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Dehalogenation and Biodegradation of Brominated Phenols and Benzoic Acids under Iron-Reducing, Sulfidogenic, and Methanogenic Conditions

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The anaerobic biodegradation of monobrominated phenols and benzoic acids by microorganisms enriched from marine and estuarine sediments was determined in the presence of different electron acceptors [i.e., Fe(III), SO_4^{2-}, or HCO_3^-]. Under all conditions tested, the bromophenol isomers were utilized without a lengthy lag period whereas the bromobenzoate isomers were utilized only after a lag period of 23 to 64 days. 2-Bromophenol was debrominated to phenol, with the subsequent utilization of phenol under all three reducing conditions. Debromination of 3-bromophenol and 4-bromophenol was also observed under sulfidogenic and methanogenic conditions but not under iron-reducing conditions. In the bromobenzoate-degrading cultures, no intermediates were observed under any of the conditions tested. Debromination rates were higher under methanogenic conditions than under sulfate-reducing or iron-reducing conditions. The stoichiometric reduction of sulfate or Fe(III) and the utilization of bromophenols and phenol indicated that biodegradation was coupled to sulfate or iron reduction, respectively. The production of phenol as a transient intermediate demonstrates that reductive dehalogenation is the initial step in the biodegradation of bromophenols under iron- and sulfate-reducing conditions.

Contamination of marine and estuarine environments by anthropogenic halogenated organic compounds, such as chlorinated pesticides, solvents and polychlorinated biphenyls, is a current environmental problem which has raised public concern. Marine and estuarine sediments serve as important sinks for these contaminants irrespective of their point of origin; therefore, it is important to understand their fate in these environments. Although many haloorganic compounds are recalcitrant, it has been observed that some are transformed or completely degraded by microorganisms under anoxic conditions (for reviews, see references 10, 29, 32, and 33). In marine and estuarine sediments, sulfate is found in abundance (4), and sulfate reduction is probably the most important electron-accepting process influencing dehalogenation and degradation of haloaromatics in these environments. Iron reduction may also serve as an important electron-accepting process for organic carbon decomposition in estuarine and marine environments (24), whereas methanogenesis is less important. The marine environment is also a rich source of naturally occurring halogenated compounds, including bromophenols (30); therefore, it is possible that microorganisms present in these environments have evolved the ability to utilize these compounds. The anaerobic degradation of brominated phenols was previously observed in estuarine sediments which contained these naturally occurring compounds (19), and a reductively debrominating bacterium has been isolated from the burrow of a bromometabolite-producing hemichordate (31).

The removal of the halogen substituent is a key step in the degradation of halogenated aromatic compounds. This may occur as an initial step via reductive, hydrolytic, or oxygenolytic mechanisms or may occur after ring cleavage at a later stage of degradation. In the absence of oxygen, reductive dehalogenation has been established as the initial step for degradation of haloaromatic compounds under methanogenic conditions (29). However, less is known about the removal of the halogen substituent and complete degradation of halogenated aromatic compounds under other conditions of biogeochemical significance. A number of studies have reported that sulfate and other sulfur oxyanions either partially or completely inhibit aryl dechlorination (1, 2, 5, 6, 8, 14, 20, 21, 27), suggesting that under sulfate-reducing conditions the degradation of halogenated aromatic compounds may not be initiated by reductive dehalogenation. Many of these studies, however, were conducted by adding sulfate or other sulfur oxyanions to methanogenic enrichment cultures.

Environments in which electron acceptors other than carbonate predominate are likely to support different microbial communities, and it is not known whether microorganisms in these anoxic habitats mediate the initial dehalogenation of halogenated aromatic compounds. Previous work has demonstrated that chlorinated phenols and benzoic acids can be degraded in the presence of different electron acceptors, including sulfate and iron (7, 11–13, 17, 18). Although degradation of these compounds and release of halogen was observed, the mechanism(s) of dehalogenation and degradation under sulfate- and iron-reducing conditions has not yet been fully characterized. In other studies, reductive dehalogenation of chlorinated aromatic compounds has been shown to occur in the presence of sulfate (7, 19, 20, 23).

In this study, enrichment cultures were established with anoxic sediments from different marine sites to determine the mechanism(s) of dehalogenation of monobrominated phenols and benzoic acids under various anaerobic conditions. By using

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monobrominated phenols and benzoic acids as model compounds, halogen (bromide) release could be measured over a large pool of chloride present in the marine sediments and the medium. This report presents evidence that reductive dehalogenation is the initial step in the biodegradation of halophenols under both sulfate-reducing and iron-reducing conditions.

MATERIALS AND METHODS

Sediment samples. Grab sediment samples were collected at low tide from the Bay of Fundy, Canada, and a decomposing seaweed mat in Lubec, Maine, and stored at 4°C in closed glass containers until used. Core samples of sediments were collected from an underwater site (approximately 10 ft. deep) in the Arthur Kill estuarine inlet located between Staten Island, N.Y., and New Jersey. Sediments from this location were rich in organic material and heavily contaminated with petroleum hydrocarbons and other contaminants (16). These sediments were transported in closed glass jars or polyvinyl chloride core liners and stored at 4°C until used. A 60-cm core was dissected into 5-cm sections, and the sulfate concentration was determined. The pore water of 1-ml sediment samples was extracted by centrifugation, and the sulfate concentration in the pore water was analyzed by ion chromatography. The core was divided into two sections on the basis of its sulfate content. The top section (0 to 35 cm), with a pore water sulfate concentration of >2.5 mM, was concluded to be predominantly sulfidogenic [designated Arthur Kill (s)], and the lower section (35 to 60 cm) was predominantly methanogenic [designated Arthur Kill (m)] due to its low sulfate concentration.

Media. The media used for methanogenic, sulfidogenic, and iron-reducing enrichments consisted of (grams per liter) KCl, 1.3; KH_2PO_4, 0.2; NaCl, 23.0; NH_4Cl, 0.5; $CaCl_2 \cdot 2H_2O$, 0.1; $MgCl_2 \cdot 6H_2O$, 1.0; and $NaHCO_3$, 2.5. In addition, the sulfidogenic medium contained 2.84 g of Na_2SO_4 per liter (20 mM), and the iron-reducing medium contained freshly precipitated amorphous iron (as ferric oxyhydroxide) prepared as previously described (17) at a final concentration of 200 mM. The medium components, with the exception of carbonate, were added to distilled water, boiled for 20 min, and allowed to cool under a stream of O_2-free N_2. After the cooling process, carbonate, resazurin (1 mg/liter), vitamins (in milligrams per liter: thiamine HCl, 0.05; Ca-D-pantothenate, 0.05; riboflavin, 0.05; pyridoxine HCl, 0.10; biotin, 0.02; folic acid, 0.02; vitamin B_{12}, 0.1; nicotinic acid, 0.05; thioctic acid, 0.05; and *p*-aminobenzoic acid, 0.05), and trace minerals (final concentration in micromolar: $FeSO_4$, 75; $MgCl_2$, 5; $CoCl_2$, 8; $ZnSO_4$, 5; H_3BO_3, 1; $NiCl_2$, 1; $CuCl_2$, 0.1; Na_2MoO_4, 1.5) were added from sterile anoxic stock solutions via a deoxygenated syringe.

Enrichment cultures. The sediment slurries (10% [vol/vol] sediment in the respective medium) were divided into 50-ml aliquots in serum flasks, capped with rubber stoppers and crimped with aluminum seals, with a 10-ml headspace of N_2-CO_2 (70:30, vol/vol). Substrates (2-bromophenol [2-BP], 3-BP, 4-BP, 2-bromobenzoate [2-BB], 3-BB, 4-BB, phenol, or benzoate [Aldrich Chemical Co., Milwaukee, Wis.]) were added from deoxygenated stock solutions in 0.1 N NaOH at initial concentrations of 100 μM to individual cultures (in triplicate). After the substrates were consumed, they were reamended to concentrations of 100 to 200 μM. Sterile controls, which were autoclaved at 121°C for 30 min on three consecutive days, were spiked with a mixture of phenol, benzoate, and the different BP and BB isomers. A second set of controls consisted of sediment slurries to which no substrates were added.

Analytical methods. For sampling, the cultures were thoroughly mixed and 1 ml of the sediment slurry was withdrawn via sterile syringes flushed with oxygen-free N_2-CO_2 as previously described (11, 12). The samples were centrifuged in a microcentrifuge at approximately 13,000 × *g* for 4 min, and the supernatants were filtered (0.45-μm-pore-size filter) into high-pressure liquid chromatography (HPLC) vials. The halophenols, halobenzoates, phenol, and benzoate were analyzed by HPLC with a model LC-10AS chromatograph (Shimadzu Corp., Kyoto, Japan) by previously described methods (12, 13) with a reverse-phase C_{18} column (Spherisorb 4.6 by 250 mm; particle size, 5 μm [Phenomenex, Torrance, Calif.]) and UV detection at 280 nm. The mobile phase consisted of methanol-water-acetic acid (60:38:2, vol/vol/vol) at a flow rate of 1.0 ml/min.

The sulfate and bromide concentrations were measured by ion chromatography with a model DX-100 ion chromatograph (Dionex, Sunnyvale, Calif.) equipped with a conductivity detector and an anion-exchange column (IonPac AS9; Dionex). The eluant was 2.0 mM Na_2CO_3–0.75 mM $NaHCO_3$ at a flow rate of 2.0 ml/min. Sulfate and bromide standards were prepared with Na_2SO_4 and KBr, respectively. The sensitivity of this method was 1 μM for sulfate or bromide. Methane was monitored by gas chromatography as described previously (18). Ferrous ion production was determined spectrophotometrically by a ferrozine method described by Lovley and Phillips (26) as modified by Kazumi et al. (17).

RESULTS AND DISCUSSION

Utilization of BP and BB isomers by enrichment cultures in the presence of different electron acceptors. The anaerobic biodegradation of bromoaromatic compounds by iron(III)-reducing, sulfidogenic, and methanogenic enrichment cultures established with sediments from pristine (Lubec and Bay of Fundy) and polluted (Arthur Kill inlet) environments is summarized in Table 1. Loss of the different substrates depended on the presence of the primary electron acceptor and varied for enrichments established with sediments from various geographical locations. Utilization of each of the BB and BP isomers was observed in the Bay of Fundy and Arthur Kill enrichments under at least one reducing condition, but BB loss was not observed in enrichments of decaying seaweed (Lubec). The rates of substrate loss were in general lower under sulfidogenic and iron-reducing conditions than under methanogenic conditions, suggesting that utilization is affected by the availability of the respective electron acceptor. Generally, the half-lives of BPs (100 μM initial concentration) were shorter (under 50 days) than those of BBs (50 to 100 days). Under all three conditions, the BPs were utilized without the lengthy lag period frequently observed in anaerobic systems (22). This suggests that these sediments may have been exposed to BPs or analogous compounds prior to the establishment of the enrichments. Background concentrations of haloorganics were below the detection level of our HPLC assay (i.e., <1.0 μM). Loss of the BB isomers was observed only after a lag period of 23 to 64 days. Phenol and benzoate were utilized under all conditions tested.

TABLE 1. Initial utilization of substrates in the presence of different electron acceptors in sediment enrichments from different locations

Condition	Substrate	t_{50}[a] (days) for substrate utilization in sediment from:			
		Bay of Fundy	Lubec, Maine	Arthur Kill (s)	Arthur Kill (m)
Methanogenic					
	2-BP	<7	11	<7	12
	3-BP	21	—[b]	15	20
	4-BP	22	17	8	10
	Phenol	11	34	45	15
	2-BB	97	—	67	73
	3-BB	50	—	50	57
	4-BB	109	—	103	48
	Benzoate	5	14	15	8
Sulfidogenic					
	2-BP	9	12	15	15
	3-BP	46	75	42	33
	4-BP	29	17	15	54
	Phenol	15	32	40	15
	2-BB	—	—	59	93
	3-BB	85	—	37	67
	4-BB	140	—	57	74
	Benzoate	4	12	9	8
Iron reducing					
	2-BP	19	17	—	20
	3-BP	—	—	—	—
	4-BP	—	—	—	54
	Phenol	29	60	54	33
	2-BB	—	—	—	—
	3-BB	—	—	—	—
	4-BB	—	—	—	—
	Benzoate	29	10	35	15

[a] Time (days) at which 50% of the substrate (initially 100 μM) was depleted (values are means of triplicate cultures). BB t_{50} values include a lag time of 23 to 64 days.
[b] —, no loss observed after 200 days.

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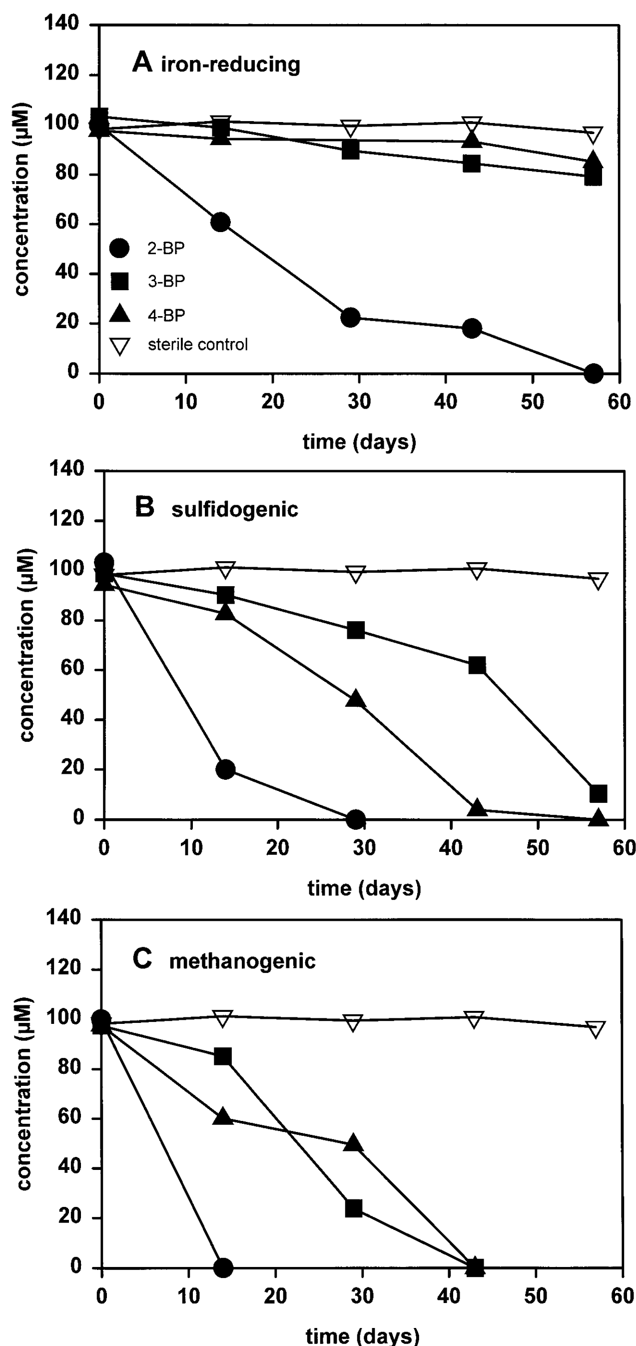


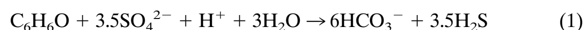
FIG. 1. Utilization of BPs in Arthur Kill (m) enrichment cultures under iron-reducing (A), sulfidogenic (B), and methanogenic (C) conditions. The results are means of three replicate cultures. The results of the sterile controls are means of triplicates containing a mixture of the three BP isomers.

The monobrominated phenol isomers were utilized in enrichment cultures of Arthur Kill (m) sediment under iron-reducing, sulfidogenic, and methanogenic conditions (Fig. 1). Under all conditions tested, 2-BP was utilized faster than 3-BP or 4-BP. Loss of 2-BP proceeded immediately without a noticeable lag period, and the initial amounts of 2-BP were consumed in less than 60, 29, and 14 days under iron-reducing, sulfidogenic, and methanogenic conditions, respectively (Fig. 1). Both 3-BP and 4-BP were utilized without a lag period but only under methanogenic and sulfidogenic conditions, and the initial loss of 100 μM 3-BP and 4-BP took 40 to 60 days. Under iron-reducing conditions, 3-BP and 4-BP were not utilized at a discernible rate compared to sterile controls. Similar substrate utilization patterns were observed with the other enrichment cultures. Regardless of the inoculum source or primary electron acceptor available, a preference for utilization of 2-BP was observed. There was no appreciable difference in the rates at which the different BP or BB isomers were utilized in enrichments started with either the sulfidogenic or the methanogenic core sections obtained from the Arthur Kill inlet.

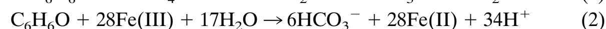
Loss of 2-BP, transient accumulation of phenol, and release of bromide. In the Arthur Kill (m) enrichments under iron-reducing, sulfidogenic, and methanogenic conditions, loss of 2-BP was followed by accumulation and subsequent utilization of phenol and release of bromide (Fig. 2). Under the three conditions, phenol accumulated to stoichiometric amounts and was subsequently consumed after all of the BP had been depleted. The amount of bromide released was equal to the amount of BP consumed. These observations indicate that BP isomers are reductively debrominated to phenol under iron-reducing, sulfate-reducing, and methanogenic conditions. Similar results of debromination and transient accumulation of phenol were also observed with 3-BP and 4-BP under sulfidogenic conditions (data not shown). Reductive debromination of 2-BP was also observed with sediment enrichments from the other sites.

Utilization of other halophenols by enrichment cultures acclimated to BP degradation. The sulfidogenic 2-BP-acclimated cultures also utilized 2-chlorophenol (2-CP) and 2-iodophenol (2-IP). 2-CP was utilized immediately, and phenol was detected as a transient intermediate, whereas 2-IP was utilized after a period of 27 days, but no intermediates were detected. Sterile controls containing the different halophenols did not show any significant dehalogenating activity under the conditions tested. This suggests that reductive dehalogenation is the initial step in degradation under sulfidogenic conditions not only of BP but also of CP. 2-Fluorophenol was not utilized by 2-BP-acclimated cultures.

Stoichiometry of phenol and BP degradation under sulfate- and iron-reducing conditions. To determine whether degradation of phenol and the BP isomers was coupled to sulfate reduction and iron reduction, the consumption of sulfate and production of Fe(II) were compared to predicted values based on the following balanced equations:



$$C_6H_6O + 3.5SO_4^{2-} + H^+ + 3H_2O \rightarrow 6HCO_3^- + 3.5H_2S \quad (1)$$



$$C_6H_6O + 28Fe(III) + 17H_2O \rightarrow 6HCO_3^- + 28Fe(II) + 34H^+ \quad (2)$$

$$C_6H_5OBr + 3.25SO_4^{2-} + 4H_2O \rightarrow 6HCO_3^- + 3.25H_2S + 0.5H^+ + Br^- \quad (3)$$

$$C_6H_5OBr + 27Fe(III) + 17H_2O \rightarrow 6HCO_3^- + 27Fe(II) + 33H^+ + Br^- \quad (4)$$

Equations 1 to 4 assume that phenol or BP is completely mineralized to CO_2. To determine the stoichiometry, cultures were fed BP or phenol repeatedly until approximately 1 or 0.7 mM substrate was utilized under sulfidogenic or iron-reducing conditions, respectively. The amount of electron acceptor consumed in the enrichments shown in Tables 2 and 3 was larger than that consumed by metabolism of the background carbon (determined from the cultures which were not spiked with substrate). Fe(II) production was approximately 105% of that expected for complete degradation of 2-BP to CO_2 and 94% of that expected for degradation of phenol. This suggests that complete mineralization of the substrates coupled to Fe(III) reduction had occurred. The consumption of sulfate in cultures which utilized the BP isomers ranged from 83 to 89% of the expected values. Methane was not produced by either sulfidogenic or iron-reducing cultures (data not shown).

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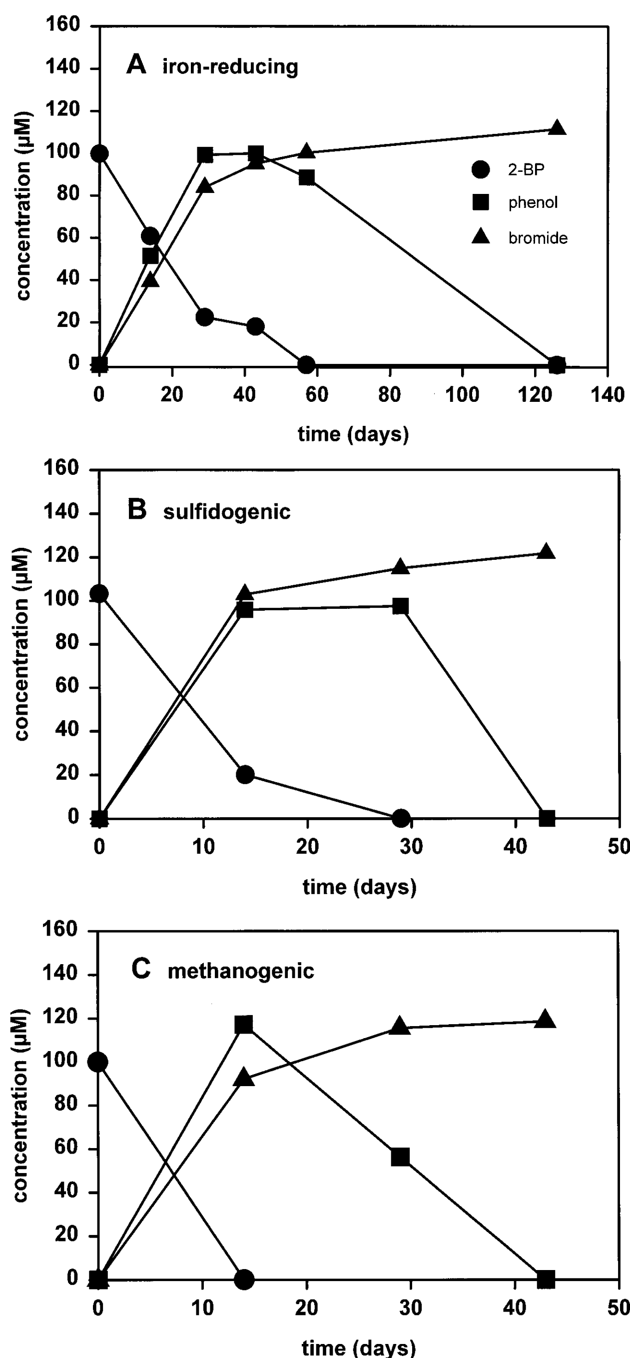

FIG. 2. Loss of 2-BP, production and utilization of phenol, and release of bromide in sediment samples under iron-reducing (A), sulfidogenic (B), and methanogenic (C) conditions in Arthur Kill (m) enrichment cultures. The results are means of triplicate cultures.

Effect of Fe^{2+} on debromination of BPs. Since it has been previously reported that oxidized forms of iron such as Fe(0) and Fe(II) can abiotically mediate the reductive dehalogenation of alkyl halides (28), enrichment cultures were incubated in the presence of both 2-BP and various concentrations of Fe^{2+} and monitored for substrate loss (data not shown). Sterilized enrichment cultures which contained either 0.1, 0.5, or 1 mM Fe^{2+} did not show any substantial loss of 2-BP, whereas the iron-reducing nonsterile culture containing 1 mM Fe^{2+} showed activity. These results indicate that 2-BP is stable in the presence of Fe^{2+} and that this form of iron does not serve as an electron donor for abiotic reductive dehalogenation of 2-BP under the conditions tested. These results also underscore the previous conclusion that the dehalogenating activity is mediated by microorganisms.

Conclusions. Our results provide the chemical evidence (e.g., production of phenol) which indicates that reductive dehalogenation is the initial step in the degradation and complete mineralization of haloaromatic compounds under iron-reducing and sulfidogenic conditions. Dehalogenation may be independent of the terminal electron-accepting process, whereas degradation of phenol is coupled to the reduction of Fe(III), sulfate, or carbonate. The dehalogenation and degradation activities observed under iron-reducing, sulfidogenic, and methanogenic conditions with enrichments established with three different marine and estuarine sediments were similar (Table 1), suggesting that dehalogenating microorganisms are widely distributed in marine sediments. In previous studies (11–13, 17, 18), it was also observed that sediment inocula from different geographical areas and environments exhibited similar degradation of chlorinated phenols and benzoic acids under either denitrifying, iron-reducing, sulfidogenic, or methanogenic conditions.

Reductive dehalogenation requires a source of reducing equivalents, and the addition of auxiliary carbon sources as an electron donor usually stimulates dehalogenation (9, 14, 15). The role of a particular electron donor such as H_2 or any other

TABLE 2. Production of Fe(II) during degradation of 2-BP or phenol in enrichments from Arthur Kill (s) sediment

Substrate	Substrate concn metabolized (mM)[a]	Fe(II) production (mM)			Measured net production/predicted value (%)
		Predicted[b]	Measured		
			Total	Net[c]	
2-BP	0.67 ± 0.11	18.1	52.0 ± 2.7	19.0 ± 2.7	105 ± 15
Phenol	0.73 ± 0.08	20.4	50.9 ± 1.8	17.9 ± 1.8	94 ± 9
None	NA[d]	NA	33.0 ± 2.0	0	NA

[a] Triplicate cultures were fed in increments of 100 to 200 µM over a period of 300 days.
[b] Based on stoichiometry, where 1 mol of 2-BP = 27 mol of Fe(II) and 1 mol of phenol = 28 mol of Fe(II).
[c] Fe(II) production (33.0 ± 2.0 mM) in background cultures was subtracted from these values.
[d] NA, not applicable.

TABLE 3. Consumption of SO_4^{2-} during degradation of BPs in enrichments from Bay of Fundy sediments

Substrate	Substrate concn metabolized (mM)[a]	SO_4^{2-} consumption (mM)			Measured net consumption/predicted value (%)
		Predicted[b]	Measured		
			Total	Net[c]	
2-BP	1.17 ± 0.13	3.8	5.1 ± 0.1	3.4 ± 0.1	89 ± 3
3-BP	1.08 ± 0.08	3.5	4.6 ± 0.2	2.9 ± 0.2	83 ± 6
4-BP	1.13 ± 0.09	3.7	4.8 ± 0.2	3.1 ± 0.2	84 ± 5
Phenol	1.04 ± 0.11	3.6	4.9 ± 0.1	3.2 ± 0.1	89 ± 3
None	NA[d]	NA	1.7 ± 0.2	0	NA

[a] Triplicate cultures were fed in increments of 100 to 200 µM over a period of 200 days.
[b] Based on stoichiometry, where 1 mol of 2-BP = 3.25 mol of SO_4^{2-} and 1 mol of phenol = 3.5 mol of SO_4^{2-}.
[c] Sulfate loss (1.7 ± 0.2 mM) in background cultures subtracted from these values.
[d] NA, not applicable.

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product of organic carbon metabolism is of particular interest. In our system, the enrichments contained 10% (vol/vol) sediment as the inoculum, and it is possible that detritus or other organic matter can provide the initial source of electrons for the reductive dehalogenation of the different BP isomers. Once fission of the phenol ring occurs, the oxidation of the substrate carbon can provide the necessary reducing equivalents for reductive dehalogenation to continue.

It remains to be seen whether dehalogenation under the different conditions is mediated by different microorganisms. It is of interest that both sulfate-reducing and iron-reducing bacteria have been shown to use aromatic compounds, including phenol, as growth substrates (3, 25). In light of our results, it is conceivable that dehalogenation and complete degradation of BPs and BBs under sulfidogenic and iron-reducing conditions could be mediated by a single organism, although the possibility of syntrophic associations cannot be ruled out.

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