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Degradation of Monochlorinated and Nonchlorinated Aromatic Compounds under Iron-Reducing Conditions

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The capacity for Fe^{3+} to serve as an electron acceptor in the microbial degradation of monochlorinated and nonchlorinated aromatic compounds was investigated in anoxic sediment enrichments. The substrates tested included phenol, benzoate, aniline, their respective monochlorinated isomers, *o*-, *m*-, and *p*-cresol, and all six dimethylphenol isomers. Phenol and 2-, 3-, and 4-chlorophenol were utilized by anaerobic microorganisms, with the concomitant reduction of Fe^{3+} to Fe^{2+}. The amount of Fe^{2+} produced in the enrichments was 89 to 138% of that expected for the stoichiometric degradation of these substrates to CO_2, suggesting complete mineralization at the expense of Fe reduction. Under Fe-reducing conditions, there was initial loss of benzoate and 3-chlorobenzoate but not of 2- or 4-chlorobenzoate. In addition, there was initial microbial utilization of aniline but not of the chloroaniline isomers. There was also initial loss of *o*-, *m*-, and *p*-cresol in our enrichments. None of the dimethylphenol isomers, however, was degraded within 300 days. Furthermore, we tested the capacity of an Fe-reducing, benzoate-grown culture of *Geobacter metallireducens* GS-15 to utilize monochlorinated benzoates and phenols. *G. metallireducens* was able to degrade benzoate and phenol but none of their chlorinated isomers, suggesting that the degradation of chlorophenols in our sediment enrichments may be due to novel Fe-reducing organisms that have yet to be isolated.

Microbial iron reduction is important in both carbon and electron flow in a number of aquatic sediments. In groundwaters of the Atlantic coastal plain, for example, sufficient Fe is available for Fe reduction to be the predominant anaerobic microbial process in aquifer sediments (4). Furthermore, in an aquifer contaminated with a landfill leachate plume, a ferrogenic redox zone was the most extensive in area within the plume; loss of organic contaminants that could not be attributed to sorption to sediments and dilution by groundwater suggested that microorganisms present in the ferrogenic zone were able to utilize these substrates (15, 16). In addition, an extensive Fe reduction zone was noted in Amazon inner shelf sediments (1), and microbial Fe reduction was estimated to be responsible for 21 to 78% of the carbon oxidized in Danish coastal sediments, where sulfidogenesis was expected to be the dominant terminal electron-accepting process (3). In some river and aquifer sediments, Fe-reducing bacteria can outcompete sulfate-reducing and methanogenic bacteria for organic substrates and electrons (4, 19). Sufficient Fe^{2+} was solubilized by microbial Fe reduction to precipitate all of the sulfide generated by dissimilatory sulfate reduction in coastal sediments (12). Microbial Fe reduction can thus affect the microbial ecology and the geochemistry of Fe, as well as S, in sedimentary environments.

Bacteria in marine and freshwater sediments can oxidize a number of organic substrates under Fe-reducing conditions (13, 29). One isolate, *Geobacter metallireducens* GS-15, can metabolize several organic contaminants, including toluene and phenol, by using Fe^{3+} as the terminal electron acceptor (17, 18). In a previous study with Nile River sediments as an inoculum source, we observed that benzoate and 2-, 3-, or 4-chlorobenzoate could be utilized with a range of electron acceptors other than oxygen and that Fe reduction was one of the processes supporting degradation (14). In this study, we investigated whether a variety of other chlorinated and nonchlorinated aromatic compounds could be utilized by microorganisms under Fe-reducing conditions. The substrates included phenol; 2-, 3-, and 4-chlorophenol (2-CP, 3-CP, and 4-CP, respectively); benzoate; 2-, 3-, and 4-chlorobenzoate (2-CB, 3-CB, and 4-CB, respectively); aniline; 2-, 3-, and 4-chloroaniline (2-CA, 3-CA, and 4-CA, respectively); *o*-, *m*-, and *p*-cresol (*o*-Cr, *m*-Cr, and *p*-Cr, respectively); and dimethylphenol isomers.

MATERIALS AND METHODS

Sediment source. Grab samples of sediments were taken from a site in the upper Hudson River near Albany, N.Y. (mile point 145 from upper New York Bay). The sediments were transferred to airtight glass jars and kept at 4°C until used. Sediments from this site were a dark grey sandy silt. Sediment samples from this site were used in a previous study examining the influence of alternate electron acceptors on chloroaromatic degradation (8).

Media. The medium for the Fe-reducing enrichments was prepared in an identical manner to that described by Lovley and Phillips (20), by using standard anaerobic techniques. Each liter of medium contained freshly precipitated amorphous Fe (as ferric oxyhydroxide), 2.5 g of $NaHCO_3$, 0.1 g of $CaCl_2 \cdot H_2O$, 0.1 g of KCl, 1.5 g of NH_4Cl, and 0.6 g of $NaH_2PO_4 \cdot H_2O$. Trace salts and vitamins were also added (25). The initial concentration of Fe in the medium was 200 mM. In a second set of enrichments used to investigate phenol, cresol, and dimethylphenol degradation under Fe-reducing conditions, the initial concentration of Fe in the medium was 400 mM.

A culture of *G. metallireducens* GS-15 was maintained in an anaerobic citrate medium (ATCC culture formulation 1768) without acetate. This medium contained, per liter, 17 g of ferric citrate, 2.5 g of $NaHCO_3$, 0.1 g of KCl, 1.5 g of NH_4Cl, and 0.6 g of $NaH_2PO_4 \cdot H_2O$. Trace salts and vitamins were also added (30). Benzoate (2 mM) was added as a growth substrate.

Enrichment preparation. Sediment slurries (1:10 [vol/vol], sediment and medium) were divided into aliquots in serum vials (50-ml nominal volume), capped with black butyl rubber stoppers, and crimped with aluminum seals. Each vial contained 50 ml of slurry and had a 10-ml headspace of N_2-CO_2 (70:30 [vol/vol]). Each substrate (benzoate; 2-, 3-, and 4-CB; phenol; 2-, 3-, and 4-CP; aniline; 2-, 3-, and 4-CA; *o*-, *m*-, and *p*-Cr; and 2,3-, 2,4-, 2,5-, 2,6-, 3,4-, and 3,5-dimethylphenol [Aldrich Chemical Co., Milwaukee, Wis.]) was maintained as a deoxygenated stock solution in 0.1 N NaOH. Each compound, with the exception of *o*-, *m*-, and *p*-Cr (or 2-, 3-, and 4-methylphenol) and the dimethylphenols, was added

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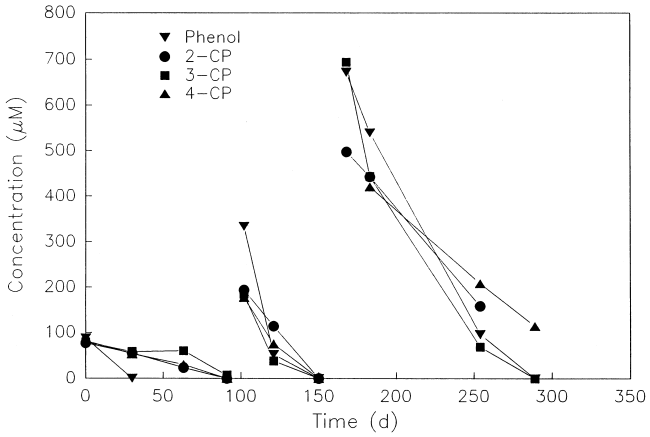

FIG. 1. Loss of phenol and chlorophenol isomers in Hudson River sediments under Fe-reducing conditions. The results are the means of three replicates. d, days.

to separate vials to a final concentration of 100 μM. In a second set of enrichments, each of the methylphenol and dimethylphenol isomers and phenol were added to a final concentration of 200 μM. Background controls were prepared in the same manner as the experimental cultures except that no substrate was added. These controls were to account for Fe^{2+} production due to metabolism of existing carbon in the sediment inoculum. Sterile controls were autoclaved three times on consecutive days before the experiment was initiated. Strict anaerobic microbial techniques were used throughout in experimental manipulations. Syringes and needles used for substrate addition and sample collection were flushed with N_2-CO_2 passed over hot reduced copper filings to remove traces of O_2. All cultures, with autoclaved controls, were made in triplicate and incubated without shaking in the dark at 30°C. With the two sets of enrichments, a total of 92 vials were established, which included the experimental cultures and background and sterile controls.

To test for the utilization of chlorinated phenols and benzoic acids by *G. metallireducens*, a dense cell culture grown on benzoate and fresh citrate medium was subdivided into 32 vials (nominal volume, 10 ml). Each vial contained 0.5 ml of culture inoculum and 7.5 ml of fresh medium. A stock solution of 2-, 3-, or 4-CB; phenol; or 2-, 3-, or 4-CP (Aldrich Chemical Co.) was added to the vials (three replicates plus one autoclaved control), each to a final concentration of 100 μM. The remaining four vials were fed benzoate (100 μM) to ensure that the culture was active in utilizing benzoate. The headspace of the vials was N_2-CO_2 (70:30). Sterile controls were autoclaved three times on consecutive days before the experiment was begun.

Analytical methods for organic substrate. At each time point for sampling, the cultures were mixed well to distribute the sediment, and 0.5 ml of the sediment-water slurry was withdrawn from the vials with a deoxygenated, sterile syringe. The samples were centrifuged, and the supernatant was filtered (0.45-μm-pore-size filter) and then frozen (−20°C) prior to analysis. Loss of substrate was monitored by injecting samples into a high-pressure liquid chromatograph (HPLC; System Gold models 126/166; Beckman, San Ramon, Calif.) equipped with a C_{18} column (25 cm by 4.6 mm, 5-μm particle size; Supelco, Bellefonte, Pa.). For benzoate, CB, phenol, and CP, a solvent system of water-methanol-acetic acid (60:38:2) was used. For cresols and dimethylphenols, the solvent system was water-methanol-acetic acid (49:49:2). For aniline and CA, the solvent system was acetonitrile–5 mM phosphate buffer (40:60). The phosphate buffer consisted of equal volumes of 5 mM KH_2PO_4 and 5 mM Na_2HPO_4 (pH 6.8). All solvents were run at a flow rate of 1.0 ml min^{-1}. The detector wavelength was set at 280 nm. Substrate concentrations were monitored with a Spectra-Physics (San Jose, Calif.) model SP4400 integrator calibrated with standards for each compound.

Analysis of Fe^{3+} reduction. Fe^{2+} production was determined by a modification of the method described by Lovley and Phillips (20) and Roden (27). The method involves adding 0.5 ml of the sediment slurry to 10 ml of 1 N HCl to extract acid-soluble Fe and shaking the sample for 1 h. The HCl sample was then centrifuged, and an aliquot (5 to 50 μl) of the supernatant was added to 2 ml of ferrozine (1 g $liter^{-1}$; Aldrich Chemical Co.). The HCl-extractable Fe^{2+} reacts with ferrozine to form a colored compound, which was then measured by spectrophotometry (UV-240 UV-visible spectrophotometer; Shimadzu Corp. Kyoto, Japan) at 562 nm. Fe^{2+} standards were made from ferrousethylenediammonium sulfate (Fluka Chemical Co., Ronkonkoma, N.Y.). The sensitivity of this method was 1 μM for Fe^{2+}. Measurements of Fe^{2+} production that could be coupled to substrate loss in the cultures were corrected for background carbon metabolism by subtracting those measurements taken from cultures to which only the electron acceptor, but no substrate, was added.

RESULTS

Utilization of chlorinated and nonchlorinated aromatic compounds by sediment enrichments. The utilization of phenol and its monochlorinated isomers by Hudson River sediments incubated under Fe-reducing conditions is shown in Fig. 1. Loss of 100 μM phenol under Fe-reducing conditions was the most rapid and was seen within 30 days. Furthermore, 2-, 3-, and 4-CP (100 μM) were initially depleted within 90 days. As shown in Fig. 1, utilization of phenol and the mono-CPs was sustained upon refeeding of the compound. Successively higher concentrations were added, and 500 to 700 μM was depleted in less than 150 days.

Figure 2A illustrates that benzoate and 3-CB (100 μM each) were utilized in the enrichments within 30 and 150 days, respectively. There was no loss of 2- or 4-CB within 180 days. Substrate utilization could be sustained by refeedings with benzoate; however, metabolism of 3-CB could not be sustained by refeeding (data not shown).

Figure 2B shows the results with aniline and its monochlorinated isomers. Aniline utilization occurred within 65 days. There was no loss, however, of any CA isomer within 180 days beyond that in sterile controls. We further observed that the activity on aniline could not be sustained by refeeding (data not shown).

In the second set of enrichments, illustrated in Fig. 3A, loss of *p*-Cr (200 μM) initially occurred within 30 days, and metabolism of phenol and *m*-Cr (200 μM) took place within 65 days. In addition, there was initial loss of *o*-Cr (200 μM) within 160 days. As seen in Fig. 3A, the activity on phenol was sustained

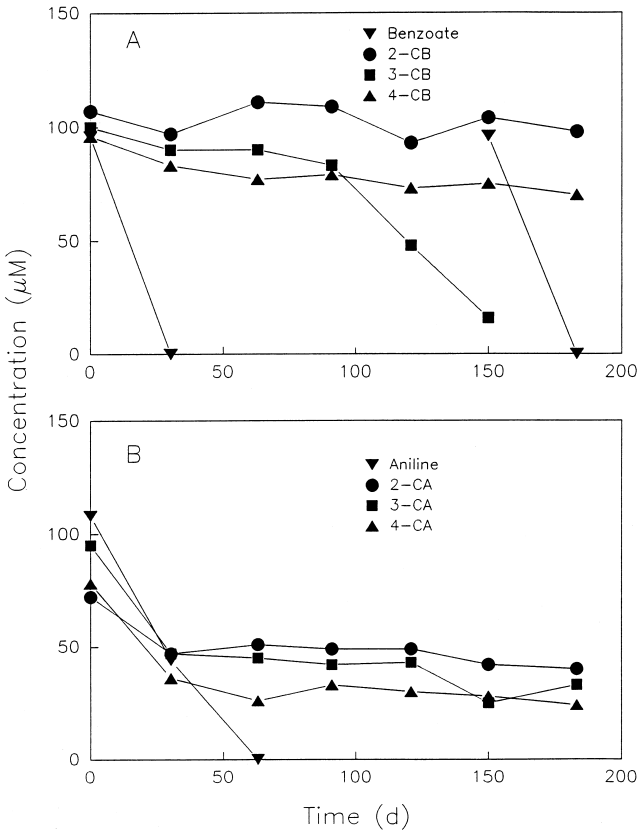

FIG. 2. Loss of benzoate and CB isomers (A) and aniline and CA isomers (B) in Hudson River sediments under Fe-reducing conditions. d, days.

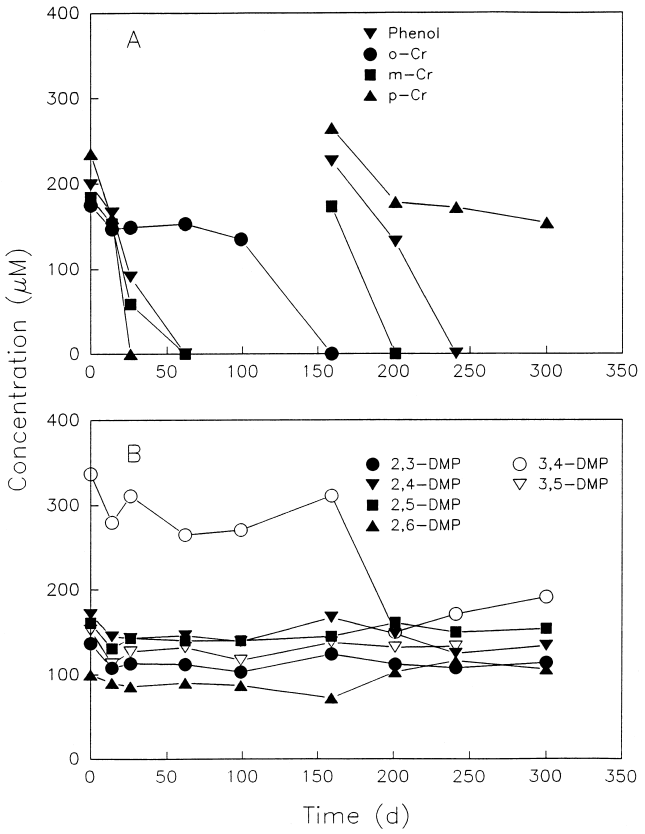

FIG. 3. (A) Loss of cresol isomers in Hudson River sediments. (B) There was no disappearance of dimethylphenol isomers. The results are the means of three replicates. d, days.

with refeeding of the substrate and continued to be sustained with repeated feedings (data not shown). The activity in the *m*-Cr enrichments was sustained with one refeeding (Fig. 3A) but could not be sustained with further refeeding (data not shown). Figure 3A shows that activity in the *p*-Cr cultures could not be sustained with refeeding. There was also loss of activity in the *o*-Cr enrichments after the initial feeding (data not shown).

Figure 3B shows that there was no loss of any of the dimethylphenol isomers tested within 300 days. For 3,4-dimethylphenol, the analytical procedures yielded values less precise than those for the other dimethylphenol isomers (for clarity, error bars are not shown).

Stoichiometry of phenol and chlorophenol degradation. In order to examine whether phenol and CP degradation was coupled to Fe reduction, predicted values based on stoichiometric equations of Fe^{2+} production were compared to those measured in phenol- and CP-degrading enrichments. In these equations, it is assumed that phenol and CP are completely mineralized to CO_2 as follows:

$$C_6H_6O + 28\,Fe^{3+} + 17\,H_2O \rightarrow 6\,HCO_3^- + 28\,Fe^{2+} + 34\,H^+ \quad (1)$$

$$C_6H_5OCl + 27\,Fe^{3+} + 17\,H_2O \rightarrow 6\,HCO_3^- + 27\,Fe^{2+} + 33\,H^+ + Cl^- \quad (2)$$

Cultures were repeatedly fed the substrates until approximately 1 mM had been utilized. In this manner, the amount of electron acceptor used for substrate degradation can be readily determined and is sufficiently above that consumed by the metabolism of background carbon in the sediment.

TABLE 1. Production of Fe^{2+} during degradation of phenol and mono-CPs in upper Hudson River sediments[a]

Substrate fed	Substrate metabolized (mM)	Fe^{2+} production (mM)		% of expected amt
		Predicted[b]	Measured[c]	
Phenol	1.00 ± 0.10	28	25 ± 1	89 ± 3
2-CP	1.04 ± 0.05	28	31 ± 1	109 ± 5
3-CP	0.94 ± 0.16	25	35 ± 6	138 ± 26
4-CP	1.13 ± 0.04	31	28 ± 2	89 ± 8

[a] Results are means ± standard deviations.
[b] Based on the stoichiometry of 1 mol of phenol = 28 mol of Fe^{2+} and 1 mol of CP = 27 mol of Fe^{2+}.
[c] The amount of Fe^{2+} produced in background control cultures (57 ± 3 mM within 300 days) was subtracted.

In Table 1, the production of Fe^{2+} in sediment enrichments utilizing phenol and 2-, 3-, and 4-CP is summarized. From the known amount of substrate utilized and the stoichiometric equations given above, the amount of Fe^{3+} required as an electron acceptor can be calculated. This amount is then compared with the measured amounts of Fe^{2+} produced. The results indicate that Fe^{2+} production was 89 to 138% of that expected for phenol and CP isomers and suggest that complete mineralization of the substrate had occurred.

Degradation of compounds by *G. metallireducens*. Of the identified bacterial isolates which can use Fe^{3+} as an electron acceptor, only *G. metallireducens* GS-15, thus far, has been shown to degrade aromatic compounds including phenol, *p*-Cr, and toluene (18). The capacity of *G. metallireducens* to degrade chlorinated aromatic compounds, however, has not been investigated. In order to compare the observed activities in our consortia with that of *G. metallireducens*, a benzoate-grown culture of *G. metallireducens* was tested in cell suspension studies on 2-, 3-, and 4-CB; phenol; and 2-, 3-, and 4-CP. Benzoate loss was also monitored to ensure that the culture was active. Figure 4 shows that *G. metallireducens* degraded benzoate within 2 days and phenol within 3 days. There was no loss, however, of any CB or CP isomer, within 11 days.

DISCUSSION

While the microbial degradation of organic contaminants under denitrifying, sulfate-reducing, and methanogenic conditions has been investigated, microbial Fe reduction coupled to organic compound utilization has not been as well examined. This study indicates that Fe^{3+} can serve as an electron acceptor in the absence of oxygen for degradation of chlorinated and nonchlorinated aromatic compounds. Microbial Fe reduction supported the utilization of phenol and 2-, 3-, and 4-CP in Hudson River sediments. The concomitant production of Fe^{2+} with the degradation of these compounds indicates that complete mineralization has taken place (Fig. 1; Table 1). In fact, CPs appear to be relatively biodegradable in the absence of oxygen. Microbial utilization of mono-CPs has been noted under denitrifying, sulfidogenic, and methanogenic conditions (8), and the degradation of 2-, 3-, and 4-CP can be coupled to sulfate reduction (9, 10), as well as to methanogenesis (8).

No UV-visible metabolites were detected in our Fe-reducing CP enrichments, suggesting that metabolism of the aromatic ring had occurred. Similarly, there was no accumulation of metabolites as detectable by HPLC in previous studies with CP-utilizing denitrifying enrichments (8) and a sulfate-reduc-

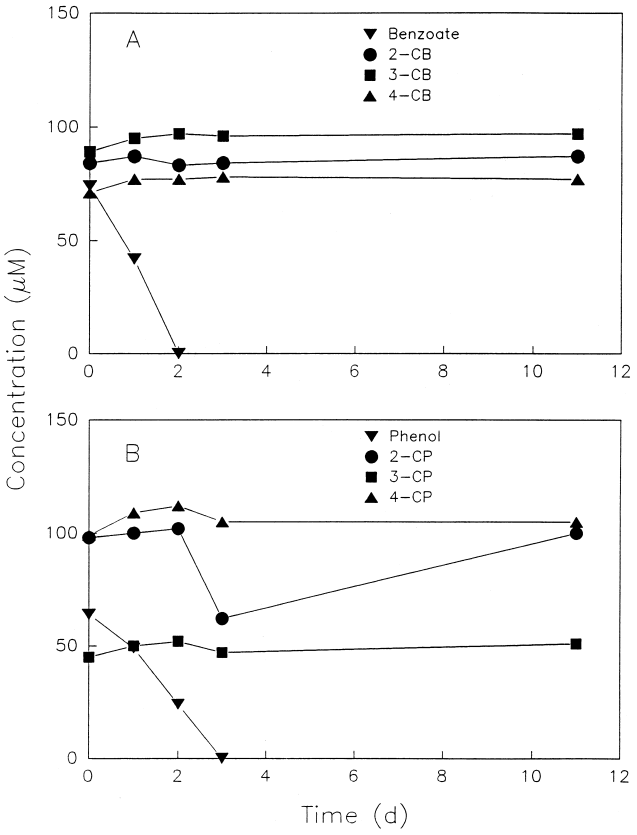

FIG. 4. Benzoate (A) and phenol (B) degradation by *G. metallireducens*. There was no utilization of any CB or CP isomers by *G. metallireducens*. d, days.

ing consortium (9, 10). In contrast, under methanogenic conditions, phenol was detected as a metabolite during the degradation of 2-CP, indicating that reductive dechlorination was an initial step in the degradation of this substrate (8). We do not know at this time, however, whether CP degradation in our Fe-reducing enrichments occurred via initial reductive dehalogenation or through some other mechanism. Recent studies have shown that iron is directly involved in the abiotic reduction of contaminant chemicals. For example, the dehalogenation of chlorinated methanes was noted in the presence of iron metal, and a proposed mechanism was the direct transfer of electrons from the Fe^0 surface to the adsorbed halogenated compound (21). In addition, nitroaromatic compounds were reported to be abiotically reduced by Fe^{2+} (11). Halogenated aromatic compounds, however, are stable in the presence of Fe^{2+} under our experimental conditions (22). Hence, abiotic dechlorination of CPs due to Fe^{2+} produced by microbial Fe reduction was not a factor.

Meyers et al. (23) reported that initial loss of benzoate, aniline, 4-hydroxybenzoate, and 3-CB occurred in enrichments to which amorphous Fe oxide was added. It is not clear from this study, however, whether oxidation of the aromatic substrates occurred at the expense of iron reduction. For example, for the amount of benzoate added (0.5 mM; as stated in reference 23), approximately 15 mM of Fe^{3+} would be required for complete degradation, assuming that 29 mol of Fe^{3+} was necessary for the stoichiometric mineralization of 1 mol of benzoate to 7 mol of CO_2. The low level of Fe^{3+} (2 mM; as stated in reference 23) relative to the amount of substrate added (0.5 mM each benzoate, aniline, 4-hydroxybenzoate, or 3-CB, as stated in reference 23) suggests that Fe was not the predominant electron acceptor in these cultures. One possible explanation for these results is that Fe was used as a required trace metal in substrate assimilation, and not as an electron acceptor, by benzoate-, aniline-, 4-hydroxybenzoate-, and 3-CB-degrading microorganisms.

We observed initial loss of 3-CB in upper Hudson River sediments, but the activity could not be sustained with refeeding (Fig. 2A). This finding is in contrast to our previous findings with Nile River sediments, in which utilization of 3-CB took place under Fe-reducing conditions and the activity was maintained through several refeedings (14). Moreover, the degradation of 3-CB in Nile River sediments was consistent with the stoichiometric production of Fe^{2+} predicted for complete mineralization of the substrate. The different observations may be due to differences in the microbial communities of the inoculum, as the methodology was the same in both studies.

Under Fe-reducing conditions, the initial loss of aniline and *o*-, *m*-, and *p*-Cr was noted; however, the activity in the enrichments could not be maintained with refeeding (Fig. 2C and 3A). This is in contrast to a previous study conducted under denitrifying, sulfidogenic, and methanogenic conditions, in which *p*-Cr degradation was maintained and was coupled to denitrification, sulfate-reduction, and methanogenesis (7). One hypothesis for the loss of degradative activity in our enrichments may be due to an inhibitory effect of accumulated metabolic products as a result of incomplete mineralization. Another possibility is that metabolism of these substrates required a cosubstrate which was subsequently depleted.

Several known bacterial isolates can reduce Fe^{3+} through enzymatic mechanisms as an electron acceptor in the oxidation of organic compounds and/or H_2. For example, *Shewanella putrefaciens* reduces Fe^{3+} to Fe^{2+} in the utilization of fermentation acids, including formate, lactate, and pyruvate (for reviews, see references 24 and 25). Another bacterial isolate, strain BrY, uses Fe^{3+} as an electron acceptor, with lactate, pyruvate, and H_2 serving as the sole electron donors (2). Fe reduction by *G. metallireducens* occurs with the oxidation of acetate, butyrate, propionate, and ethanol, as well as some aromatic compounds (17). Furthermore, Fe^{3+} is utilized as an electron acceptor by some sulfate-reducing bacteria. For example, *Desulfuromonas acetoxidans* can utilize Fe^{3+} in the metabolism of acetate, ethanol, propanol, and pyruvate (28), and it has been suggested that *Desulfovibrio desulfuricans* can reduce Fe^{3+} with H_2 serving as the electron donor (5). A sulfidogenic consortium utilizing 4-CP (10), was, however, not able to degrade CP when Fe^{3+} was provided as the sole electron acceptor (6).

Of the identified bacterial isolates which can use Fe^{3+} as an electron acceptor, only *G. metallireducens* GS-15 has been shown thus far to utilize a number of aromatic compounds including benzoate, phenol, toluene, and *p*-Cr (17, 18). From our studies, it appears that *G. metallireducens*, however, cannot utilize any of the mono-CB or mono-CP isomers (Fig. 4). This suggests that if *G. metallireducens* was present in our enrichments, it was not solely responsible for mediating the observed CB (14) and CP degradation. It should be noted that syntrophic associations may be involved, with one or more organisms dehalogenating the substrate and other organisms utilizing the aromatic ring and ring degradation products.

It is evident that microbial Fe reduction can be an important anaerobic process in a number of environments, affecting both the microbial ecology and the geochemistry of aquatic sediments. The abundance of Fe in some sediments can promote microbial Fe reduction at the expense of other, competing

anaerobic processes in organic matter and electron flow, including sulfate reduction and methanogenesis. In addition, the microbially mediated solubilization of bound Fe, for example iron hydroxides, with the subsequent precipitation and formation of Fe sulfides, can affect the geochemistry of Fe as well as S. Studies thus far indicate that Fe^{3+} can serve as an electron acceptor in the microbial oxidation of organic substrates, including fermentation acids, as well as some aromatic monochlorinated and nonchlorinated compounds. Considering the environmental significance of Fe, however, its role as an electron acceptor in the microbial degradation of organic contaminants is relatively unknown and unexplored.

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