

Report for 2004GA59B: REDUCTIVE BIOTRANSFORMATION OF POLYCHLORO-NITROBENZENES UNDER IRON- REDUCING CONDITIONS

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Project Report

**REDUCTIVE BIOTRANSFORMATION OF
POLYCHLORONITROBENZENES UNDER IRON-REDUCING
CONDITIONS**

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May 2005

TABLE OF CONTENTS

	<u>Page</u>
EXECUTIVE SUMMARY	3
1. INTRODUCTION	4
2. BACKGROUND	5
3. RESEARCH METHODOLOGY	7
3.1. Target Compounds	7
3.2. Scope/Hypothesis	7
3.3. Research Objectives	9
3.4. Materials and Methods	9
3.4.1. Culture development	9
3.4.1.1. Iron-reducing enrichment cultures (Cultures A, B, and C)	9
3.4.1.2. Fermentative/methanogenic enrichment culture (Culture D)	10
3.4.2. PCNB biotransformation assays	10
3.4.3. Assessment of the effect of iron reduction on PCA dechlorination	10
3.4.4. Analytical methods	11
4. RESULTS AND DISCUSSION	12
4.1. Iron-reducing enrichment cultures	12
4.1.1. Development and activity of iron-reducing enrichment cultures (Cultures A, B, and C)	12
4.1.2. PCNB biotransformation in an iron-reducing enrichment culture (Culture A)	16
4.2. Fermentative/methanogenic enrichment cultures	17
4.2.1. PCNB biotransformation by the fermentative/methanogenic enrichment culture (Culture D)	17
4.2.2. Effect of iron reduction on PCA dechlorination by the fermentative/ methanogenic enrichment culture (Culture D)	18
5. CONCLUSIONS	22
6. DISSEMINATION OF PROJECT RESULTS	23
7. REFERENCES	23

EXECUTIVE SUMMARY

Persistent anthropogenic, hydrophobic organic compounds in the environment pose a chronic threat to the health and safety of humans and wildlife. Despite the fact that chloronitroaromatics are extensively used synthetic compounds in industry and agriculture (e.g., pesticides, fungicides, pharmaceuticals, dyes) and found worldwide in surface and subsurface soils, as well as streambed sediments, studies on their fate and transformation, especially for polychloronitro-substituted compounds, have been rare as compared to either chlorinated or nitroaromatic compounds. To address and explore this paucity of information, the focus of this research was to elucidate the reductive biotransformation of polychloronitrobenzenes, and their corresponding chloroanilines (CAs) under iron-reducing conditions. Several of these compounds are classified as either carcinogenic, potentially carcinogenic, or as methemoglobinemia-causing chemicals. The main parent compound used in the present research was pentachloronitrobenzene (PCNB), a powerful organochlorine fungicide commonly used to combat phytopathogenic fungi. Although PCNB is used in several States, most of its usage is found in the Southeastern US because of its effectiveness against phytopathogenic fungi associated with crops predominantly found in this region.

Our research hypothesis was that reductive dehalogenation of polychlorinated organic compounds under iron-reducing conditions may take place co-metabolically where both Fe(III) and the chlorinated organic compounds merely serve as electron acceptors (primary and secondary electron acceptors, respectively), and/or abiotically, using biologically produced Fe(II) as the terminal electron donor (i.e., as a reductant). The specific objectives of this study were to: a) assess the potential for the microbial reductive transformation of PCNB and CAs under dissimilatory iron-reducing conditions; b) evaluate the effect of iron bioavailability on the amination/dechlorination process; and c) assess the role of humic substances in the iron reduction and PCNB biotransformation processes.

Overall, the results of this study indicate that biotransformation of PCNB to pentachloroaniline (PCA) is very fast under both methanogenic and iron reducing conditions. Reduction of the nitro-group of PCNB and production of PCA also takes place under abiotic, reductive conditions. However, the rate of this conversion is highly enhanced under biotic conditions. Further dechlorination of PCA under abiotic, reductive conditions, e.g., in the presence of sulfide and/or Fe(II), was not observed in the present study. Under biotic conditions, the rate of PCNB to PCA biotransformation was higher under methanogenic conditions as compared to iron-reducing conditions. PCA dechlorination did not take place when Fe(III)-EDTA was reduced at a relatively fast rate under biotic conditions. In contrast, PCA dechlorination and iron reduction took place simultaneously when the iron source was less bioavailable (e.g., FeOOH). Addition of anthraquinone 2,6-disulfonate (AQDS), a humic acid model compound, to the culture, increased the rate and extent of FeOOH reduction, which in turn resulted in a significant decrease of the rate and extent of PCA dechlorination. Thus, fast iron reduction suppresses and delays the onset of the reductive dechlorination of PCA.

The results of this study have significant implications relative to the fate and biotransformation of PCNB and PCA under anoxic/anaerobic conditions encountered in most subsurface soil and sediment environments. Based on the observed fast and facile conversion of PCNB to PCA, PCNB is not expected to be persistent in natural systems. However, the bioavailability of iron in natural systems, which in turn is controlled by soluble humic material, has a pronounced effect on the rate and extent of PCA dechlorination and thus on its persistence in natural systems.

1. INTRODUCTION

Persistent anthropogenic, hydrophobic organic compounds in the environment pose a chronic threat to the health and safety of humans and wildlife. Despite the fact that chloronitroaromatics are extensively used synthetic compounds in industry and agriculture (e.g., pesticides, fungicides, pharmaceuticals, dyes) and found worldwide in surface and subsurface soils, as well as streambed sediments, studies on their fate and transformation, especially for polychloronitro-substituted compounds, have been rare as compared to either chlorinated or nitroaromatic compounds. To address and explore this paucity of information, the focus of this research was to elucidate the reductive biotransformation of polychloronitrobenzenes (polyCNBs), and their corresponding chloroanilines (CAs) under iron-reducing conditions. Several of these compounds are classified as either carcinogenic, potentially carcinogenic, or as methemoglobinemia-causing chemicals. The main parent compound used in this research was pentachloronitrobenzene (PCNB). PCNB ($C_6Cl_5NO_2$), a powerful fungicide commonly used to combat phytopathogenic fungi, is a registered organochlorine fungicide used as a seed dressing or soil treatment to control a wide range of fungi species in crops such as cotton, potatoes, wheat, onions, lettuce, tomatoes, tulips, garlic, and others, as well as on grass, lawn flowers, ornamental crops, shrubs and in gardens (EXTOXNET, 1996; U.S. EPA, 2003). PCNB is included in U.S. EPA's toxicity class III and is among the thirty chemicals included in the U.S. EPA's list of "Waste Minimization Priority Chemicals". The U.S. EPA has classified PCNB as a possible cancer causing substance (U.S. EPA, 2003).

During 2000 alone, 23,500 pounds of PCNB were used in six participating States (CA, FL, MI, OR, PA, and TX) in nursery and floriculture, whereas, 5,400 pounds were used in 2002 in the production of a single vegetable (fresh snap beans) in five participating States (FL, GA, NY, NC, and TN)(USDA, 2002; USDA, 2003). Although PCNB is used in several States, most of its usage is found in the Southeastern US because of its effectiveness against phytopathogenic fungi associated with crops predominantly found in this region. For example, in 2000, from the total reported PCNB usage of 543,500 lbs of active ingredient, 331,100 lbs (i.e., 61%) were used in seven States in the region (Table 1).

Table 1. PCNB Use for Two Selected Crops in the Southeastern U.S.
(active ingredient in lbs; 2000 data; NASS, 2003)

State	Snap Fresh Beans	Cotton Upland
Alabama	NA	74,000
Florida	6,900	NA
Georgia	7,200	NA
Louisiana	NA	48,000
Mississippi	NA	109,000
North Carolina	NA	17,000
South Carolina	NA	NA
Tennessee	NA	69,000
Total	> 14,100	> 317,000

NA, data not available

PCNB is extensively used as a fungicide for the prevention and control of certain soil borne diseases on golf courses, sod farms, home lawns and institutional areas where turf is grown. In a recently published report by the USGS on the occurrence of semivolatile organic compounds in streambed sediments of twenty major river basins across the United States, PCNB

was detected at a maximum concentration of 180 µg/kg (Lopes and Furlong, 2001). Therefore, as the above-mentioned, PCNB-related activities are more intense in the Southeastern US, the fate of PCNB and its biotransformation products is of regional interest.

2. BACKGROUND

Polyhalogenated organic compounds tend to be resistant to biodegradation in aerobic environments. Such compounds are more oxidized than their nonhalogenated counterparts due to the presence of highly electronegative halogen substituents, which provide molecular stability. Therefore, as the degree of halogenation increases, reduction of these compounds is more likely to occur than oxidation (Pavlostathis *et al.*, 2003). However, polyhalogenated compounds can be used as electron acceptors in thermodynamically favorable reactions (Dolfing and Beurskens, 1995; Fantroussi *et al.*, 1998; Fetzner and Lingens, 1994; Fetzner, 1998; Middeldorp *et al.*, 1999; Häggblom *et al.*, 2000). Under anaerobic conditions, the reductive transformation of nitroaromatic compounds proceeds with the reduction of one or more aryl nitro groups (-NO₂) and the formation of aryl amines.

Nitrate, sulfate, and carbonates are alternative electron acceptors commonly considered for anaerobic subsurface processes (i.e., nitrogen reduction, sulfate reduction, and methanogenesis) and bioremediation applications. Relatively recent studies however have demonstrated the significant role of iron and manganese reducing bacteria in the cycling of organic matter and the biotransformation of organic contaminants (Lovley, 1991). The role of dissimilatory iron-reducing bacteria in the reduction of nitrobenzenes has been elucidated (Heijman *et al.*, 1995; Klausen *et al.*, 1995). This transformation involves two coupled reactions, one biotic and mediated by the iron-reducing bacteria, the other abiotic, where reduced iron [Fe(II)] serves as the intermediate electron donor and nitrobenzenes serve as the electron acceptor. The interdependence between the reduction of the organic pollutants and microbial iron reduction in aquifers was demonstrated. The biotic iron reduction step -- which provides the required reducing power for the transformation of the contaminants -- was found to be the rate-limiting process. On the other hand, the reduction of the contaminants leads to a continuous regeneration of the relatively limited, microbially-available Fe(III) pool.

Based on published reduction potentials of key redox couples of Fe(III)/Fe(II) and organic compounds (Haderlein and Schwarzenbach, 1995), polychlorinated organic compounds can be reduced by a number of Fe(II)-bearing compounds. Iron-mediated, abiotic dechlorination has been demonstrated for compounds such as carbon tetrachloride and chloroform (Matheson and Tratnyek, 1994; Kriegman-King and Reinhard, 1992; Kriegman-King and Reinhard, 1994). Microbial dechlorination under iron-reducing conditions has been reported for a number of halogenated compounds, including carbon tetrachloride (Picardal *et al.*, 1993), monochlorinated isomers of phenol, and benzoate (Kazumi *et al.*, 1995a; Kazumi *et al.*, 1995b), and vinyl chloride (Bradley and Chapelle, 1996).

Despite the fact that chloronitroaromatics are extensively used synthetic compounds in industry and agriculture (e.g., pesticides, fungicides, pharmaceuticals, dyes) and found worldwide in surface and subsurface soils, as well as streambed sediments, studies on their fate and transformation, especially for polychloronitro-substituted compounds, have been rare as compared to either chlorinated or nitroaromatic compounds. An exhaustive literature review did not reveal any information on the definitive reductive dechlorination of polyCNBs under iron-reducing conditions which was the focus of this research. To address and explore this paucity of information, the focus of this research was to elucidate the reductive biotransformation of polyCNBs, and their corresponding chloroanilines (CAs).

We have developed methanogenic, mixed cultures from a sediment historically contaminated with chlorinated benzenes and hexachloro-1,3-butadiene (Prytula and Pavlostathis, 1996; Booker and Pavlostathis, 2000; Yeh and Pavlostathis, 2001) as well as dissimilatory iron-reducing, mixed cultures from a non-contaminated soil sample and the contaminated sediment (Doikos, 1998). Iron was provided in the form of ferric-EDTA in order to circumvent limitations related to iron bioavailability. Research carried out in our laboratory with the iron-reducing cultures on the reductive dechlorination of hexachlorobenzene revealed a low rate and extent of dechlorination as compared to those achieved with methanogenic cultures (Doikos, 1998). These results may be an outcome of competition for available electrons by the two simultaneous processes, namely, iron-reduction and dechlorination, or due to complexation of Fe(II) with EDTA and possible inhibition of the dechlorination process. The proposed research expanded the scope of the previously conducted research by the use of PCNB as well as different forms of Fe(III).

The role of humic substances has been recognized in electron-transfer reactions in general and more specifically in the biotransformation of recalcitrant compounds (Bradley *et al.*, 1998; Lovley *et al.*, 1998; Scott *et al.*, 1998). Humics could serve as carbon and energy sources in mixed, dissimilatory iron-reducing cultures, as electron-transfer mediators between Fe(II) and halogenated organic compounds (Haderlein and Schwarzenbach, 1995), as well as electron shuttles in the microbial reduction of insoluble Fe(III) compounds (He and Sanford, 2003; Komlos and Jaffr 2004). The role of humic substances in the biotransformation process of PCNB and PCA under dissimilatory, iron-reducing conditions was also assessed in this study.

Most of the early studies on the biodegradation of PCNB focused on using fungi and were conducted under aerobic/anoxic conditions. Production of extracellular phenoloxidases by a number of fungal strains was not correlated with their capacity to degrade PCNB (Seiglemurandi *et al.*, 1992; Steiman *et al.*, 1992; Guiraud *et al.*, 1999). Removal of more than 50% of 100 mg/L PCNB by three fungal species was attributed to biosorption and subsequent biodegradation (Lièvrement *et al.*, 1996a). The best PCNB degradation by the fungal species *Sporothrix cyanescens* was observed under carbon and nitrogen limitation (Lièvrement *et al.*, 1996b). During the biotransformation of PCNB by four soil micromycetes, six metabolites were identified according to three metabolic pathways (Mora Torres *et al.*, 1996): PCNB → pentachloroaniline (C₆Cl₅NH₂) → tetrachloroaniline (C₆Cl₄HNH₂); PCNB → pentachlorothiophenol (C₆Cl₅SH) → pentachlorothioanisole (C₆Cl₅SCH₃); and PCNB → pentachlorophenol (C₆Cl₅OH) → pentachloroanisole (C₆Cl₅OCH₃).

PCNB degradation was observed in a large number of soil samples screened for microorganisms capable of degrading PCNB, irrespective of previous application of PCNB to the field. An isolate with a relatively fast PCNB degradation rate was identified as *Pseudomonas aeruginosa*. This isolate degraded PCNB best under anoxic conditions and the principal metabolic product of PCNB was pentachloroaniline, thus demonstrating that in anoxic soil environments the main degradation pathway of PCNB is its reduction to pentachloroaniline (Tamura *et al.*, 1995). Co-metabolic mineralization of monochloronitrobenzenes was achieved by a coculture of *Pseudomonas putida* and a *Rhodococcus* sp. The former species converted the chloronitrobenzenes to chloro-hydroxyacetanilides by partial reduction and subsequent acetylation, and the later species mineralized the chloro-hydroxyacetanilides to CO₂, NH₄⁺ and Cl⁻ (Park *et al.*, 1999). Sequential dehalogenation of CAs in microcosms developed with aquifer material was achieved under methanogenic, but not sulfidogenic conditions. Dechlorination at the *para* and *ortho* position of 2,3,4,5-TeCA resulted in the formation of 2,3,5-TrCA and eventually 3,5-DCA, whereas 3,4-DCA was transformed to 3-CA (Kuhn and Suflita, 1989).

Chloronitrobenzenes (3-CNB, 3,4-DCNB, 2,3,4-TrCNB, and PCNB) were transformed in anaerobic, sulfidogenic estuarine sediments with pseudo-first order rate constants ranging from 0.216 to 0.866 day⁻¹. Their transformation proceeded with reduction of the nitro group resulting in the formation of the corresponding chloroanilines, which were further transformed via *ortho* and *para* dechlorination pathways (Susarla et al., 1996). In contrast, chloroanilines were sequentially dechlorinated in the same estuarine sediments with pseudo-first order rate constants ranging from 0.005 to 0.012 day⁻¹ (Susarla et al., 1997). The degradation of 3,4-dihaloanilines by an anaerobic enrichment culture and *Rhodococcus* sp. strain derived from this culture under nitrate reducing conditions was recently reported to proceed by reductive deamination and formation of 1,2-dihalobenzene (Travkin et al., 2002). Further dehalogenation of the produced dihalobenzene was also observed.

3. RESEARCH METHODOLOGY

3.1. Target Compounds

As mentioned above, the focus of this research was primarily on the biotransformation of PCNB and its transformation products (i.e., CAs). The following compounds can theoretically be produced as a result of the sequential reductive dechlorination of pentachloroaniline: tetrachloroanilines (3); trichloroanilines (6); dichloroanilines (6); monochloroanilines (3); and aniline (see Figure 1). Reduction of the nitro group and formation of the electron-donating amino group will lead to electronic and steric effects, which in turn are expected to affect the reductive dechlorination of CAs. It has been shown that substitution of an electron-withdrawing nitro group by an electron-donating amino group strongly decreases the formation of electron donor-acceptor complexes by nitroaromatic compounds (Haderlein et al., 2000).

3.2. Scope/Hypothesis

Our research hypothesis was that reductive dehalogenation of polychlorinated organic compounds under iron-reducing conditions may take place by either one or both of the following mechanisms (Figure 2):

- a) Co-metabolic biotransformation: both Fe(III) and the chlorinated organic compounds merely serve as electron acceptors (primary and secondary electron acceptors, respectively)(Mechanism I, Figure 2);
- b) Abiotic transformation: the reductive dechlorination is mediated abiotically, using Fe(II) as the terminal electron donor (i.e., as a reductant) which is biotically produced from the reduction of Fe(III)(Mechanism II, Figure 2).

The difference between these two mechanisms is that in the first one, Fe(III) is reduced to Fe(II) without regeneration of Fe(III), whereas, according to the second mechanism, the reduction of the chlorinated organic contaminant(s) regenerates and continuously provides microbially available Fe(III) (see Figure 2). In the case of humic substances involvement in the reductive biotransformation process, these substances may serve as an electron shuttle between Fe(II) and the target chloroorganic compound, especially if the Fe(II) is surface-bound. In addition, humic substances may accelerate the biological reduction of amorphous Fe(III) oxyhydroxide. Such possible effects were assessed in the present study.

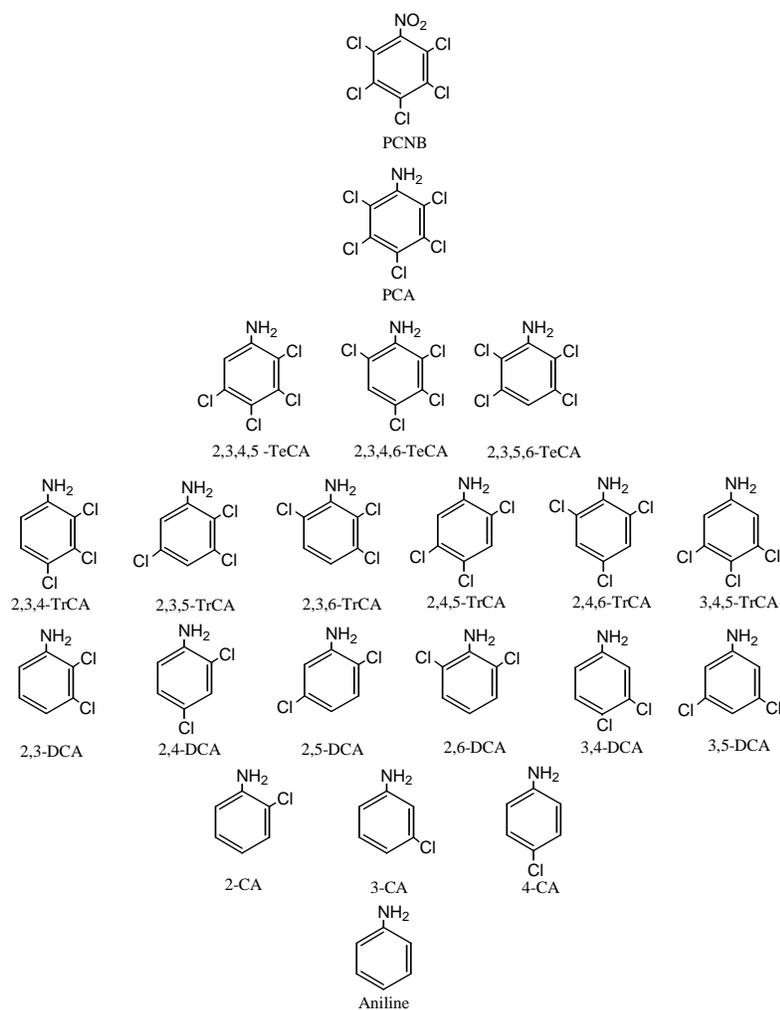


Figure 1. Possible compounds formed by the reductive transformation of PCNB.

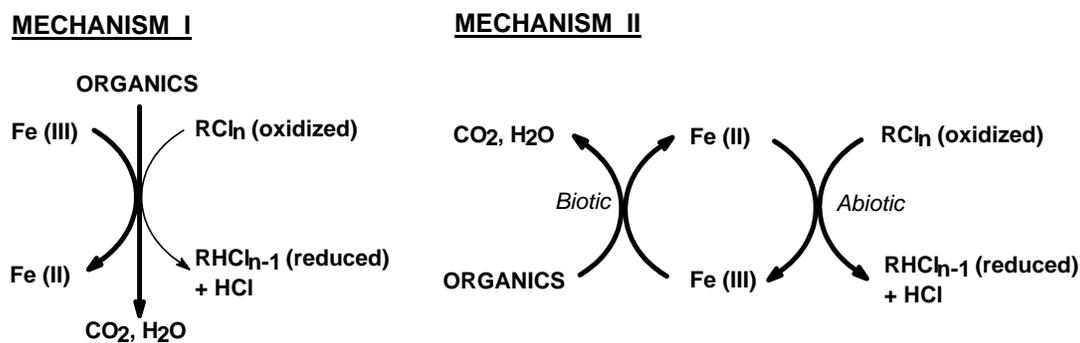


Figure 2. Mechanisms potentially involved in the reductive dechlorination of polychlorinated organic compounds under iron-reducing conditions.

3.3. Research Objectives

The specific objectives of this study were to:

1. Assess the potential for the microbial reductive transformation of PCNB and CAs under dissimilatory iron-reducing conditions.
2. Evaluate the effect of iron bioavailability on the amination/dechlorination process.
3. Assess the role of humic substances in the iron reduction and PCNB biotransformation processes.

3.4. Materials and Methods

3.4.1. Culture development

In order to meet the above-stated research objectives, a number of cultures were developed using a contaminated estuarine sediment as inoculum and monitored for various parameters over several feeding cycles (Table 2). Acetate or glucose and methanol were used as electron donors and Fe(III)-EDTA, Fe(III)-citrate, amorphous ferric hydroxide (FeOOH) and PCNB or PCA were used as electron acceptors.

Table 2. Enrichment cultures and their characteristics.

Culture	Electron acceptor-Target Compound	Electron donor	Iron form
A	Fe(III)	Acetate	Fe(III)-EDTA
B	Fe(III)	Acetate	Fe(III)-citrate
C	Fe(III)	Acetate	FeOOH
D	Fermentative/methanogenic-PCNB & PCA	glucose/methanol	None

The contaminated sediment was obtained from Bayou d'Inde, a tributary of Calcasieu River near Lake Charles, Louisiana, USA. Industrial wastes, including petroleum hydrocarbons, polycyclic aromatic hydrocarbons, and polychlorinated aromatic and aliphatic organic compounds have been discharged into this canal. The pH of the sediment sample was 6.6 and the total organic carbon was 5.74 ± 0.02 (expressed as % on a dry weight basis). The location of sediment and details on the sediment sampling have been reported elsewhere (Prytula and Pavlostathis, 1996; Gess and Pavlostathis, 1997). All cultures were developed and incubated in a constant temperature room at 22°C in the dark to better simulate natural conditions and were mixed once a day by hand.

3.4.1.1. Iron-reducing enrichment cultures (Cultures A, B, and C)

Three iron-reducing cultures were setup in 500 mL serum bottles as follows. Sediment samples (16 g wet basis) were added to the serum bottles, which were capped with butyl rubber stoppers, flushed with N₂ and the sediment sample was then diluted to 400 mL with iron-reducing culture mineral media. The media had the following composition (in mg/L): KH₂PO₄, 1000; NH₄Cl, 500; CaCl₂·2H₂O, 100; MgSO₄·7H₂O, 200; MgCl₂·6H₂O, 100; MnCl₂·4H₂O, 100; NaHCO₃, 2,500. Also, 0.2 mL/L vitamin stock solution (Wolin et al., 1963) and 1 mL/L trace metal stock solution (Mah and Smith, 1981) were added to the media. The cultures were fed with acetate (5 mM) as an electron donor and yeast extract (20 mg/L) and three different iron sources (Fe-citrate, Fe-EDTA and FeOOH) with a Fe(III) concentration of 25 mM. The pH in each culture

was kept between 6 and 7 with the addition of NaHCO_3 . A volume of each culture was wasted once a week and replaced with fresh media, and an aliquot of electron donor and Fe(III) was added, resulting in a culture retention time of 52 days. Microbial activity in these cultures was monitored by measuring total gas and gas composition, acetate, Fe(II) and total iron concentration. After several feeding cycles, sediment-free cultures were developed by diluting 50 mL of the first generation culture with 1.5 L mineral media in a nitrogen-flushed 2-L glass flask reactor (second generation culture). Because of the complexity of the process, as discussed in the Results and Discussion section, the Fe-citrate fed culture was not transferred to a new reactor.

3.4.1.2. Fermentative/methanogenic enrichment culture (Culture D)

This enrichment culture had been developed from the above-mentioned contaminated estuarine sediment as follows. A sediment sample (80 g wet basis) was added and diluted with 1.8 L of mineral media in a N_2 -flushed, 2-L glass flask reactor, capped with a Teflon-lined stopper. The media had the following composition (in mg/L): K_2HPO_4 , 900; KH_2PO_4 , 500; NH_4Cl , 500; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 100; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 200; $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$, 100; $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$, 500; NaHCO_3 , 1,200; resazurin (redox indicator), 2. Also, 0.2 mL/L vitamin stock solution (Wolin et al., 1963) and 1 mL/L trace metal stock solution (Mah and Smith, 1981) were added to the media. The culture was fed weekly with glucose and a PCNB/methanol solution. Glucose and methanol served as the carbon source and electron donor. At the beginning of each seven-day feeding cycle, glucose, yeast extract, and PCNB in methanol were added resulting in initial concentrations of 333 mg/L, 17 mg/L, 0.089 μM and 53 mg/L, respectively. The culture was kept in the dark in a 22°C constant temperature room, was stirred once a day using a Teflon-coated stirring bar over a magnetic stir plate, and its pH was kept around 7 with NaHCO_3 addition. After several feeding cycles, a culture transfer took place by diluting 100 mL of the first generation culture in 1.8 L mineral media in a N_2 -flushed, 2-L glass flask reactor (second generation culture). Finally, after several weekly wasting/feeding cycles, a similar culture transfer took place in 2 L of mineral media in a N_2 -flushed, 9-L glass reactor, and its contents were gradually increased up to 6 L (third generation culture). The retention time of this enrichment culture was 84 d. All batch experiments with the fermentative/methanogenic culture reported here were performed with the sediment-free, third generation mixed culture.

3.4.2. PCNB biotransformation assays

The biotransformation of PCNB by the enriched cultures was investigated using 160 mL serum bottles amended with acetate (300 mg/L), yeast extract (20 mg/L) and methanol (246 mg/L). The enriched culture D was able to quickly transform PCNB to PCA and further dechlorinate PCA to predominantly dichloroanilines under methanogenic conditions. Because of the relatively fast transformation of PCNB to PCA under both methanogenic and iron reducing conditions, in subsequent batch experiments, PCA dechlorination rather than PCNB biotransformation was investigated.

3.4.3. Assessment of the effect of iron reduction on PCA dechlorination

The impact of Fe(III) on the reductive dechlorination of PCA was investigated using the sediment-derived, third generation mixed culture developed under fermentative/methanogenic conditions (culture D). Fe(III)-EDTA (25 mM) and FeOOH (25 mM) were used as a source of iron in order to assess the rate and extent of PCA dechlorination and iron reduction. Aliquots of culture D were anaerobically transferred to 160 mL serum bottles which were previously capped with Teflon-lined septa and flushed with N_2 . The cultures were amended with 0.5 μM PCA

dissolved in methanol. The Fe(III)-EDTA amended cultures were fed twice with acetate in order to avoid lack of electron donor during the prolonged incubation. Possible inhibitory effects of EDTA (25 mM) and sodium (50 mM) resulting from the disodium salt of EDTA ($\text{Na}_2\text{-EDTA}$) were also tested. AQDS (200 μM), a humic acid model compound was added to some iron-amended cultures in order to increase the availability and thus the reduction rate of ferric iron. Another control was set up to test for any possible effect of AQDS on the reductive dechlorination of PCA in the absence of iron. Because of the potential of several culture media components (i.e., sulfide, Fe(II), vitamin B_{12}) to abiotically mediate the reductive transformation of PCA, two additional controls were setup, one with only autoclaved culture media and another with autoclaved media plus 25 mM ferrous iron ($\text{FeCl}_2\cdot 4\text{H}_2\text{O}$), which matched the iron concentration in the culture series amended with either Fe(III)-EDTA or FeOOH (see above).

3.4.4. Analytical methods

Liquid/liquid extraction was performed to analyze and quantify PCNB and its biotransformation products. Aliquots of 10 mL samples were extracted in glass tubes with 2 mL isooctane which contained 0.5 mg/L 1,3,5-tribromobenzene (TBB) as an internal standard. The tubes were capped with Teflon-lined stoppers and aluminum crimps, mixed vigorously for 2 min and centrifuged at 3000 rpm for 20 min. The solvent phase was transferred to amber glass autosampler vials, and sealed with Teflon-lined septa and aluminum crimps. The efficiency of the solvent extraction procedure was assessed at a concentration range of 0.03-40 μM PCNB, PCA and the less chlorinated anilines for 2 min, 1 h, 1 d and 3 d extraction period. It was determined that the efficiency was greater than 90% and did not change between 2 min and 3 days extraction period. Analysis of PCNB and biotransformation products was performed with an HP 6890 Series gas chromatograph (GC) equipped with an electron capture detector (ECD) and a 75-m 0.53-mm ID column (J&W Scientific, Folsom, CA, USA). The temperature program used was as follows: 100°C for 4 min, increased by 2°C/min up to 210°C and held for 60 min. Nitrogen was used as the carrier gas at a constant flow rate of 10 mL/min. The detection limits for PCNB, PCA and less chlorinated anilines were as follows: PCNB, 7 nM; PCA, 4 nM; tetrachloroanilines, 9 nM; trichloroanilines, 25 nM; dichloroanilines, 0.15 μM ; monochloroanilines 16 nM. Because of the structural similarity which led to coelution, 2,5-DCA and 2,4-DCA could not be separated during the GC/ECD analysis.

Because of detection limitations in GC/ECD, aniline, monochlorinated and dichlorinated anilines were also measured with a high performance liquid chromatography (HPLC) unit (Hewlett Packard Model 8453, Hewlett Packard Co., Palo Alto, CA) which was equipped with a Zorbax SB-C18 column (3 x 250 mm, 5 μm) (Agilent Technologies, New Castle, DE). The column compartment temperature was set at 40°C and the injection volume was 10 μL . The eluent mixture was an aqueous solution of 25 mM phosphate buffer (pH = 2.5) (52%) and methanol (48%) and the flow rate was 0.35 mL/min. Spectrometric detection was set at 203 nm. Samples used in HPLC analyses were filtered through 0.20 μm PTFE syringe filters (Cole-Parmer, Vernon Hills, IL) using glass syringes. The detection limits in HPLC for dichlorinated and monochlorinated anilines were 4 μM . 2,5-DCA and 2,4-DCA could also be separated during HPLC analysis.

Gas production was measured by connecting the culture headspace via a needle to an acid-brine solution filled graduated buret and recording the volume of displaced solution, after correcting to atmospheric pressure. Gas composition was determined by a gas chromatography (GC) unit equipped with a thermal conductivity detector. Methane was separated with a 25 m Chrompack Molsieve 5A fused silica 0.53 mm ID column and carbon dioxide was separated

with a 25 m Chrompack PoraPlot Q fused silica 0.53-mm ID column. Both columns were operated with helium as the carrier gas with a constant flow rate of 5 mL/min. The 10:1 split injector was maintained at 150°C and the detector temperature was set at 150°C. H₂ was measured with a GC (HP 5980 Series) equipped with a thermal conductivity detector and a 10 m Chrompack Molsieve 5A fused silica 0.53-mm ID column. The carrier gas was N₂ at a constant pressure of 70 psi. All gas samples were analyzed by injecting a 100 µL gas sample.

Methanol and acetate were measured in culture samples filtered through 0.20 µm syringe filters (PVDF-Whatman, Springfield Mill, England) using an HP 5890 Series II GC equipped with a flame ionization detector (FID) and a 35 m Stabilwax-DA 0.53-mm ID column (Restek, Bellefonte, PA, USA). Glucose was measured with a HP Series 1050 HPLC (Hewlett Packard, Palo Alto, CA) equipped with an Aminex HPX-87H-resin packed column (Bio-Rad, Richmond, CA). The column was maintained at 65°C in an external column heater. The eluent was 0.01 N H₂SO₄ and the flow rate was 0.6 mL/min.

The oxidation-reduction potential (ORP) was measured following the procedure outlined in *Standard Methods* (APHA, 1995) using an Orion Research digital pH/milivolt meter model 611 in conjunction with a Sensorex electrode (platinum electrode with an Ag/AgCl reference in a 3.5 M HCl gel). The meter was calibrated prior to use with a shorting lead to establish meter reading at zero millivolts and by using Light's standard solution [Fe(NH₄)₂(SO₄)₂·6H₂O, 0.1 M; H₂SO₄, 1 M]. Fe(II) was quantified with a modification of the method described by Kazumi et al. (1995a, b). Aliquots of 1 mL well-mixed culture were added to 20 mL 0.5 N HCl in 28 mL serum tubes. After 10 min of acidification, an aliquot of 100 µL was filtered and added to a serum tube containing 5 mL of ferrozine solution [1 g/L in 50 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer (pH = 7)]. This solution was vortexed for 10 s to develop the characteristic magenta color of the Fe(II)-ferrozine complex. The absorbance of this solution was measured using an HP 8453 UV-Visible spectrophotometer (Hewlett Packard, Palo Alto, CA) with DI water as a blank.

4. RESULTS AND DISCUSSION

4.1. Iron-reducing enrichment cultures

4.1.1. Development and activity of iron-reducing enrichment cultures (Cultures A, B, and C)

These cultures were fed with acetate (5mM) and yeast extract (20 mg/L) and three different iron sources (Fe-citrate, Fe-EDTA and FeOOH). The initial Fe(III) concentration was 25 mM which served as the electron acceptor. Development of the enrichment cultures took place without any contaminant (i.e., PCNB, or PCA) amendment. Significant gas production was observed during the development of the Fe-citrate and Fe-EDTA amended cultures (Figure 3A and 3B). After 25 days of incubation, methane was observed in the Fe-citrate amended culture, which also used citrate as an electron donor after a few days of incubation, resulting in significant acetate accumulation (Figure 4A). Significant carbon dioxide and methane production was not observed in the reactor amended with FeOOH (Figure 3A and 3B), although after 65 days of incubation, traces of methane were observed. Significant consumption of acetate was not observed in the culture amended with FeOOH (Figure 4A).

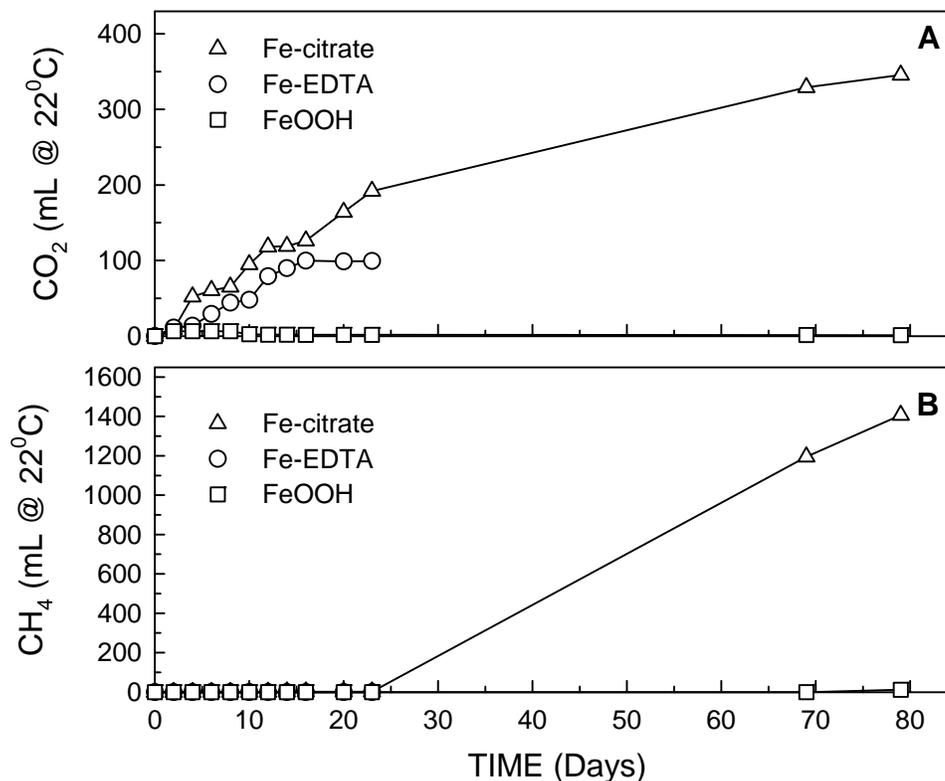


Figure 3. Cumulative gas production and composition: CO₂ (A) and CH₄ (B) in the sediment-derived, iron-reducing cultures (first-generation; multiple feeding cycles).

During all feeding cycles, the pH in the Fe-EDTA amended culture remained between 5 and 7. After feeding, the pH increased up to 6.5-7, as a result of media and alkalinity addition and gradually decreased due to the reduction of the newly added Fe(III) (Figure 4B). The pH drop can be explained by the following stoichiometric equation of iron reduction and acetate oxidation: $\text{CH}_3\text{COO}^- + 8 \text{Fe}^{3+} + 3 \text{H}_2\text{O} \rightarrow \text{CO}_2 + \text{HCO}_3^- + 8 \text{Fe}^{2+} + 8 \text{H}^+$. In the FeOOH amended culture, the pH increased from 7 to 8 over 23 days of incubation, which also indicates that iron reduction was very slow in this culture (Figure 4B).

Iron reduction in all three cultures was monitored by the measurement of Fe(II). Fast Fe(III) reduction was observed in both the Fe-citrate and Fe-EDTA amended cultures. However, Fe(III) reduction was much slower in the FeOOH amended culture (Figure 4C and 4D). Fe(III) reduction was the fastest in the Fe-EDTA amended culture. Because of the observed consumption of citrate and accumulation of a high concentration of acetate in the Fe-citrate amended culture, this culture was not transferred to create a second generation culture. After several feeding cycles, 50 mL inocula from the Fe-EDTA and FeOOH amended cultures were transferred to 2-L glass reactors and were maintained with the same feeding procedure. Headspace gas analysis showed production of CO₂ in the Fe-EDTA amended culture, but gas production was not observed during all feeding cycles in the second generation, FeOOH amended culture (Figure 5A). Similar to the first generation culture, the pH increased in the FeOOH amended culture during the incubation. After 15 days of incubation, although the pH was adjusted to 7, CO₂ production did not increase in the FeOOH amended culture (Figure 5B).

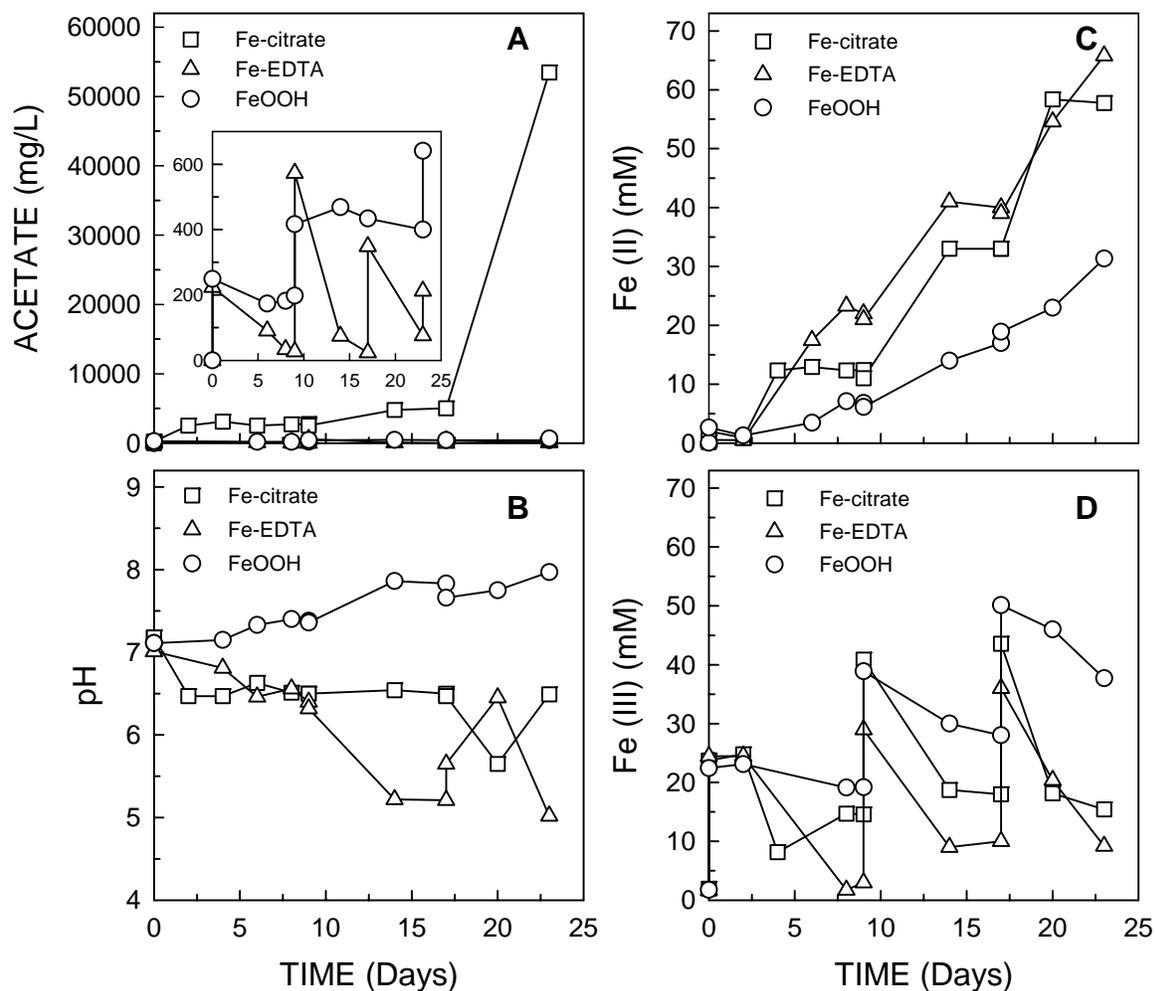


Figure 4. Acetate consumption (A), pH variation (B), Fe(II) production (C), and Fe(III) reduction (D) in the sediment-derived, iron-reducing cultures (first-generation; multiple feeding cycles).

Acetate consumption was much slower in the second generation FeOOH amended culture as compared to the Fe-EDTA amended culture (Figure 5C), which combined with the observed very low gas production, indicates very low microbial activity when the electron acceptor was FeOOH. In contrast, acetate consumption was much faster in the second generation Fe-EDTA amended culture (Figure 5C) and Fe(III) reduction approached almost 100% in all feeding cycles (Figure 5D). In contrast, the rate and extent of iron reduction in the FeOOH amended culture was much lower (Figure 5D). After pH adjustment of the FeOOH amended culture, a modest increase in the iron reduction rate was observed. In order to assess the effect of iron concentration on iron reduction in FeOOH-amended cultures, two batch cultures were setup with inoculum taken from the second generation FeOOH-reducing culture. In this setup, the initial Fe(III) concentrations of 40 mM and 80 mM were 1.6 and 3.2 times higher than the normal culture feeding conditions. After 7 days of incubation, about 45% of initially added FeOOH was reduced in both cultures (Figure 6). A higher iron reduction rate was observed in the 80 mM amended culture as compared to the 40 mM amended culture. Magnetite (Fe_3O_4) production was observed in the culture amended with 80 mM FeOOH.

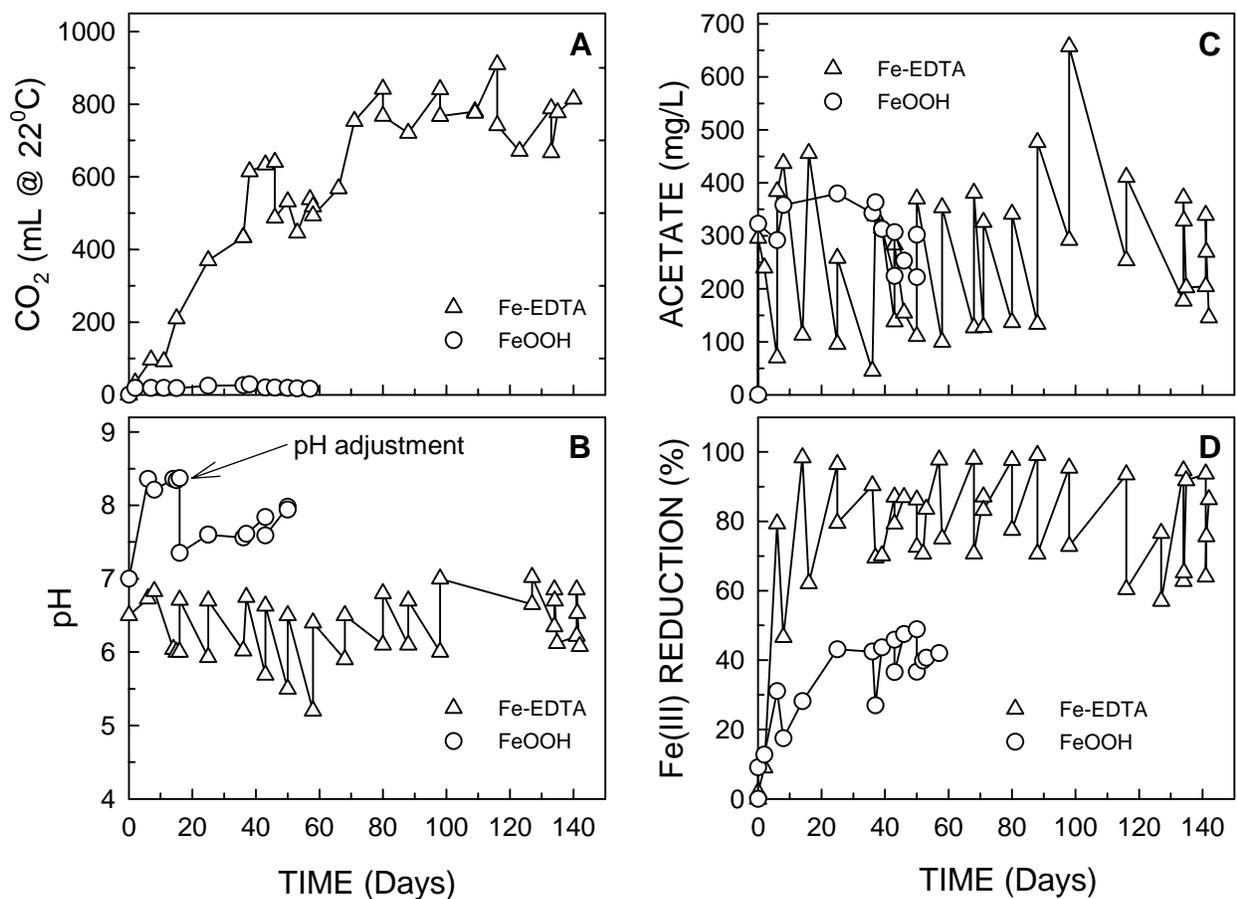


Figure 5. Cumulative CO₂ production (A), pH profile (B), acetate consumption (C), and extent of iron reduction (D) in the sediment-derived, iron-reducing cultures (second-generation; multiple feeding cycles).

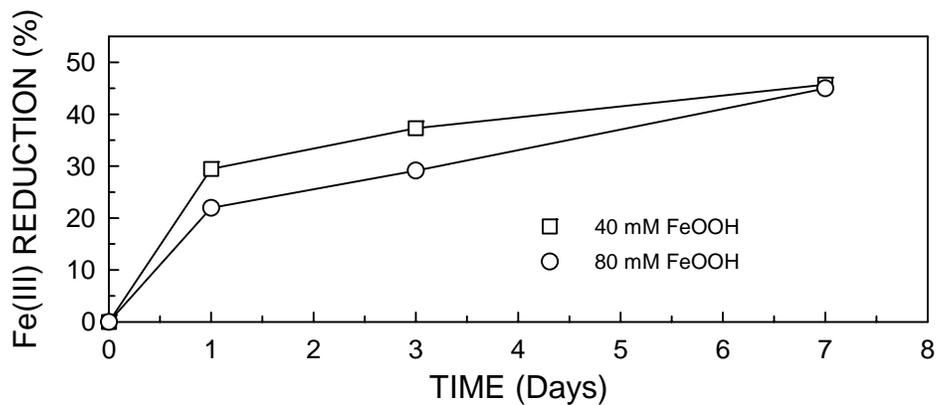


Figure 6. Extent of iron reduction in sediment-derived, FeOOH-amended, second generation, batch cultures.

4.1.2. PCNB biotransformation in an iron-reducing enrichment culture (Culture A)

A batch PCNB biotransformation assay was conducted with the sediment-derived, second generation culture developed under iron reducing conditions (Fe-EDTA). Two sub-cultures were developed: one with Fe(III)-EDTA addition and one without any new iron addition. About 78 mM Fe(II) was present in these cultures at the beginning of the incubation contributed by the inoculum (Figure 7A and 7D). A delay in the transformation of PCNB to PCA was observed in the culture amended with Fe(III)-EDTA. In contrast, PCNB to PCA transformation was complete within 2 days in the culture that was not amended with any Fe(III). In both cultures, PCA was sequentially transformed to TeCAs and TrCAs (Figure 7A and 7D). However, the observed transformation rates in both cultures were significantly slower compared to those observed with the mixed methanogenic, PCNB-dechlorinating enrichment culture (see Section 4.2.1, below). After 10 days of incubation, methane production was observed in both cultures (Figure 7C and 7F). Acetate consumption and CO₂ production was higher in the culture amended with Fe(III), compared to that in the culture which was not amended with Fe(III). Transformation of PCNB to PCA was not observed during the 43-day of incubation period in the abiotic control which was setup with autoclaved iron-reducing culture media (data not shown).

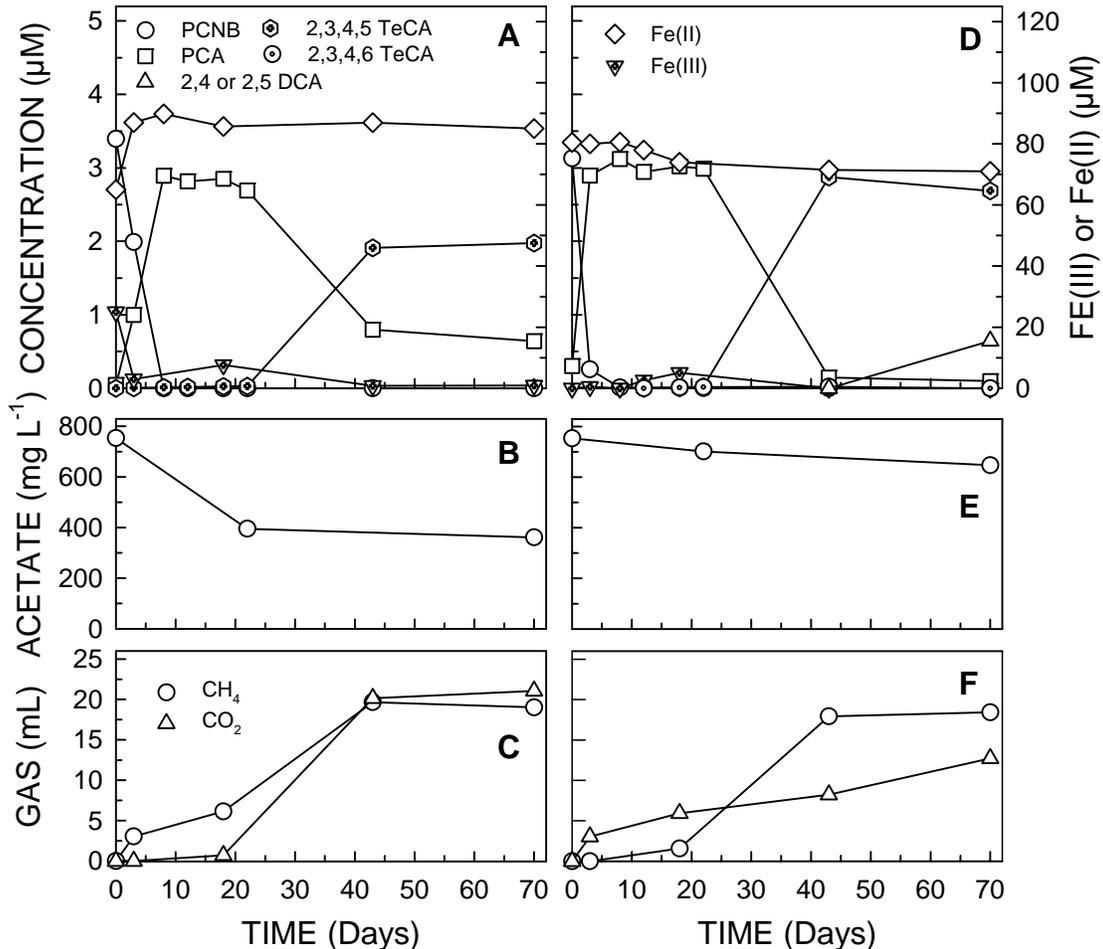


Figure 7. PCNB transformation, acetate consumption and gas production (at 22°C) in the sediment-derived, second generation Fe-EDTA reducing cultures (A, B, and C: culture with Fe(III)-EDTA amendment; D, E, and F: culture without Fe(III)-EDTA amendment).

4.2. Fermentative/methanogenic enrichment culture

4.2.1. PCNB biotransformation by the fermentative/methanogenic enrichment culture (Culture D)

Complete transformation of PCNB to PCA in the sediment-free, fermentative/methanogenic culture occurred in less than one day (Figure 8). Batch assays performed with the sediment-free enrichment culture resulted in the biotransformation of PCNB to PCA, which was then sequentially dechlorinated as follows: PCA \rightarrow 2,3,4,5- and 2,3,5,6-tetrachloroaniline (TeCA) \rightarrow 2,4,5- and 2,3,5-trichloroaniline (TrCA) \rightarrow 2,4-, 2,5- and 3,5-dichloroaniline (DCA) \rightarrow 3- and 4-chloroaniline (CA) (low levels). The predominant end product was 2,5-DCA (Figure 9).

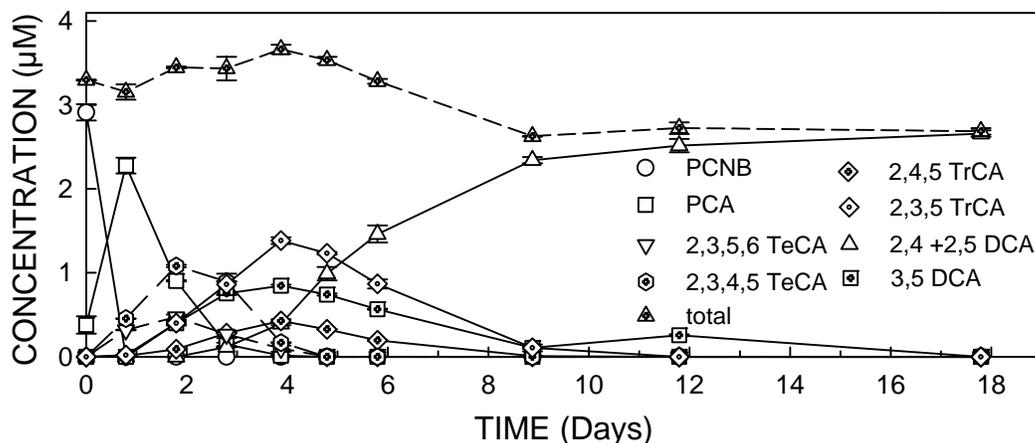


Figure 8. Time course of PCNB and its biotransformation products during a typical batch biotransformation assay conducted with the PCNB-enriched, mixed methanogenic culture (Error bars represent mean values \pm one standard deviation).

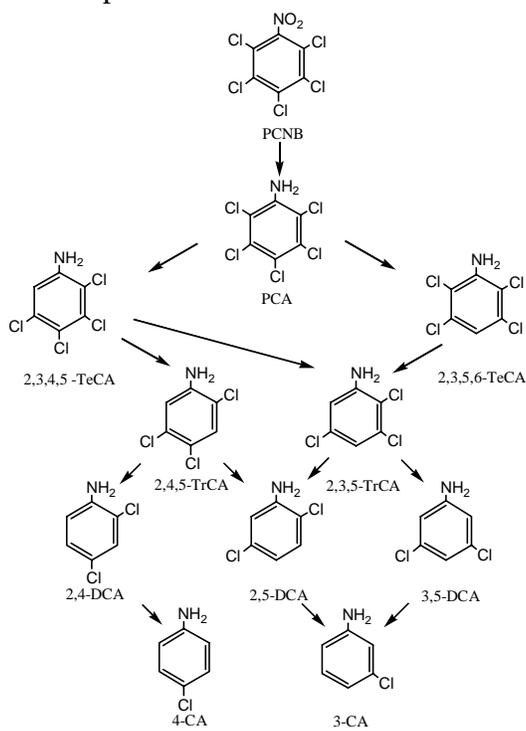


Figure 9. PCNB biotransformation products observed in the fermentative/methanogenic enrichment culture.

4.2.2. Effect of iron reduction on PCA dechlorination by the fermentative/methanogenic enrichment culture (Culture D)

As mentioned in the Materials and Methods section, above, complete and fast PCNB transformation to PCA was observed under any anoxic/anaerobic conditions. Therefore, all subsequent assays were conducted with PCA as opposed to PCNB. The initial biomass concentration in all cultures in this assay was 310 ± 20 mg C/L (measured as particulate organic carbon). The pH and ORP values in the iron-free, control culture were 7.0 ± 0.3 and -170 ± 5 mV, respectively (mean \pm standard deviation; $n = 3$). The initial and final ORP values in the cultures amended with Fe(III)-EDTA or FeOOH were $+138 \pm 2$, -120 ± 4 mV, and -60 ± 14 , -120 ± 70 mV, respectively. The higher decrease in the ORP value of the Fe(III)-EDTA amended cultures was mainly due to the exhaustion of Fe(III). Fetzner and Conrad (1993) found no inhibition of methanogenesis at ORP values as high as $+420$ mV. Therefore, inhibition of methanogenesis due to the relatively high redox potential seems unlikely in all of these cultures. The initial and final pH values in the Fe(III)-EDTA amended cultures were similar to those of the control culture. However, the pH increased from 7.2 ± 0.1 to 8.1 ± 0.4 in the FeOOH amended cultures within 60 days of incubation. The increase in pH was also observed in the sediment-derived FeOOH reducing cultures as mentioned above.

PCA was dechlorinated to 3,5 DCA and 2,4+2,5 DCA and all acetate was consumed in the iron-free, control culture under methanogenic conditions within 10 days of incubation without any delay (Figure 10). However, dechlorination of PCA was not observed in the cultures amended with either Fe(III)-EDTA or Fe(III)-EDTA and AQDS until 30 days of incubation when more than 90% of the added Fe(III) was reduced (Figure 11). The PCA dechlorination pathway was similar to that of the control culture after 30 days of incubation in both the Fe(III)-EDTA and Fe(III)-EDTA plus AQDS amended cultures (Figure 11A and 11B). On the other hand, PCA dechlorination as well as acetate consumption were not observed during 60 days of incubation in the culture amended with $\text{Na}_2\text{-EDTA}$ (Figure 12C). Therefore, EDTA had an inhibitory effect on the dechlorinating and methanogenic culture. In contrast, the presence of iron in the case of Fe-EDTA eliminated the inhibitory effect of EDTA, more likely because of binding with the resulting Fe(II). Straub et al. (2001) suggested that the inhibitory effect of EDTA could be because of its interaction with divalent cations such as Ca^{2+} or Mg^{2+} , causing an imbalance in the supply of these ions. Such interactions may be detrimental, especially for gram-negative bacteria, which depend on such cations in their outer membranes. PCA dechlorination was not observed in the presence of only culture media or culture media amended with 25 mM ferrous iron (Figure 12A and 12B). PCA dechlorination and acetate consumption in the culture which was amended with 50 mM Na^+ proceeded as in the iron-free, control culture without any inhibitory effect (Figure 13A and 13B). AQDS did not have any inhibitory or stimulatory effect on the dechlorination of PCA in the absence of an iron source (Figure 13B).

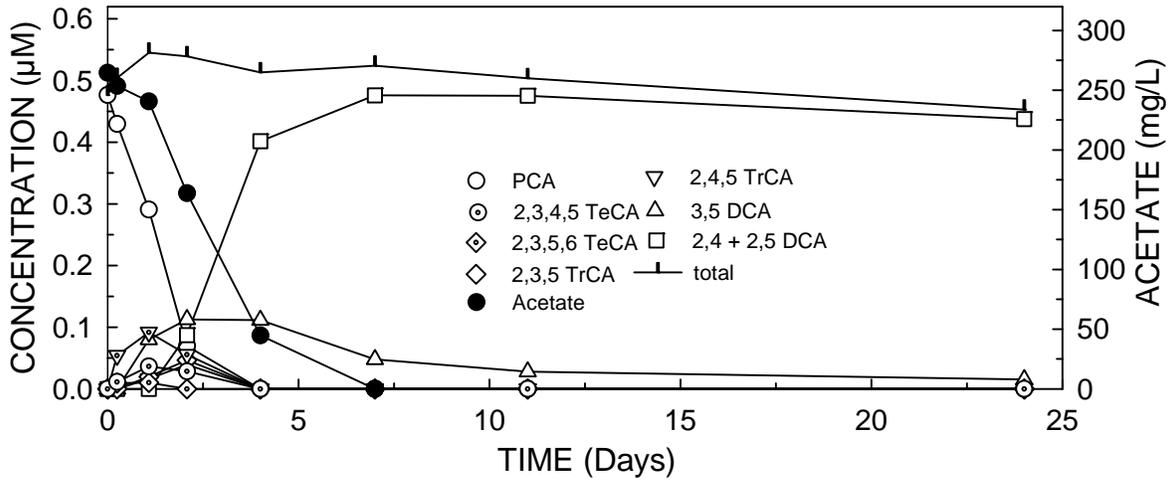


Figure 10. Time course of PCA and its dechlorination products, as well as acetate consumption in the iron-free, control methanogenic culture (sediment-free culture)

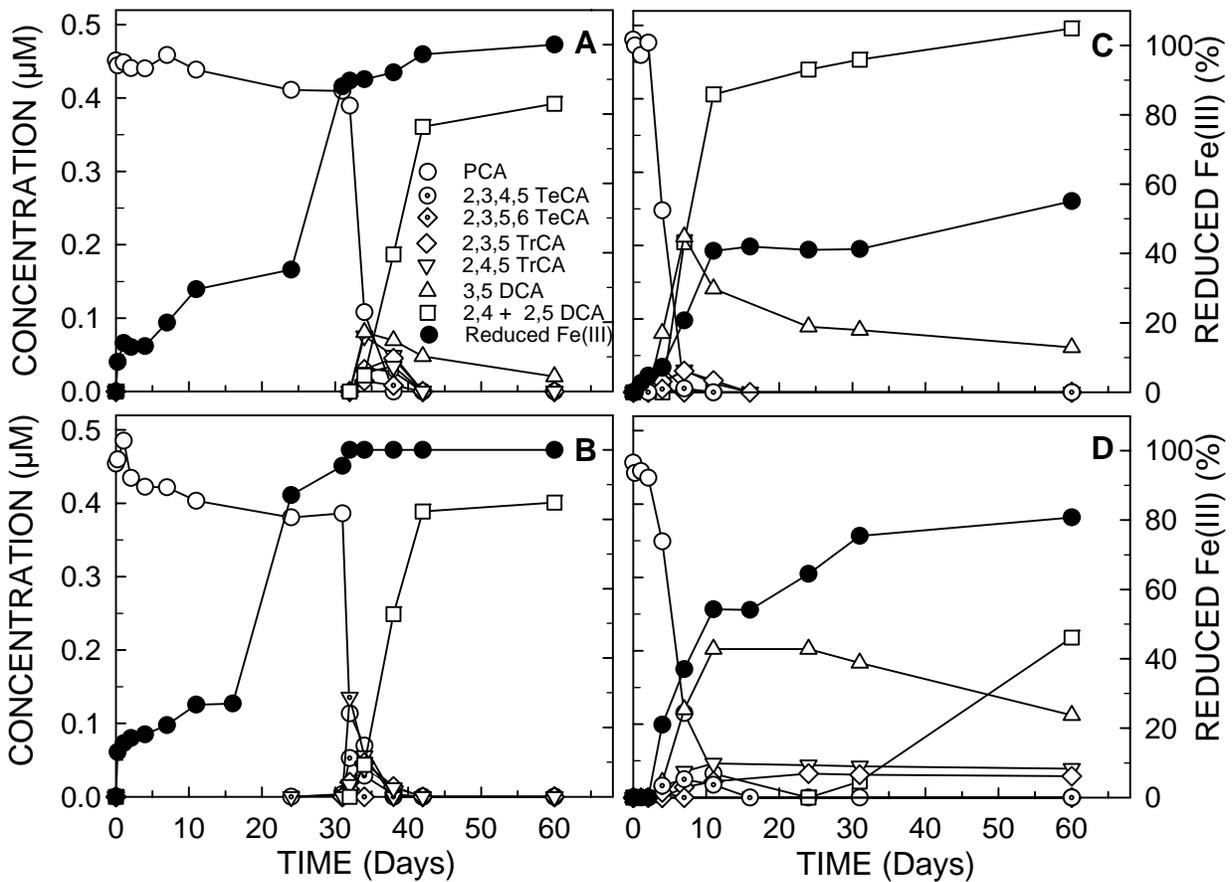


Figure 11. Iron reduction and PCA dechlorination in cultures amended with Fe(III)-EDTA (A), Fe(III)-EDTA and AQDS (B), FeOOH (C), FeOOH and AQDS (D).

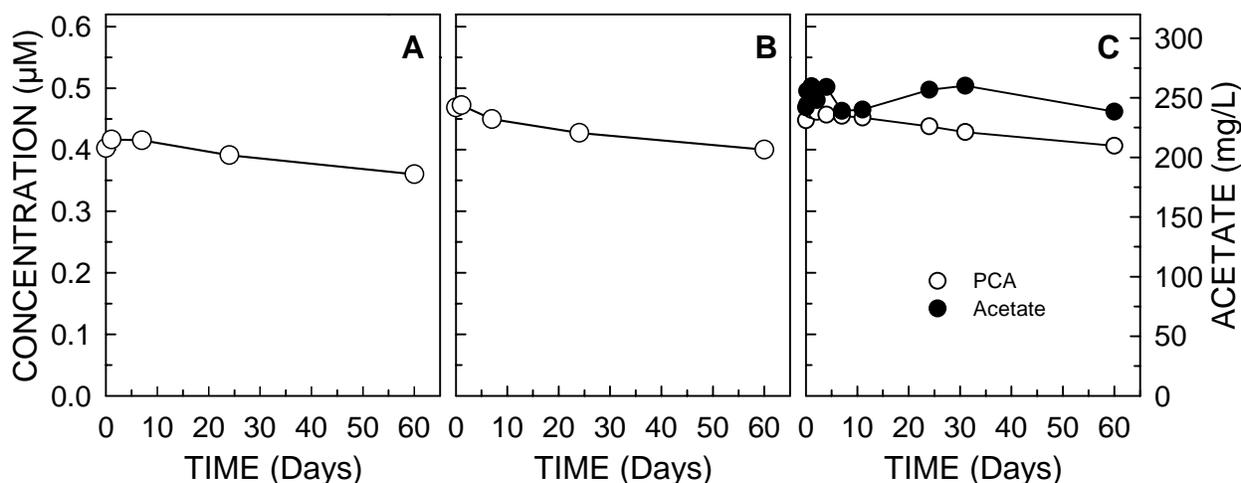


Figure 12. PCA in culture media (A), culture media amended with Fe(II) (B), and methanogenic culture amended with Na₂-EDTA (C).

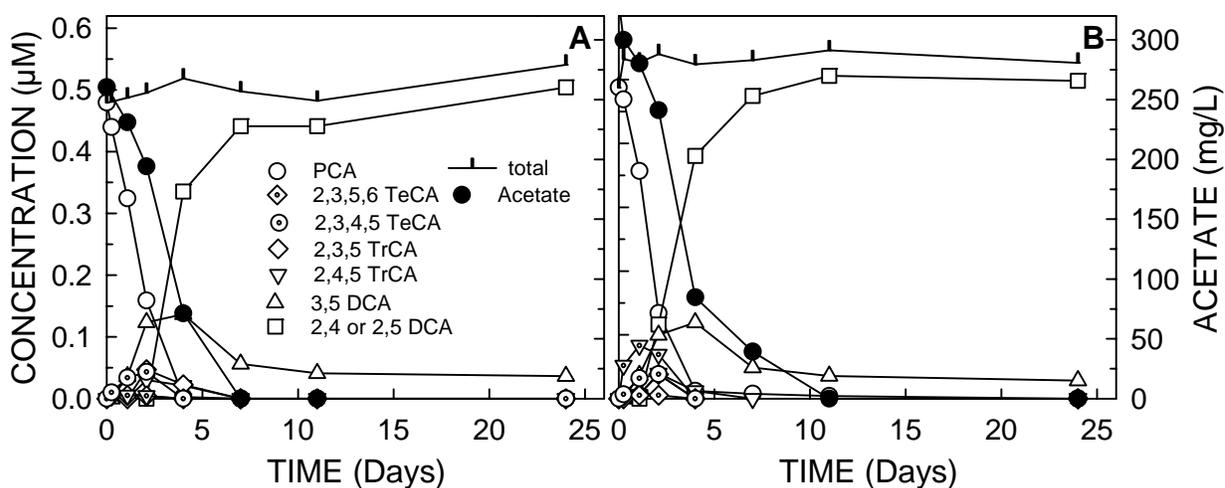


Figure 13. PCA dechlorination and acetate consumption in methanogenic cultures amended with either Na⁺ (A) or AQDS (B).

Dechlorination of PCA started after 2 days of lag period in the culture amended with FeOOH (Figure 11C), but the dechlorination rate was lower than that observed in the control culture. Dechlorination of PCA was slower in the FeOOH and AQDS amended culture (Figure 11D) as compared to the culture amended with only FeOOH. Lovley et al. (1998) showed that the humics analog, AQDS, can be reduced by microorganisms to anthrahydroquinone-2,6-disulfonate (AHQDS) which then abiotically reduces Fe(III). Similar to our results, He and Sanford (2003) reported inhibition of 2-chlorophenol dechlorination by the *Anaeromyxobacter dehalogenans* strain 2CP-C when the iron source was soluble (ferric pyrophosphate), whereas insoluble FeOOH did not have a significant effect on the dechlorination of 2-chlorophenol.

Gas production by all cultures during the incubation period is shown in Figure 14. AQDS did not have any inhibitory or stimulatory effect on the methane production of the dechlorinating, mixed fermentative/methanogenic culture. A slight decrease in the rate and

extent of methane production was observed in the culture amended with sodium chloride. An earlier study reported sodium concentrations ranging from 150 to 240 mM to be moderately and 347 mM to be strongly inhibitory to methanogens at mesophilic temperatures (McCarty, 1964). In another study, sodium chloride concentrations over 15.2 mM were inhibitory for *Methanobacterium thermoautotrophicum* (Patel and Roth, 1977). Similar to the dechlorination activity, methane production was observed after a lag period of 2 days in the cultures amended with FeOOH. Very low methane production and relatively high carbon dioxide production was observed in the cultures amended with Fe(III)-EDTA until 30 days of incubation. However, significant methane production and PCA dechlorination was observed after 30 days of incubation when more than 90% of Fe(III) was reduced to Fe(II). High carbon dioxide production shows that iron-reduction was the main metabolic process in these cultures. The cumulative carbon dioxide production was very similar in the two Fe(III)-EDTA amended cultures. Methane production was lower in the culture amended with both FeOOH and AQDS compared with that of the culture amended with only FeOOH. van Bodegom et al. (2004) reported direct inhibition of methanogenesis in the presence of ferric iron, and found that methanogens grown on H₂/CO₂ were more sensitive to ferric iron than methanogens grown on acetate. A relatively low carbon dioxide production was observed in the cultures amended with FeOOH compared to the Fe(III)-EDTA amended cultures. Both methane and carbon dioxide production was very low in the culture amended with Na₂-EDTA, showing very low biological activity in this culture.

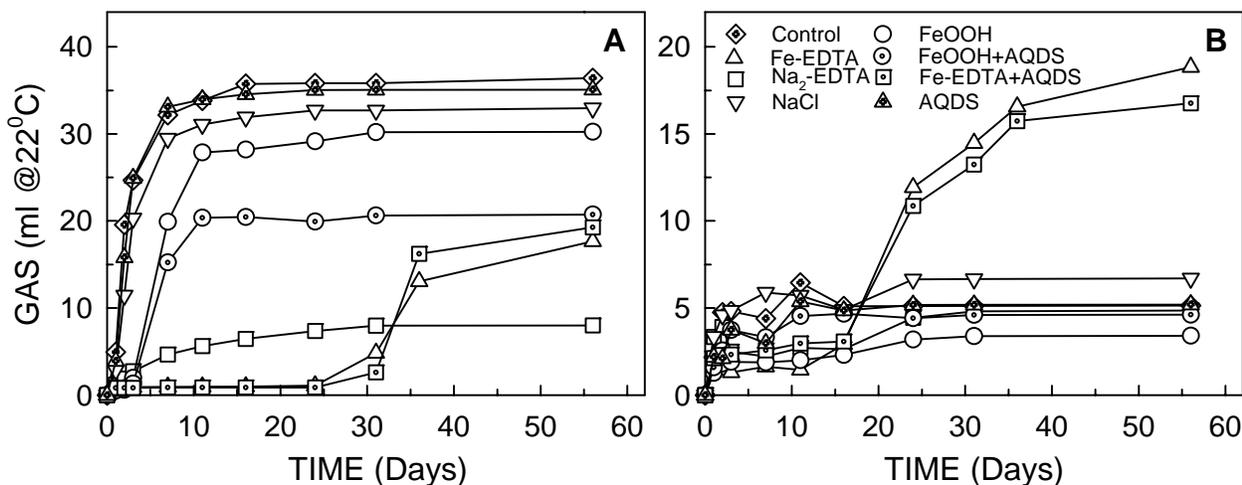


Figure 14. Gas production in all PCA-amended cultures. (A) methane; (B) carbon dioxide.

AQDS addition increased the Fe(III) reduction rate to some degree in the Fe(III)-EDTA amended culture, but the Fe(III) reduction rate was considerably increased in the FeOOH amended culture (Figure 15). Complete iron reduction was observed in the Fe(III)-EDTA plus AQDS amended culture after 30 days of incubation, whereas complete iron reduction in the culture without AQDS amendment was observed after 60 days of incubation (Figure 15A). Iron reduction was not observed for the first two days of incubation in the culture amended with FeOOH and AQDS, but after 10 days of incubation, the extent of Fe(III) reduction was significantly higher than in the culture without AQDS amendment (Figure 15B). Similar to our findings, Lovley et al. (1996) reported acceleration of the reduction of amorphous Fe(III) oxyhydroxide by the presence of a very low concentration of AQDS. The role of the humic acid analog AQDS as an electron shuttle provides a strategy for Fe(III) reducers to access insoluble

Fe(III) compounds (Lovley et al., 1996). Complete iron reduction was not observed in the cultures amended with FeOOH (Figure 15B). Similarly, it has been demonstrated that even the most available iron oxide -- amorphous hydrous ferric oxide -- cannot be totally consumed during microbial iron reduction (Lovley and Phillips, 1986a and b; Roden and Zachara, 1996).

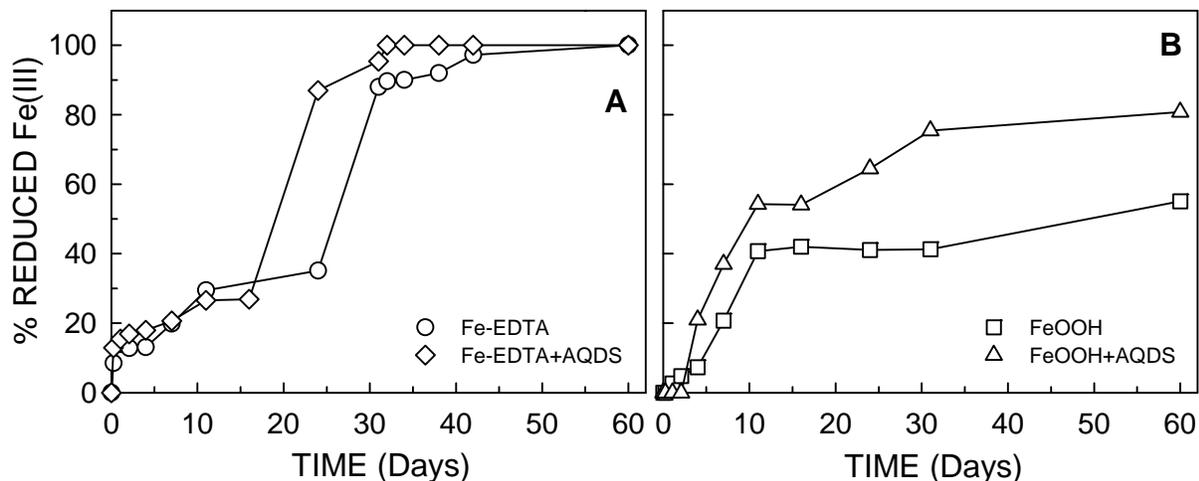


Figure 15. Iron reduction in cultures amended with Fe-EDTA (A) and FeOOH (B)(The effect of AQDS on the rate and extent of iron reduction is also shown).

5. CONCLUSIONS

Based on the results of the present study, the following conclusions are drawn:

- 1) Enrichment of dissimilatory, iron-reducing cultures using amorphous Fe(III) oxyhydroxide as an electron acceptor is extremely slow as compared to cultures enriched with either Fe(III)-citrate or Fe(III)-EDTA. Thus, the bioavailability of Fe(III) oxyhydroxide at circumneutral pH values is very low.
- 2) Reduction of the nitro-group of PCNB and production of pentachloroaniline (PCA) is taking place under both abiotic and biotic, reductive conditions. However, the rate of this conversion is highly enhanced under biotic conditions. Under biotic conditions, the rate of PCNB to PCA biotransformation was higher under methanogenic conditions as compared to iron-reducing conditions.
- 3) Dechlorination of PCA under abiotic, reductive conditions, e.g., in the presence of sulfide and/or Fe(II), was not observed in the present study.
- 4) PCA dechlorination did not take place when Fe(III)-EDTA was reduced at a relatively fast rate by an enriched, PCA-dechlorinating methanogenic culture, but dechlorination took place after iron reduction was almost complete. The PCA dechlorination pathway was similar to that of the iron-free, control culture.
- 5) PCA dechlorination and iron reduction took place simultaneously when the iron source was less bioavailable (e.g., FeOOH). However, addition of anthraquinone 2,6-disulfonate (AQDS), a humic acid model compound, to the culture, increased the rate and extent of

FeOOH reduction, which in turn resulted in a significant decrease of the rate and extent of PCA dechlorination. Thus, fast iron reduction suppresses and delays the onset of the reductive dechlorination of PCA.

The results of this study have significant implications relative to the fate and biotransformation of PCNB and PCA under anoxic/anaerobic conditions encountered in most subsurface soil and sediment environments. Based on the observed fast and facile conversion of PCNB to PCA, PCNB is not expected to be persistent in natural systems. However, the bioavailability of iron in natural systems, which in turn is controlled by soluble humic material, has a pronounced effect on the rate and extent of PCA dechlorination and thus on its persistence in natural systems.

6. DISSEMINATION OF PROJECT RESULTS

The results of this project are being disseminated as follows:

Okutman-Tas, D. and S. G. Pavlostathis. Microbial Reductive Transformation of Pentachloronitrobenzene under Methanogenic Conditions. Manuscript submitted to *Environmental Science and Technology*.

Okutman-Tas, D. and S. G. Pavlostathis. Microbial Reductive Transformation of Pentachloronitrobenzene under Nitrate-reducing Conditions. Abstract to be presented at the 105th General Meeting, American Society for Microbiology, Atlanta, GA, June 5-9, 2005.

Okutman-Tas, D., and S. G. Pavlostathis. The Influence of Iron Reduction on the Reductive Biotransformation of Pentachloronitrobenzene. Paper to be presented at the Third European Bioremediation Conference, Chania, Greece, July 4-7, 2005.

Okutman-Tas, D. and S. G. Pavlostathis. The Influence of Iron Reduction on the Sequential Microbial Reductive Dechlorination of Pentachloroaniline. Manuscript in preparation.

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