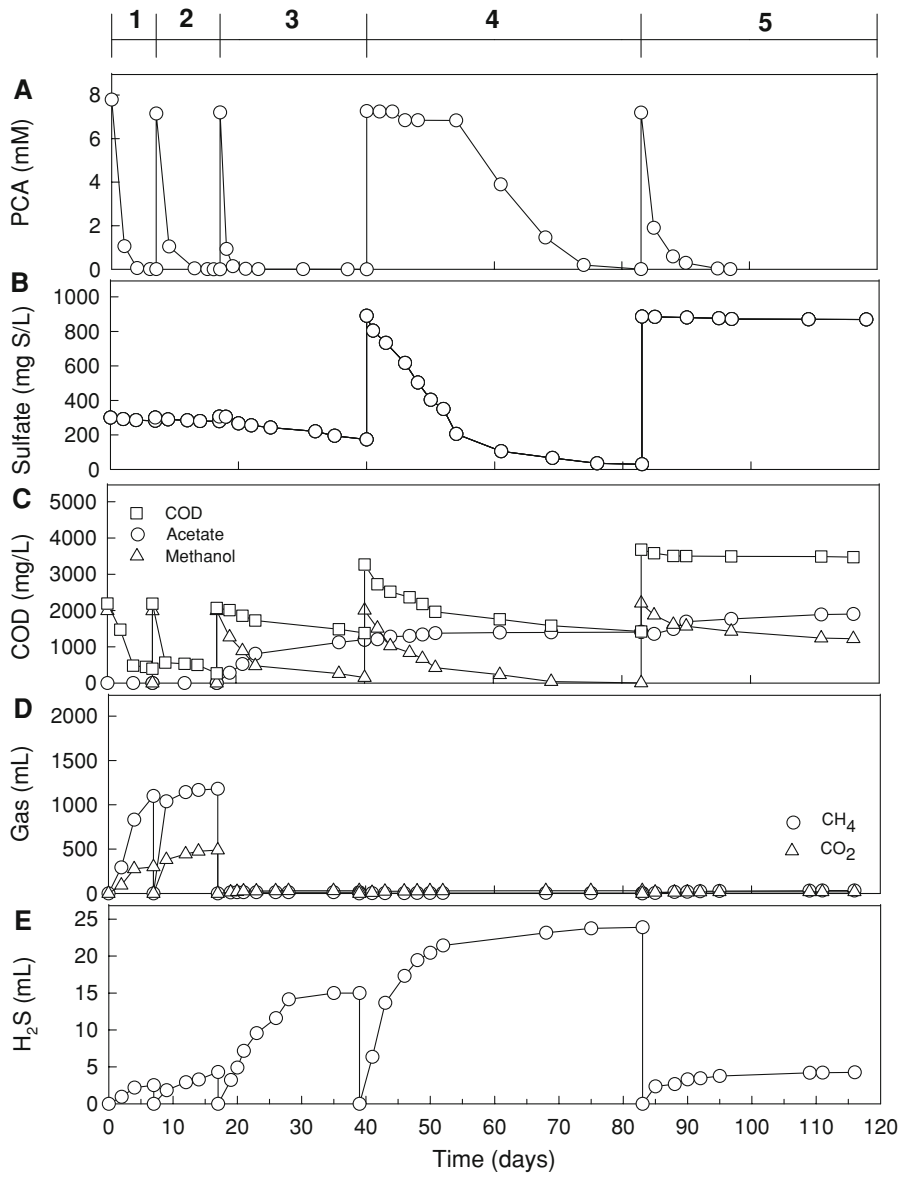

Fig. 3 Long-term PCA dechlorination assay. **a** PCA dechlorination, **b** sulfate reduction, **c** methanol utilization, acetate production, and COD, **d** CH_4 and CO_2 production, and **e** H_2S production (five successive feedings cycles; cycles 1 and 2: culture amended with sulfate; cycle 3: culture amended with sulfate and BES; cycles 4 and 5: culture amended with sulfate)

during the first 15 days of incubation of the fourth feeding cycle and then resumed when the sulfate concentration dropped to about 100 mg S/L. It is noteworthy that the methanol utilization in this cycle proceeded at a slower rate compared to the previous three cycles, which is due to the cessation of methanol utilization by methanogens. In addition, acetate continued to increase, albeit at a lower rate (Fig. 3c). In a recent study by Aulenta et al. (2008), addition of sulfate (120 mg S/L) to a mixed dechlorinating culture resulted in a drastic decrease of the dechlorination rate of 1,1,2,2-tetrachloroethane compared to an identical culture devoid of sulfate. Previous reports on the effect of sulfate reduction on microbial dechlorination have shown either an absence of inhibition (Bagley and Gossett 1990; DeWeerd et al. 1991; Hoelen and Reinhard 2004; Pavlostathis and Zhuang 1991), partial inhibition (Cabirol et al. 1998), or complete inhibition (Nelson et al. 2002). The difference among the above cited studies with respect to the effect of sulfate reduction on dechlorination may be due to the microbial diversity and relative population size of the different physiological groups resulting from difference in inocula origin and enrichment conditions (e.g., relative

levels of electron donor, sulfate and chlorinated compounds, as well as type of electron donor).

In the absence of methanogenesis in the sulfate-amended mixed culture after the addition of BES, the possible metabolic processes related to methanol utilization are: (a) direct methanol utilization by sulfate reducing bacteria; and (b) conversion of methanol by non-sulfate reducing bacteria to intermediates such as H_2/CO_2, formate, and acetate, which can then serve as electron donors for sulfate reduction. Cord-Ruwisch et al. (1988) reported the intermediary production of H_2 from methanol and the consumption of H_2 by hydrogenotrophic sulfate reducers. It is also known that in the presence of bicarbonate, homoacetogenic bacteria can convert methanol to acetate (Weijma and Stams 2001). However, homoacetogenic bacteria are more predominant and active at thermophilic as opposed to mesophilic conditions. The production of H_2 from methanol is of particular significance relative to microbial reductive dechlorination. It is believed that methanol, as well as other carbon sources such as low molecular weight fatty acids, used to support reductive dechlorination in either pure or mixed cultures may merely serve as precursors for the formation of an intermediate H_2 pool (Aulenta et al. 2008; DiStefano et al. 1992; Fennell et al. 1997; Kassenga and Pardue 2006; Löffler et al. 1999). Duhamel and Edwards (2007) reported conversion of methanol to acetate and hydrogen during the reductive dechlorination of chlorinated ethenes and the dihaloelimination of 1,2-dichloroethane in a mixed anaerobic culture.

In the present study, the observed, transient cessation of PCA dechlorination at the beginning of the fourth cycle and its relationship to the accelerated sulfate reduction is explained based on the following reasoning. Reported H_2 threshold values for halorespirers and sulfidogens are below 0.3–2 and 1–15 nM, respectively (Cord-Ruwisch et al. 1988; Löffler et al. 1999). Therefore, based on the threshold concentration consideration, halorespirers are expected to outcompete sulfidogens and therefore PCA dechlorination should not have been impacted at the beginning of the fourth cycle. A plausible explanation for the cessation of PCA dechlorination is that methanol was directly utilized by sulfate reducers, and while active sulfate reduction was maintained, the pool of H_2 derived from methanol by non-sulfate reducing bacteria dropped below the halorespirers' H_2 threshold value. It is noteworthy that at the time PCA dechlorination resumed in the forth cycle, a significant methanol concentration was still present (Fig. 3c). Therefore, as the sulfate reduction became slower when the sulfate concentration dropped to about 100 mg/L (around day 50), the formation of H_2 from methanol may have increased, which in turn was able to support a relatively fast PCA dechlorination rate (Fig. 3a). Throughout the 5 cycles, low H_2 concentrations must have existed as a result of H_2 utilization by both dechlorinating and non-dechlorinating species, which explains the lack of H_2 detection in the culture head space (detection limit 500 ppmv).

In the fifth cycle, the initial sulfate concentration was similar to that in the fourth cycle (886 mg sulfate-S/L), but a very low rate of sulfate reduction was observed in this cycle (Fig. 3b), which was attributed to the accumulation of sulfide (see below). In contrast, the rate of PCA dechlorination was as fast as in the first 3 cycles and its pathway not affected. These results further reinforce the findings of Okutman Tas et al. (2006) that the sequential dechlorination of PCA by the mixed enrichment culture is mediated by dechlorinating bacteria. It is noteworthy that in the fifth cycle, the rate of methanol utilization was slow, about 55% of the initial methanol was not utilized by the end of the cycle, and about 50% of the methanol used was converted to acetate (Fig. 3c).

Electron equivalence calculations were performed at the end of each feeding cycle during the long-term assay (Table 3). During the five feeding cycles, the total meeq accounted for ranged from 85.4 to 93.3% of the methanol-derived meeq, and the balance was attributed to biomass growth. In the first and second feeding cycle, the meeq fraction used by sulfate reducers was 2%, respectively, as a result of the relatively low population size of sulfate reducers relative to methanogens and competition for electron equivalents as discussed above. In the third feeding cycle, as a result of BES addition, the meeq fraction used by the methanogens dropped to 5.3%, while the meeq fraction used by the sulfate reducers increased to 80% due to the low demand for electron equivalents by the methanogens. In the fourth feeding cycle, the meeq fraction used by the sulfate reducers and methanogens was 93 and 0.2%, respectively, as a result of enrichment of sulfate reducers and inhibition of methanogens by BES. In the fifth feeding cycle,

Table 3 Electron balance for the long-term PCA dechlorination and sulfate reduction assay

Culture cycle[a]	Total electron equivalents used (meeq/L)[b]	Electron equivalents (meeq/L) used for[c]			Total electron equivalents accounted for (meeq/L)	Recovery (%)
		Methano genesis	Sulfate reduction	Dechlorination		
First	224.8	201.7	4.5	0.03	206.23	91.7
Second	239.8	216.1	5.0	0.03	221.13	92.2
Third	41.6	2.2	33.3	0.03	35.53	85.4
Fourth	231.1	0.5	215.0	0.03	215.53	93.3
Fifth	11.8	5.9	4.3	0.03	10.23	86.7

Culture initial conditions: PCA, 7.1–7.8 μM; methanol, 1,318 mg/L (=371 meeq/L), yeast extract, 17 mg/L; sulfate, 300 mg S/L (75 meeq/L; first to third cycle), 890 and 886 mg S/L (223 and 222 meeq/L; fourth and fifth cycle)

[a] First and second cycle, BES-free; third to fifth cycle, BES-amended

[b] Based on soluble COD measurements and net soluble COD destruction

[c] Ignores microbial growth

the meeq fraction used by the sulfate reducers dropped to 36%, whereas the meeq fraction used for methane production increased to 50% indicating a slow recovery of methanogenesis overtime. BES was added to the culture only at the beginning of the third cycle and the reduced effectiveness of BES in inhibiting the methanogens over the long incubation period may be attributed to culture dilution as a result of periodic culture media addition and/or possible partial BES degradation. Based on the total meeq utilized, the meeq fraction diverted to the sequential dechlorination of PCA to dichloroanilines was very small (ca. 0.013%) in the first, second, and fourth cycle, whereas this fraction was 0.07 and 0.25% in the third and fifth cycle.

Effect of sulfide on PCA dechlorination and sulfate reduction

At the end of the fifth feeding cycle (118 days total incubation period), the total and soluble measured sulfide was 27.7 ± 0.3 mM (886 mg S/L ± 8.9) and 18.2 ± 0.2 mM (583 mg S/L ± 5.5), respectively. In spite of this significant sulfide accumulation during the course of the long-term assay, inhibition of the sequential dechlorination of PCA was not observed. This observation was further confirmed by the results of a batch assay performed with the stock, PCNB-transforming culture amended with a total sulfide concentration of 700 mg S/L. In this batch assay, the rate and pathway of PCA dechlorination were similar to that obtained in the short-term assay (Fig. 1).

The low sulfate reduction rate during the fifth feeding cycle was attributed to sulfide toxicity. Low levels of sulfide are necessary for growth, but it has been reported that excess sulfide can be toxic to sulfate reducers (Okabe et al. 1992; Roychoudhury and McCormick 2006; Vavilin et al. 1994). Reis et al. (1992) suggested that sulfide toxicity of sulfate reducers can result either through direct toxicity of enzymes involved in sulfate reduction or indirectly as a result of iron precipitation, leading to iron deprivation needed for cell constituents such as ferredoxin and cytochrome C, and/or reaction with cytochrome iron adversely impacting the electron transport system. Hilton and Oleskiewick (1988) reported that inhibition of sulfate reduction in a batch culture was proportional to the total sulfide concentration, not to the unionized H_2S concentration at pH values between 6 and 8. Reis et al. (1992) stated that hydrogen sulfide produced from sulfate reduction in a batch culture had a direct and reversible toxicity effect on sulfate reducers growing on lactate and incubated at a pH range of 5.8–7.0.

To further test our conclusion that the low sulfate reduction rate during the fifth cycle was due to sulfide accumulation, a batch assay was conducted with a culture aliquot obtained at the end of the fifth cycle, diluted with fresh culture media, and amended with 900 mg sulfate-S/L. A relatively fast sulfate reduction rate (25.1 mg sulfate-S/L-day) was observed, which supports the conclusion that accumulated sulfide led to the low sulfate reduction rate during the fifth cycle.

In order to calculate all sulfur species in the sulfate and BES-amended culture, the MINEQL + version 4.5 chemical equilibrium software was used (Schecher and McAvoy 1992). Calculations were performed for a closed system under the following conditions: pH 7, temperature 22°C, ionic strength 0.263 M, alkalinity 4 g/L as $CaCO_3$ and metal concentrations those in the culture media listed above. Based on MINEQL+ calculations, the values of aqueous and precipitated sulfide were 24 and 9.1 mM, respectively, which are relatively close to the measured sulfide concentration in the culture. According to MINEQL+ speciation modeling, chalcopyrite ($CuFeS_2$) and covellite (CuS) were the dominant precipitated sulfur species in the sulfate-amended culture at concentrations of 7.5 and 1.29 nM, respectively and $H_2S(aq)$, HS^-, $ZnS(HS)^-$ and $Zn(HS)_3^-$ were the dominant dissolved sulfur species at concentrations 11.2, 13.5, 2.73×10^{-4}, and 5.19×10^{-3} mM, respectively.

The production and accumulation of acetate during the course of the third, fourth and fifth feeding cycles in the sulfate and BES-amended culture may also have contributed to the inhibition of sulfate reducers. Lens et al. (2002) pointed out that acetate production is a major drawback of sulfate reduction because sulfate reducers cannot completely oxidize acetate even at excess sulfate levels. Reis et al. (1990) found that undissociated acetic acid at a concentration of approximately 54 mg/L was inhibitory to sulfate reducing bacteria growing under H_2S stripping conditions and pH values between 5.8 and 7.0. In the present study, at the end of the fifth cycle, the total acetate + acetic acid was 1,900 mg COD/L, which for pH = 7 and a $pK_a = 4.76$, corresponds to an undissociated acetic acid concentration of about 10 mg/L (0.17 mM). Regardless of the exact cause of the observed inhibition of sulfate reduction in the fifth cycle, it is noteworthy that both the rate and extent of PCA reductive dechlorination were not impacted.

Conclusions

The rate and pathway of PCA dechlorination by a PCA-dechlorinating, mixed anaerobic culture in the presence of sulfate was assessed. Overall, the results of this study show that the sequential dechlorination of PCA takes place simultaneously with sulfate reduction at a relatively low sulfate reduction rate. Therefore, the presence of low sulfate concentrations (≤300 mg sulfate-S/L) in anaerobic environments is not expected to negatively affect the dechlorination of PCA. At relatively higher sulfate reduction rates, PCA dechlorination was halted until the sulfate concentration was reduced to or below 100 mg sulfate-S/L. Accumulation of sulfide and acetate in excess of 800 mg S/L and 1,900 mg COD/L, respectively, resulted in a drastic decline of the sulfate reduction rate. However, the rate and pathway of PCA dechlorination were not affected under these conditions. We cannot emphasize enough that in mixed culture environments, the outcome of the competition between sulfate reducers and dechlorinators does not simply depend on the sulfate concentration but rather on the actual sulfate reduction rate. The same is true for the observed competition between methanogens and sulfate reducers, where methanogens outcompeted sulfate reducers because of their higher abundance and/or activity.

As stated before (Okutman Tas and Pavlostathis 2005), because of a 1,800–9,800-fold increase in aqueous solubility and a 55–340-fold decrease in the octanol-water partition coefficient between PCA and di- or mono-chloroanilines, respectively, microbial reductive dechlorination leads to an increased mobility of the PCA biotransformation products. Thus, the findings of the present study have significant environmental implications relative to the fate and transport of PCA and its dechlorination products in sulfate-laden subsurface systems.

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