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Influence of Alternative Electron Acceptors on the Anaerobic Biodegradability of Chlorinated Phenols and Benzoic Acids

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Nitrate, sulfate, and carbonate were used as electron acceptors to examine the anaerobic biodegradability of chlorinated aromatic compounds in estuarine and freshwater sediments. The respective denitrifying, sulfidogenic, and methanogenic enrichment cultures were established on each of the monochlorinated phenol and monochlorinated benzoic acid isomers, using sediment from the upper (freshwater) and lower (estuarine) Hudson River and the East River (estuarine) as source materials. Utilization of each chlorophenol and chlorobenzoate isomer was observed under at least one reducing condition; however, no single reducing condition permitted the metabolism of all six compounds tested. The anaerobic biodegradation of the chlorophenols and chlorobenzoates depended on the electron acceptor available and on the position of the chlorine substituent. In general, similar activities were observed under the different reducing conditions in both the freshwater and estuarine sediments. Under denitrifying conditions, degradation of 3- and 4-chlorobenzoate was accompanied by nitrate loss corresponding reasonably to the stoichiometric values expected for complete oxidation of the chlorobenzoate to CO_2. Under sulfidogenic conditions, 3- and 4-chlorobenzoate, but not 2-chlorobenzoate, and all three monochlorophenol isomers were utilized, while under methanogenic conditions all compounds except 4-chlorobenzoate were metabolized. Given that the pattern of activity appears different for these chlorinated compounds under each reducing condition, their biodegradability appears to be more a function of the presence of competent microbial populations than one of inherent molecular structure.

It is well documented that methanogenic conditions can promote the biodegradation of a number of halogenated aromatic compounds (5, 13). As a consequence, they are no longer considered recalcitrant under anaerobic conditions. Degradation of chlorinated phenols and benzoic acids by methanogenic consortia is initiated by reductive dechlorination (3, 8, 11, 12, 15, 17–21, 24, 26, 28, 35, 37), which is followed by cleavage of the aromatic ring and ultimately mineralization to CH_4 and CO_2 (3, 8, 11, 12, 30). Methanogenic consortia capable of aryl-dehalogenation have been enriched from a variety of environments, such as sewage sludge, freshwater and marine sediments, soils, and groundwater aquifers (3, 8, 10, 12, 18, 19, 24, 26, 35). One obligately anaerobic bacterium, *Desulfomonile tiedjei* DCB-1, capable of reductive aryl-dehalogenation was obtained from a methanogenic enrichment, yet it is a sulfidogen (7, 34). It can utilize nonaromatic carbon sources, but the mineralization of 3-chlorobenzoate (3-CB) required mutualism among the dechlorinating, benzoate-oxidizing, and methanogenic members of the consortium (9, 34). 3-CB-induced cell suspensions of *D. tiedjei* were recently shown to dehalogenate *meta*-chlorine substituents of chlorophenols (30). Recently, a *Clostridium*-like bacterium that reductively dechlorinates chlorophenols was isolated from a 2,4,6-trichlorophenol-dechlorinating consortium enriched from sewage sludge (29).

Under reducing conditions other than methanogenic conditions, less information on the fate of halogenated aromatic compounds is available. Aside from carbonate (methanogenesis), a wide variety of alternative electron acceptors for respiration can be used by bacteria, including nitrate, sulfate, manganese, and iron. Denitrification can be significant in regions of high nitrate input from agricultural runoff or sewage discharge (4). In anaerobic freshwater environments, sulfate concentrations tend to be low, and carbonate reduction to methane serves as the predominant electron sink for microbial processes. On the other hand, in marine systems, sulfate concentrations tend to be high, and sulfate reduction or sulfidogenesis serves as the major electron-accepting process (5). Sulfate has, however, been shown to inhibit reductive dechlorination by methanogenic consortia (2, 6, 15, 25, 27). This inhibition was thought to be due to competition for reducing equivalents by sulfate (12). Dechlorination of chlorophenols in the presence of sulfate, as well as degradation of chlorophenols coupled to sulfate reduction, has nonetheless been demonstrated (10, 16, 23, 25). Nitrate was reported to stimulate 3-CB degradation in anaerobic enrichment cultures, though it was not certain whether denitrification took place (10). In addition, anaerobic utilization of 2-fluorobenzoate by a benzoate-degrading, denitrifying bacterium was observed, but chlorinated benzoates were not metabolized (33). Whether the anaerobic degradation of a chloroaromatic compound can be coupled to denitrification remains unclear, and to learn this was one of the objectives of this study.

We carried out a systematic evaluation of the anaerobic biodegradability of monochlorinated phenols and benzoic acids coupled to denitrification, sulfidogenesis, and methanogenesis and demonstrated that these alternative electron acceptors, nitrate, sulfate, and carbonate, can affect degradation rates and substrate specificities. Sediment from two environmentally different sites (freshwater and estuarine) of

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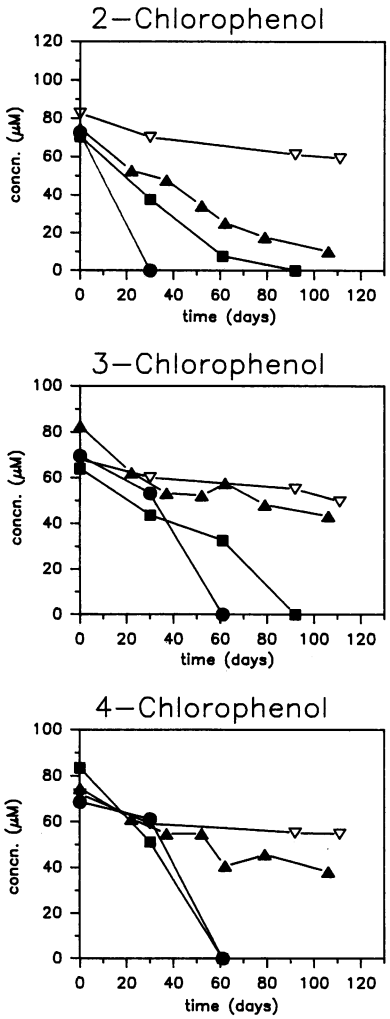

FIG. 1. Degradation of 2-CP, 3-CP, and 4-CP by methanogenic (●), sulfidogenic (■), and denitrifying (▲) enrichment cultures of UH sediment. Datum points are the means of two replicate cultures. Sterile controls (▽) are the means of methanogenic and sulfidogenic cultures.

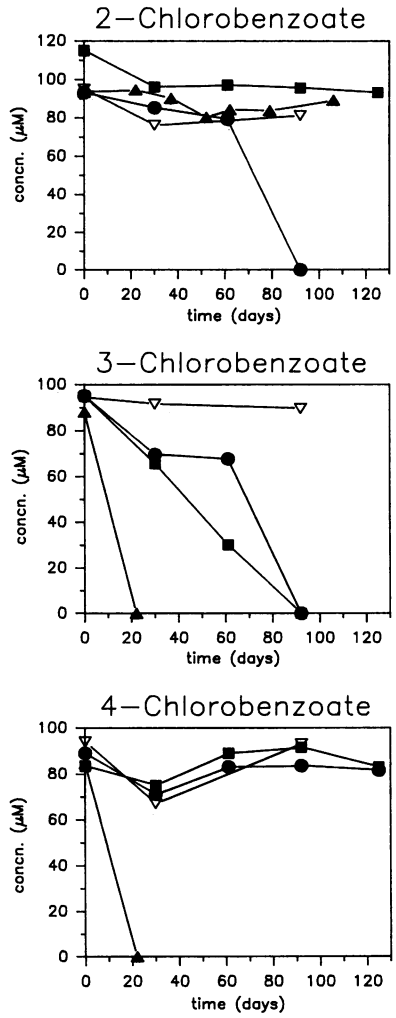

FIG. 2. Degradation of 2-CB, 3-CB, and 4-CB by methanogenic (●), sulfidogenic (■), and denitrifying (▲) enrichment cultures of UH sediment. Datum points are the means of two replicate cultures. Sterile controls (▽) are the means of methanogenic and sulfidogenic cultures.

the Hudson River and sediment from the East River, an estuarine intertidal strait, were used as inocula.

MATERIALS AND METHODS

Source of inoculum. Sediment samples were collected from the Hudson River, at New York City (lower Hudson [LH], mile point 4 from Upper New York Bay), near Albany, N.Y. (upper Hudson [UH], mile point 145), and from the East River (ER), an intertidal strait separating Manhattan from Long Island. The LH and ER sediments were estuarine (salinity, 23‰), while the UH sediment was a freshwater site. Sediment samples were collected by grab sampling from the upper 20 cm of the sediment layer. Glass jars were filled to capacity with sediment, sealed, transported to the laboratory, and stored at 4°C until used. The UH sediment was a sandy silt, while the LH and ER sediments consisted of black silty mud. The heavy-metal concentration of the sediments was determined as described previously (1), and the following concentrations (micrograms per gram of dry weight) of heavy metals were observed: UH, Cr = 74, Pb = 72, Cd = 2, and Zn = 186; LH, Cr = 181, Pb = 195, Cd = 2, and Zn = 288; ER, Cr = 340, Pb = 301, Cd = 5, and Zn = 429.

Establishment of cultures. Strict anaerobic techniques for medium preparation, culture handling, and sampling were followed throughout the study. A 10% (vol/vol) sediment slurry was prepared in either a methanogenic (14), sulfidogenic (20 mM sulfate) (16), or denitrifying (30 mM nitrate) (14) medium supplemented with trace elements and vitamins (14, 16) and was dispensed to a final culture volume of 50 ml into 65-ml serum bottles that had been flushed with O_2-free N_2/CO_2 or argon. For the estuarine sediment samples, LH and ER cultures, the respective media received an additional 23.0 g of NaCl and 1.0 g of $MgCl_2$ $liter^{-1}$ on the basis of the measured salinity of the lower Hudson and the East Rivers. The bottles were sealed with thick butyl rubber stoppers and aluminum crimps under a headspace of N_2/CO_2 (70:30) for the methanogenic and sulfidogenic cultures and argon for the

TABLE 1. Initial utilization of chlorinated phenols and benzoic acids under denitrifying, sulfidogenic, and methanogenic conditions[a]

Inoculum	Enrichment condition	Days required for initial utilization of:					
		2-CP	3-CP	4-CP	2-CB	3-CB	4-CB
UH	D	106[b]	—	—	133[c]	<21	<21
	S	92	92	61	—	92	—
	M	<30	61	61	92[c]	92	—
LH	D	—	—	—	—	100	79
	S	125	92	211	—	167[c,d]	—
	M	135	135[c]	>200	240	210	—
ER	D	164[d]	—	—	—	133	106

[a] Chlorophenols or chlorobenzoates were added to a concentration of 100 μM. Enrichment cultures were sampled at 2- to 4-week intervals for up to 1 year. D, denitrifying; S, sulfidogenic; M, methanogenic. —, no degradation observed for up to 1 year.
[b] There was activity in only one culture upon refeeding.
[c] Only one culture was active.
[d] Activity could not be sustained upon refeeding.

denitrifying cultures. 2-Chlorophenol (2-CP), 3-chlorophenol (3-CP), 4-chlorophenol (4-CP), 2-chlorobenzoate (2-CB), 3-CB, or 4-chlorobenzoate (4-CB) (Aldrich Chemical Co., Milwaukee, Wis.) was added to separate cultures to an initial concentration of 100 μM. The cultures were established in duplicate with background (no substrate added) and sterile controls (autoclaved for 1 h on three consecutive days prior to feeding with substrate). All cultures (a total of 140) were incubated without shaking at 30°C, in the dark, and monitored over a period of more than 2 years. Substrate depletion rates in acclimated cultures were determined by linear regression analysis of the substrate concentration over time.

Analysis. Liquid samples were taken periodically for analysis with sterile syringes, which were flushed with N_2/CO_2 or argon before sampling. The volume of gas produced was measured with a water-lubricated glass syringe that was preflushed with N_2/CO_2 or argon (32). CH_4 and N_2 in the headspace were analyzed with a gas partitioner as described previously (14). Chlorophenols and chlorobenzoates were quantified by high-performance liquid chromatography (HPLC) and UV detection as described before (14, 16). Metabolites, when present, were identified by comparison of their retention times to those of authentic compounds.

Nitrate and sulfate were analyzed by ion chromatography with conductivity and UV detection, using a Waters Ion Chromatography unit (600E Sample Controller, 715 Ultra Wisp Sample Processor, 590 Solvent Delivery Module, 431 Conductivity Detector, 484 Tunable Absorbance Detector; Waters Associates, Milford, Mass.) equipped with an IC-Pak A HC column (150 by 4.6 mm; Waters Associates) and a Minichrom Version 1.5 Chromatography Handling System (VG Data Systems Ltd., Chesire, United Kingdom). A borate-gluconate solvent system (20 ml of a concentrate consisting of 7.2 g of LiOH monohydrate, 25.5 g of boric acid, 13.2 ml of a 50% [wt/vol] solution of gluconic acid, 94 ml of 95% glycerol, and deionized and demineralized water added to 1,000 ml was mixed with 120 ml of acetonitrile, with deionized and demineralized water added to 1,000 ml) filtered through a 0.22-μm-pore-size Millipore membrane was used at a flow rate of 2 ml/min.

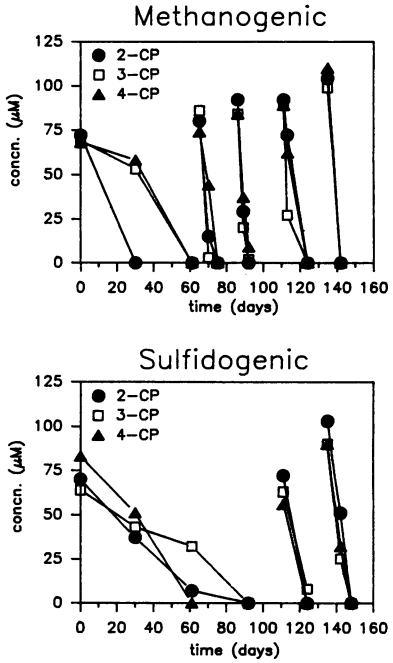

FIG. 3. Degradation of monochlorophenols in methanogenic and sulfidogenic enrichment cultures of UH sediment.

RESULTS

Degradation of chlorophenols and chlorobenzoates. Methanogenic, sulfidogenic, and denitrifying enrichment cultures were established with each of the monochlorinated phenols and benzoate isomers with UH and LH sediments as inocula. Denitrifying enrichment cultures were also established with ER sediment. Since these cultures are all enrichments, the terms methanogenic, sulfidogenic, and denitrifying are used to describe culture conditions only, not to imply that methanogens, sulfidogens, or denitrifyers per se are responsible for degradation of the substrates.

The initial depletion of the chlorophenols and chlorobenzoic acids in the UH enrichment cultures is shown in Fig. 1 and 2, respectively. Utilization of each of the chlorophenol and chlorobenzoate isomers was observed under at least one reducing condition; however, the pattern of substrate utilization was distinct. While 2-CP was degraded under all three reducing conditions, loss of 3-CP and 4-CP was not observed under denitrifying conditions. 3-CB was also utilized under all three reducing conditions, but degradation of 2-CB was only observed in the methanogenic cultures, and 4-CB was only degraded under denitrifying conditions. Phenol was detected as a transient metabolite during degradation of 2-CP in methanogenic cultures (not shown), indicating that reductive dechlorination was an initial step in degradation of this isomer. No metabolites, however, were detected by HPLC (UV, A_{280}) in the sulfidogenic or denitrifying cultures. Loss of chlorobenzoates in autoclaved controls was limited to less than 35% in 260 days, indicating that metabolism in the active cultures was indeed biologically mediated.

Table 1 summarizes the time required for substrate utilization in UH, LH, and ER cultures upon initial incubation of the samples. A similar pattern of susceptibility of the chlorophenols and chlorobenzoates was observed in both the UH and LH sediment cultures. The initial biodegradation rates were substantially slower, however, for the LH cul-

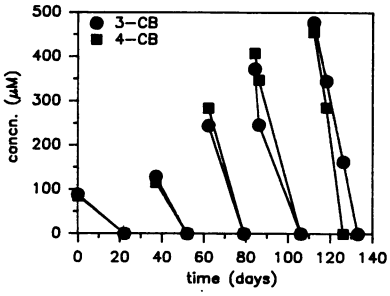

FIG. 4. Degradation of 3-CB and 4-CB under denitrifying conditions in enrichment cultures of UH sediment.

tures. Similar to that observed in the UH enrichment cultures, both 3-CB and 4-CB were utilized in LH and ER cultures under denitrifying conditions. In addition, ER sediment also yielded initial utilization of 2-CP under denitrifying conditions.

When the respective substrates were completely depleted, the cultures were refed to the original concentration. Utilization of the monochlorophenols under methanogenic and sulfidogenic conditions is shown in Fig. 3. Utilization of 2-CP in the UH cultures under denitrifying conditions, although initially observed, could not be sustained with refeeding (not shown). In acclimated cultures, 2-CP, 3-CP, and 4-CP were metabolized under methanogenic conditions at rates of 32 ± 3, 53 ± 6, and 21 ± 1 μmol/liter/day, respectively, and under sulfidogenic conditions at rates of 8.4 ± 2.3, 7.0 ± 3.4, and 11 ± 1.2 μmol/liter/day, respectively. That no UV (A_{280})-absorbing material accumulated in the cultures suggests that the aromatic ring was metabolized.

After 3-CB and 4-CB had been depleted in the denitrifying cultures, they were similarly repeatedly refed their respective substrate (Fig. 4). The two chlorobenzoate isomers were degraded in 20 days or less upon refeeding, and substrate concentrations were increased in a stepwise fashion fivefold. At approximately 500 μM, 3-CB and 4-CB were metabolized in acclimated cultures at rates of 26 ± 2 and 32 ± 1 μmol/liter/day, respectively. Again, no metabolites detectable by HPLC (UV, A_{280}) accumulated, indicating cleavage of the aromatic ring.

Stoichiometry of anaerobic degradation. The mineralization of the chlorophenols and chlorobenzoates coupled to denitrification, sulfidogenesis, or methanogenesis can be described by the stoichiometric equations listed below.

For chlorophenols:

$$C_6H_5OCl + 5.2NO_3^- + 4.2H^+ \rightarrow 6CO_2 + 2.6N_2 + 4.6H_2O + Cl^- \quad (1)$$

$$C_6H_5OCl + 3.25SO_4^{2-} + 4H_2O \rightarrow 6HCO_3^- + 3.25H_2S + 0.5H^+ + Cl^- \quad (2)$$

$$C_6H_5OCl + 4.5H_2O \rightarrow 3.25CH_4 + 2.75CO_2 + H^+ + Cl^- \quad (3)$$

For chlorobenzoates:

$$C_7H_5O_2Cl + 5.6NO_3^- + 4.6H^+ \rightarrow 7CO_2 + 2.8N_2 + 4.8H_2O + Cl^- \quad (4)$$

$$C_7H_5O_2Cl + 3.5SO_4^{2-} + 5H_2O \rightarrow 7HCO_3^- + 3.5H_2S + H^+ + Cl^- \quad (5)$$

$$C_7H_5O_2Cl + 5H_2O \rightarrow 3.5CH_4 + 3.5CO_2 + H^+ + Cl^- \quad (6)$$

To determine the relationship of substrate utilization to the reduction of each of the electron acceptors, the consumption of the terminal electron acceptors or the production of reduced end products was analyzed. Acclimated cultures were repeatedly fed their respective substrates until 0.6 to 1.2 mM had been consumed, after which loss of nitrate or sulfate, or production of methane, was determined.

Nitrate consumption in the 3-CB and 4-CB UH enrichments is shown in Table 2. The predicted amount of nitrate consumed is based on the cumulative concentration of chlorobenzoate added to the cultures and calculated from equation 4 (above). Assimilation of chlorobenzoate to cell carbon is not taken into account. As noted in Table 2, the measured amounts of nitrate utilized compared favorably with the predicted amounts, indicating that mineralization of the chlorobenzoates coupled to denitrification has occurred. Nitrite was transiently detected, but did not accumulate, and nitrogen gas was produced in excess of background controls.

TABLE 2. Consumption of nitrate during degradation of monochlorobenzoates in UH enrichment cultures

Sediment source	Substrate fed	Substrate metabolized (mM)	NO_3^- consumption (mM)		
			Predicted[a]	Measured[b]	% of expected
UH	3-CB	1.13 ± 0.02	6.3	5.4 ± 2.6	84 ± 40
UH	4-CB	1.12 ± 0.01	6.3	6.1 ± 0.01	98 ± 0.2

[a] Based on stoichiometry, 1 mol of chlorobenzoate = 5.6 mol of NO_3^-.
[b] Consumption of NO_3^- (2.7 ± 0. mM) in background control cultures was subtracted.

Sulfate loss was observed in all sulfidogenic enrichment cultures. Sulfate reduction in 2-CP-, 4-CP-, and 3-CB-degrading cultures is shown in Table 3. Degradation resulted in loss of sulfate above background controls corresponding to 57 to 114% of that expected on the basis of the stoichiometry for mineralization given by the equations listed above. The variability between measured and predicted sulfate loss may be due to differences in the amount of sediment inoculum in the different cultures or to incomplete degradation. No methane was produced in these cultures, supporting the observation that sulfate served as the main electron acceptor during chlorophenol and chlorobenzoate degradation.

Formation of CH_4 in the methanogenic enrichments from the UH sediment fed 2-CP, 3-CP, or 4-CP is shown in Table 4. All three chlorophenol isomers were degraded, yielding stoichiometric amounts of CH_4. The measured CH_4 production corresponded to 98 to 120% of that expected on the basis of values calculated for the complete oxidation of monochlorophenol to CH_4 and CO_2, indicating that they were mineralized under methanogenic conditions.

DISCUSSION

Our results indicate that chlorinated phenols and benzoic acids are biodegradable under a variety of anaerobic conditions. For each of the three monochlorophenols and three monochlorobenzoates, utilization was observed under at least one reducing condition (Table 1; Fig. 1 and 2). No single reducing condition permitted the metabolism of all six compounds examined, and the pattern of substrate specificity was distinct for each electron acceptor. Metabolism depended on both the electron acceptor available and the position of the chlorine substituent.

Particularly noteworthy is the initial degradation of 3-CB and 4-CB, which was rapid and occurred without a lag in the denitrifying enrichments compared with the other reducing conditions. Denitrifying cultures degrading 3-CB and 4-CB were enriched from all three sediments studied. Furthermore, for these two compounds, activity was sustained with repeated feeding of the substrates, and the chlorobenzoate degradation correlated reasonably with stoichiometric reduction of nitrate to N_2 gas (Fig. 4; Table 2). Since no metabolites were detected, we do not know at this point

TABLE 3. Consumption of sulfate during degradation of monochlorophenols and monochlorobenzoates in UH and LH enrichment cultures

Sediment source	Substrate fed	Substrate metabolized (mM)	SO_4^{2-} consumption (mM)		
			Predicted[a]	Measured[b]	% of expected
UH	2-CP[c]	1.24	4.0	2.3	57
LH	4-CP	1.0 ± 0.02	3.25	3.7 ± 0.2	114 ± 5
UH	3-CB[c]	0.61	2.2	1.6	74

[a] Based on stoichiometry, 1 mol of chlorophenol = 3.25 mol of SO_4^{2-}; 1 mol of chlorobenzoate = 3.5 mol of SO_4^{2-}.
[b] Consumption of SO_4^{2-} (UH, 0.7 ± 0.2 mM; LH, 1.7 ± 0.04 mM) in background control cultures was subtracted.
[c] Data from one culture only.

whether 3-CB and 4-CB were degraded via initial reductive dechlorination or through some other mechanism.

To our knowledge, this is the first report demonstrating degradation of a chlorinated aromatic molecule coupled to denitrification. This was first examined by Genthner et al. (10, 11), who reported that 3-CB was degraded in the presence of nitrate and that nitrate stimulated 3-CB degradation. Whether denitrification occurred was not clear. Anaerobic degradation of 2- and 4-fluorobenzoate mediated by denitrifying pseudomonads has also been reported, but chlorobenzoates were not metabolized (33, 36). Resting cells of a coryneform bacterium, strain NTB-1, convert 4-CB to 4-hydroxybenzoate under anaerobic conditions in the presence of nitrate (12a, 12b). The authors suggested that nitrate was needed to drive an energy-dependent uptake and hydroxylation of 4-CB. 4-Hydroxybenzoate, however, was not degraded further in the absence of oxygen (12b). Recently, anaerobic phototrophic metabolism of 3-CB by *Rhodopseudomonas palustris* strains was demonstrated (22). 3-CB was converted to CO_2 and biomass in the presence of benzoate and light.

All three chlorophenol isomers and 3-CB were metabolized under sulfidogenic conditions with reduction of sulfate in excess of background controls (Table 3). This supports our previous report showing degradation of each of the three monochlorophenol isomers coupled to sulfate reduction (16). In addition, we believe that this is the first report of 3-CB undergoing degradation under sulfidogenic conditions. As with the denitrifying enrichments, whether dechlorination is the initial step in degradation could not be determined.

The most notable difference between the cultures established from the different sediment sources was the rate of initial substrate utilization. For each of the compounds which were metabolized, the UH sediment generated much more rapid utilization than the LH or ER sediment (Table 1). Since all three anaerobic conditions were affected in the same way, it is possible that the LH and ER sediments contain other toxic or inhibitory compounds. Results from the heavy-metal analyses of the sediment (see Materials and Methods) indicate that the concentrations of Cr^{2+}, Pb^{2+}, Cd^{2+}, and Zn^{2+} totalled 334 μg/g (dry weight) for the UH and 666 and 1,075 μg/g (dry weight) for the LH and ER sediments, respectively, which indeed may have had an inhibitory effect.

Because the differences in initial rates were so prominent among the UH, LH, and ER cultures, any effects due to the freshwater environment of the UH and the marine estuarine environment of the LH and ER sediments are difficult to evaluate. The UH and LH cultures under sulfidogenic and methanogenic conditions yield the most information (Table 1). If the relative rates of initial substrate utilization are compared, there is a trend which suggests that metabolism in sulfidogenic cultures is faster than in methanogenic cultures in estuarine LH sediment, while the opposite is true in the UH cultures. Further work would be needed to substantiate this.

It appears that, in general, the patterns of substrate utilization are similar with the three sediment samples (Table 1). In some cases, e.g., 2-CP with nitrate (UH and ER), although substrate loss was initially observed, it could not be sustained and, hence, could not be confirmed. Thus, the three monochlorophenols appear to be recalcitrant under denitrifying conditions, but readily susceptible to biodegradation under sulfidogenic and methanogenic conditions. On the other hand, methanogenic conditions have been documented to provide a variety of dehalogenating activity, yet our results indicate that 4-CB was not metabolized. Furthermore, 2-CB, susceptible under methanogenic conditions, could not be utilized under either denitrifying or sulfidogenic conditions. Evidence for reductive dechlorination as the initial step in metabolism was only seen for 2-CP under methanogenic conditions. Since all six compounds tested could be utilized under at least one condition, it appears that it is not the position of the halogen substituent but the competency of the microbial population that determines biodegradation. That the three different sediment inocula exhibited essentially similar patterns of activities under the various reducing conditions suggests that these patterns are fundamental. Since contaminated sites usually have mixtures of wastes, bioremediation efforts may need to take into account the activities of diverse anaerobic communities to carry out effective treatment of all components.

TABLE 4. Formation of methane during degradation of monochlorophenols in UH enrichment cultures

Chlorophenol fed	Amt metabolized (μmol)	CH_4 formation (μmol)		
		Predicted[a]	Measured[2]	% of expected
2-CP	68 ± 3	221 ± 10	234 ± 28	106 ± 8
3-CP	66 ± 2	215 ± 6	256 ± 9	120 ± 8
4-CP	65 ± 2	214 ± 6	208 ± 21	98 ± 13

[a] Based on stoichiometry, 1 mol of chlorophenol = 3.25 mol of CH_4.
[b] Formation of CH_4 (16 ± 0.8 μmol) in background control cultures was subtracted.

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