APPLIED AND ENVIRONMENTAL MICROBIOLOGY, July 1996, p. 2317–2323 Vol. 62, No. 7
0099-2240/96/$04.00+0

Effect of Added Heavy Metal Ions on Biotransformation and Biodegradation of 2-Chlorophenol and 3-Chlorobenzoate in Anaerobic Bacterial Consortia

CHUN-WEI KUO[1] AND BARBARA R. SHARAK GENTHNER[2]*

Department of Biology[1] and Center for Environmental Diagnostics and Bioremediation,[2] University of West Florida, Pensacola, Florida 32514

Received 31 January 1996/Accepted 2 May 1996

The effect of added Cd(II), Cu(II), Cr(VI), or Hg(II) at 0.01 to 100 ppm on metabolism in anaerobic bacterial consortia which degrade 2-chlorophenol (2CP), 3-chlorobenzoate (3CB), phenol, and benzoate was examined. Three effects were observed, including extended acclimation periods (0.1 to 2.0 ppm), reduced dechlorination or biodegradation rates (0.1 to 2.0 ppm), and failure to dechlorinate or biodegrade the target compound (0.5 to 5.0 ppm). 3CB biodegradation was most sensitive to Cd(II) and Cr(VI). Biodegradation of benzoate and phenol was most sensitive to Cu(II) and Hg(II), respectively. Adding Cr(VI) at 0.01 ppm increased biodegradation rates of phenol (177%) and benzoate (169%), while Cd(II) and Cu(II) at 0.01 ppm enhanced biodegradation rates of benzoate (185%) and 2CP (168%), respectively. Interestingly, with Hg(II) at 1.0 to 2.0 ppm, 2CP and 3CB were biodegraded 133 to 154% faster than controls after an extended acclimation period, suggesting adaptation to Hg(II). Metal ions were added at inhibitory, but sublethal, concentrations to investigate effects on metabolic intermediates and end products. Phenol accumulated to concentrations higher than those in controls only in the 2CP consortium with added Cu(II) at 1.2 ppm but was subsequently degraded. There was no effect on benzoate, and little effect on acetate intermediates was observed. In most cases, methane yields were reduced by 23 to 97%. Thus, dehalogenation, aromatic degradation, and methanogenesis in these anaerobic consortia showed differential sensitivities to the heavy metal ions added. These data indicate that the presence of heavy metals can affect the outcome of anaerobic bioremediation of aromatic pollutants. In addition, a potential exists to use combinations of anaerobic bacterial species to bioremediate sites contaminated with both heavy metals and aromatic pollutants.

Hazardous halogenated aromatic compounds which have been used by agriculture and industry and discharged into the environment for several decades often accumulate in anaerobic sediments, soils, and aquifers. Anaerobic bioremediation has been proposed as an inexpensive method for in situ removal of organic contaminants in the environment. However, heavy metal contamination from both natural (erosion, fires, leaching, volcanic activity, and microbial transformation) and anthropogenic (industrial waste, dumping of sewage, burning of fossil fuels, etc.) sources also results in the accumulation of metals in anoxic environmental niches. Little is known about the effect on anaerobic bioremediation when both types of contamination are present. Therefore, anaerobic bioremediation of chlorinated xenobiotic compounds in the environment must take into consideration the effect of metals on anaerobic bacterial processes and of anaerobic bacterial species on the metals themselves.

The toxicity of a heavy metal to anaerobic bacteria depends upon a number of factors, including chemical form, bioavailability, and the presence of bacterial species that are resistant to or can detoxify the metal (11, 12). In addition, hydrogen sulfide and organic compounds produced by anaerobic bioprocesses may precipitate metals, reducing toxicity, while highly reducing conditions may change the valence of the metal ion, resulting in a change in the toxicity of a heavy metal (7, 12, 13, 25). To add to the complexity of the situation, anaerobic biodegradation often occurs through interdependent cooperation of several types of anaerobic bacteria (23). This requires an understanding of the effect the heavy metal has on several functional groups of bacteria within the population, particularly those groups occupying central positions and playing key metabolic roles in anaerobic ecosystems, e.g., dehalogenating, syntrophic (24, 26), or methanogenic species.

Only one preliminary study previously investigated the effect of heavy metals on reductive dechlorination (17). That investigation found increased lag phases, reduced rates, and complete inhibition of dechlorination for various chlorophenols in unadapted sediment slurries to which high concentrations (20 to 100 ppm) of Cd(II) or Cr(VI) were added. Because of the high organic content of sediment slurries and the high concentrations of metal ions required for inhibition, these data suggested that a major portion of the metal ion was not bioavailable.

The objective of our work was to examine the effects of Cd(II), Cu(II), Cr(VI), and Hg(II), heavy metals listed as priority pollutants (15), on the reductive dechlorination of two model chlorinated compounds, 2-chlorophenol (2CP) and 3-chlorobenzoate (3CB), in anaerobic bacterial consortia derived from aquatic sediment and specifically adapted to completely degrade these compounds in a mineral medium containing no sediment but supplemented with yeast extract (0.02%), resulting in a low-organic background (32, 33). To investigate the effect of added metal ions on dechlorination versus aromatic biodegradation, consortia which were derived

* Corresponding author. Mailing address: Center for Environmental Diagnostics and Bioremediation, University of West Florida, 11000 University Parkway, Pensacola, FL 32514. Phone: (904) 474-3362. Fax: (904) 474-3130.

from the 2CP consortium and which degraded either phenol (34) or benzoate (31) were also studied.

MATERIALS AND METHODS

Consortia and growth conditions. The 2CP and 3CB consortia were derived from aquatic sediment enrichments in which the original sediment was diluted out as a result of several sequential transfers to fresh anaerobic medium (33). The phenol (34) and benzoate (31) consortia were derived from the original 2CP consortium by transferring inocula from the 2CP consortia to phenol or benzoate medium. These derivative consortia were refed phenol or benzoate when the substrate was depleted and passed (10%) to fresh medium every 2 months for over 1 year. After this adaptation, both derivatized consortia lost the ability to dechlorinate 2CP. The benzoate consortium also lost the ability to degrade phenol.

The anaerobic cultivation and sampling techniques and anaerobic defined mineral medium used were previously described (33, 34), except that 0.02% yeast extract was added and the Na_2S reducing agent was deleted. The 2CP, phenol, benzoate, and 3CB consortia were adapted to this medium by passing several sequential transfers (10%) from a stock consortium to the medium containing the target compound, i.e., 2CP (500 μM), phenol (1 mM), benzoate (2 mM), or 3CB (800 μM). This diluted Na_2S from the stock consortium. Incubations were done at 30°C. Cultures were grown in serum bottles (160 ml) with 100 ml of medium.

Anaerobic degradation studies in the presence of added metals. Concentrated metal salt stock solutions of $CdCl_2$, $CuCl_2 \cdot 2H_2O$, K_2CrO_4, and $HgCl_2$ were prepared with sterile H_2O, equilibrated with N_2, sealed in acid-washed serum bottles, and autoclaved. An initial study monitored the biodegradation rates of target compounds in consortia after addition of the metal salt to yield Cd(II), Cu(II), or Cr(VI) final concentrations of 0.01 to 100 ppm. Hg(II) was added to 0.1 to 100 ppm. A sublethal concentration of each metal, which reduced the biodegradation rate by approximately 50%, was selected for each consortium, and the effect of added metal ions on the formation and degradation of metabolic intermediates and formation of methane as an end product was monitored. Dechlorination and degradation rates were calculated as the slope of the best-fit line. When dechlorination of 2CP and degradation of the resulting phenol occurred simultaneously, the concentrations of 2CP and phenol were summed at each sampling point. This allowed a best-fit line to be generated for the phenol biodegradation rate that accounted for the additional phenol forming via dechlorination. The acclimation period was the number of days that passed before a decline in the initial concentration of the target compound was detected. Test cultures, control cultures without added metals, and uninoculated control cultures were prepared in triplicate.

Analytical methods. Aromatic compounds were quantified by high-performance liquid chromatography. Samples (1 ml) were filtered (Acrodisc CR PTFE, 13-mm diameter, 0.2-μm pore size) and analyzed immediately or stored (−20°C) until analysis on a Hewlett-Packard 1050 high-performance liquid chromatograph equipped with a Hypersil octyldecyl silane column (5 μm, 100 by 2.1 mm), an autosampler, and a Hewlett-Packard Chemstation. Injected samples (2 μl) were analyzed at 207 nm. The mobile phase (0.5 ml/min) was 50 mM K_2HPO_4 (pH 3.5)–CH_3CN, with the CH_3CN increasing in an isogradient from 41 to 65%. Target compounds and dechlorinated intermediates were identified and quantified by comparing retention times and areas under the curves with those of authentic standards.

Pentafluorobenzyl derivatives (14) of volatile fatty acids, including acetate, were prepared from filtered (Acrodisc CR PTFE, 0.2-μm pore size) culture supernatant. Derivatized volatile fatty acids were identified and quantified by comparing retention times and areas under the curves to those of a derivatized mixture of authentic standards. An Ultra 2 (5% phenylmethyl siloxane) column (Hewlett-Packard) on a Hewlett-Packard 5890 gas chromatograph equipped with an electron capture detector was used. Heptanoate was added to each sample before derivatization as an internal derivatization control.

Total methane was calculated from the percent methane (33) and total gas volume (24) as previously described, with a Hewlett-Packard 5840 gas chromatograph. The methane yield was determined by dividing the total amount of methane produced in consortia with added metal ions by the total amount of methane produced in control cultures (27).

The concentration of dissolved metal ions was determined on filtered (0.45-μm pore size) culture samples. Cu, Cr, or Cd was determined with a Varian spectrAA-300/400 atomic absorption graphite spectrophotometer equipped with hollow cathode lamps (Starna Cells, Inc., Atascadero, Calif.) specific for each metal. Dissolved Hg was determined by cold vapor fluorescence spectroscopy with a Leemann PS200 mercury analyzer as previously described (30).

The effect of added metal ions on the redox potential of uninoculated medium was determined with a Fisher platinum-Ag-AgCl combination electrode (8). The electrode was rinsed in anaerobic medium and placed in an opened serum bottle of anaerobic medium with stirring under the anaerobic gas phase while increasing amounts of concentrated anaerobic metal salt solutions were added anaerobically. The E_h was recorded with each addition once equilibrium was reached. Light's (+430 mV) and Zobell's (+183 mV) solutions were used as standards (8). Medium pH was determined in a similar manner with a pH electrode. Cu(I) and Cu(II) were differentiated by using the Bathocuproine method with and without hydroxylamine (8) under an anaerobic headspace of N_2 to maintain the same Cu(I)-to-Cu(II) ratio as in the anaerobic medium. Cr(VI) was quantified by using method 3500-Cr D (8) without addition of the oxidizing agent required to convert Cr(III) to Cr(VI). This assay was performed aerobically.

TABLE 1. Effect of added Cd(II), Cr(VI), Cu(II), or Hg(II) on the rate of 2CP, phenol, benzoate, or 3CB biodegradation

Metal and concn (ppm)	%V[a] (acclimation time [days])[b]			
	2CP	Phenol	Benzoate	3CB
Cd(II)				
0.00[c]	100 (7)	100 (2)	100 (0)	100 (0)
0.01	118 (7)	101 (2)	185 (0)	83 (0)
0.1	124 (5)	138 (2)	127 (0)	100 (2)
0.5	74 (5)	80 (5)	110 (2)	0
1.0	59 (15)	29 (7)	35 (2)	
2.0	0	0	0	
Cr(VI)				
0.00[c]	100 (7)	100 (2)	100 (0)	100 (0)
0.01	123 (7)	177 (2)	169 (0)	93 (0)
0.1	98 (7)	106 (2)	131 (0)	126 (5)
0.5	76 (13)	70 (5)	60 (2)	0
1.0	41 (13)	68 (5)	58 (2)	
2.0	37 (13)	70 (5)	58 (2)	
5.0	0	0	0	
Cu(II)				
0.00[c]	100 (7)	100 (2)	100 (0)	100 (0)
0.01	168 (7)	101 (2)	109 (0)	96 (0)
0.1	90 (7)	102 (2)	91 (0)	93 (0)
0.5	71 (13)	44 (5)	112 (2)	98 (0)
1.0	64 (15)	31 (5)	0	74 (4)
2.0	0	0		23 (8)
5.0				0
Hg(II)				
0.0[c]	100 (3)	100 (5)	100 (3)	100 (0)
0.1	30 (6)	75 (3)	149 (3)	105 (6)
0.5	23 (10)	79 (3)	131 (5)	103 (6)
0.7		58 (0)		
1.0	133 (14)	0	71 (9)	105 (6)
1.7				154 (33)
2.0	142 (14)		0	69 (30)

[a] %V = degradation rate with added metals ÷ degradation rate in controls lacking metals × 100.
[b] Acclimation time is the time before biodegradation was observed.
[c] Control lacking added metal.

RESULTS

Determination of sublethal concentrations of heavy metal ions. Acclimation times and biodegradation rates for 2CP, phenol, benzoate, and 3CB in the presence of added metal ions are presented in Table 1. Biodegradation was not observed in any consortia with added metals at 25 to 100 ppm during a 30-day incubation, despite complete degradation in control consortia (data not shown). Acclimation times for 2CP, phenol, benzoate, and 3CB biodegradation in control consortia were 3 to 7, 2 to 5, 0 to 3, and 0 days, respectively. 2CP, phenol, benzoate, and 3CB biodegradation was complete in controls between 20 and 26, 5 and 14, 7 and 10, and 11 and 15 days, respectively. Consortia containing added metals and showing no biodegradation were incubated at least twice as long as it took for biodegradation to be completed in control consortia.

Cadmium. 3CB biodegradation was the most sensitive to added Cd(II). The acclimation time increased from 0 to 2 days at 0.1 ppm, and biodegradation was not observed at ≥0.5 ppm (Table 1). 2CP, phenol, and benzoate biodegradations were

similarly sensitive to added Cd(II) when acclimation time, metal concentration at which biodegradation rates started to decline, and concentration at which biodegradation failed to occur were considered. Acclimation times increased at 0.5 ppm by 2 and 3 days, respectively, for benzoate and phenol biodegradation and by 10 days at 1.0 ppm for 2CP biodegradation. Although biodegradation rates of 2CP and phenol declined to 80 to 29% of control rates from 0.5 to 1.0 ppm, benzoate biodegradation rates did not decline until 1.0 ppm. 2CP, phenol, and benzoate were similar in not being degraded at 2.0 ppm or greater. Interestingly, the presence of Cd(II) at 0.01 ppm increased benzoate degradation rates to 185% of the control rates.

Chromium. As with the addition of Cd(II), 3CB biodegradation was most sensitive to added Cr(VI). The acclimation time increased from 0 to 5 days at 0.1 ppm, and biodegradation also failed to occur at ≥0.5 ppm (Table 1). 2CP, phenol, and benzoate biodegradations showed similar sensitivities to added Cr(VI) but were less sensitive to added Cr(VI) than to added Cd(II). 2CP, phenol, and benzoate acclimation times increased by 6, 3, and 2 days, respectively, and biodegradation rates decreased to 76 to 37% of control rates from 0.5 to 2.0 ppm. Biodegradation of 2CP, phenol, and benzoate was not observed at 5.0 ppm. The presence of added Cr(VI) at 0.01 ppm increased the biodegradation rates of both phenol and benzoate to 177 and 169% of the control rates, respectively.

Copper. Benzoate biodegradation was most sensitive to added Cu(II), and biodegradations of 2CP and phenol were similarly sensitive. In contrast to Cd(II) and Cr(VI), 3CB biodegradation was less sensitive to added Cu(II) than was biodegradation of the other three compounds. Acclimation times for 2CP, phenol, and benzoate biodegradation increased by 6, 3, and 2 days, respectively, at 0.5 ppm. Benzoate biodegradation rates were not affected by 0.5 ppm, while 2CP and phenol biodegradation rates declined to 71 to 31% of the control rates between 0.5 and 1.0 ppm. However, benzoate biodegradation was not observed at 1.0 ppm, compared with 2.0 ppm for 2CP and phenol. Interestingly, adding Cu(II) at 0.01 ppm increased the biodegradation rate of 2CP to 168% of the control rate. The acclimation time for 3CB biodegradation increased from 0 to 4 and 8 days, respectively, at 1.0 and 2.0 ppm. Biodegradation rates declined between 1.0 and 2.0 ppm, while biodegradation was not observed at 5.0 ppm.

Mercury. Phenol biodegradation was most sensitive to added Hg(II), followed by benzoate biodegradation. Although phenol acclimation times decreased from 5 to 0 days between 0.1 and 0.7 ppm, biodegradation rates also decreased between these concentrations. Phenol was not degraded at 1.0 ppm. Acclimation times for benzoate biodegradation increased from 3 to 9 days between 0.1 and 1.0 ppm, but biodegradation rates did not decrease until 0.7 ppm. Benzoate biodegradation was not observed at 2.0 ppm.

The 2CP and 3CB biodegradation curves were unusual in the presence of added Hg(II). Between 0.1 and 0.5 ppm, acclimation times increased by 3 to 7 days for 2CP and the overall biodegradation rates were reduced by 70 to 80%. However, at these metal concentrations biodegradation rates actually increased over time. For example, the overall 2CP biodegradation rate at 0.5 ppm was 10.8 μM/day, but the rate was 6.8 μM/day for the first 37 days and then increased sharply to 32.7 μM/day until 2CP was completely degraded. Interestingly, at 1.0 and 2.0 ppm after a 14-day acclimation period, rapid onset of 2CP biodegradation was observed at linear rates that were 133 and 142% of the control rates, respectively. Although acclimation times for 3CB biodegradation increased from 0 to 6 days between 0.1 and 1.0 ppm, 3CB biodegradation rates were unaffected. However, after an extended acclimation period of 33 days at 1.7 ppm, 3CB biodegradation rates increased to 154% of the control rate, as had been observed for 2CP biodegradation. After an acclimation period of 30 days at 2.0 ppm, and in contrast to that of 2CP, the biodegradation rate of 3CB decreased to 69% of the control rate.

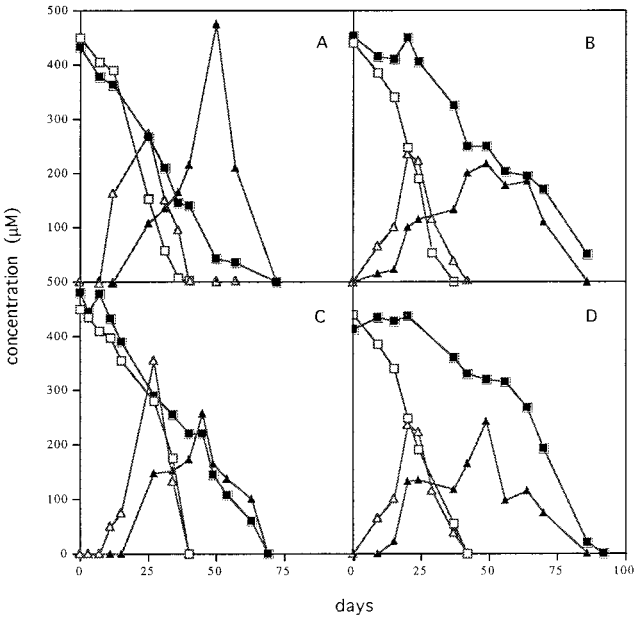

FIG. 1. Degradation of 2-CP (□) and formation and degradation of phenol (△) in the presence of a sublethal concentration of Cu(II) (1.2 ppm) (A), Cr(VI) (2.5 ppm) (B), Cd(II) (0.8 ppm) (C), or Hg(II) (0.3 ppm) (D). Open symbols represent control consortia, and closed symbols represent metal-amended consortia.

Effect of sublethal concentrations of metal ions. To study the effect of added heavy metals on biodegradative intermediates and end products, sublethal concentrations of each metal were selected which reduced the biodegradation rate of target compounds by approximately 50%.

2CP. 2CP dechlorination rates were 7.7 μM/day (Fig. 1A) with added Cu(II) at 1.2 ppm, or 18.9 μM, compared with 13.4 μM/day in controls, a decline of 42%. Phenol, the dechlorination product, accumulated stoichiometrically in the Cu(II)-amended consortia to 473 μM for the first 50 days, indicating little or no degradation of phenol. Phenol biodegradation was observed after 50 days (22.3 μM/day), which coincided with a sharp reduction in the 2CP dechlorination rate (2.0 μM/day). Both 2CP and phenol were completely degraded by 72 days in the Cu(II)-amended consortia. Phenol accumulated stoichiometrically to 266 μM in controls, but phenol biodegradation started on day 25 and was somewhat faster (27.7 μM/day) than in Cu(II)-amended consortia. Control consortia completely degraded 2CP and phenol in 40 days. There was no acclimation period for either control or Cu(II)-amended consortia.

Adding Cr(VI) to 2.5 ppm or 48.1 μM, had a marked effect on 2CP dechlorination rates, decreasing them by 65% to 4.6 μM/day, compared with 13.0 μM/day in control consortia (Fig. 1B). Phenol accumulated in metal-amended consortia for 49 days, followed by degradation at 10.9 μM/day, or 40% slower than in control consortia (27.5 μM/day), which accumulated phenol for only 20 days before phenol biodegradation was initiated. Degradation of 2CP and phenol was complete by 40 days in control consortia, compared with ≥87 days for Cr(VI)-amended consortia. There was no discernible acclimation pe-

TABLE 2. Effects of heavy metal ions on final methane ratios in 2CP, phenol, benzoate, and 3CB consortia

Metal	Consortium[a]			
	2CP	Phenol	Benzoate	3CB
Cd(II)	0.29[b] (7.1)[c]	0.34 (7.1)	0.76 (8.9)	0.25 (2.7)
Cr(VI)	0.65 (48.1)	0.38 (19)	0.97 (32.7)	0.77 (5.8)
Cu(II)	0.30 (18.9)	0.21 (7.9)	0.63 (9.4)	0.23 (15.7)
Hg(II)	0.94 (1.5)	0.52 (3.5)	0.03 (5.0)	0.32 (7.5)

[a] Phenol and benzoate consortia were derived from the 2CP consortium.
[b] Methane ratio = final [methane] with metals ÷ final [methane] in controls lacking metals.
[c] Added metal ion concentration (micromoles per liter).

riod in control consortia, but an acclimation period of 20 to 24 days was observed with metal-amended consortia.

In the presence of Cd(II) at 0.8 ppm, or 7.1 μM, the overall dechlorination rates in control and metal-amended consortia (Fig. 1C) were 11.0 and 7.3 μM/day, respectively, which is a 34% decrease. However, dechlorination rates in control consortia were similar to those of Cd(II)-amended consortia (6.9 μM/day) for the first 27 days and increased to 21.4 μM/day thereafter, while the rates in Cd(II)-amended consortia remained unchanged. Phenol accumulated in control consortia until day 27, after which it was rapidly degraded (48.7 μM/day). Thus, in control consortia the onset of phenol biodegradation coincided with an increase in 2CP dechlorination rates. Phenol accumulated for a longer period (45 days) in Cd(II)-amended consortia and was degraded at 17.3 μM/day thereafter, 64% more slowly than in controls. 2CP and phenol biodegradation was complete by 40 days in control consortia but took 75 days in Cd(II)-amended consortia. There was a 7-day acclimation period in metal-amended consortia but none in control consortia.

With Hg(II) at 0.3 ppm, or 1.5 μM, 2CP dechlorination was much slower than in control consortia. First, there was an acclimation period of at least 20 days in the Hg(II)-amended consortia but none in controls (Fig. 1D). Second, once initiated, the overall 2CP dechlorination rate in Hg(II)-amended consortia was 6.8 μM/day versus 11.0 μM/day in control consortia, a 38% decrease. However, in Hg(II)-amended consortia, the 2CP dechlorination rate was only 3.6 μM/day between 20 and 56 days and the rate increased to 10.2 μM/day between 56 and 86 days. Increasing dechlorination rates over time in the presence of Hg(II) were also observed in the initial study discussed above. Finally, 2CP dechlorination was complete by 40 days in control consortia but took 92 days in Hg(II)-amended consortia. Phenol, the dechlorination product, accumulated for 49 days in Hg(II)-amended consortia before being degraded at 14.1 μM/day, and degradation was complete by 86 days. In contrast, phenol accumulated for only 20 days in control consortia and was degraded at 23.3 μM/day, 65% faster than in Hg(II)-amended consortia. Degradation was complete by 40 days.

Benzoate, the transformation product of phenol in our consortia, was not detected in any of the 2CP control or metal-amended consortia, indicating rapid biodegradation of benzoate under all conditions. Acetate, the organic product of benzoate ring cleavage, was detected at somewhat higher concentrations in metal-amended consortia (138 to 536 μM) than in control consortia (0 to 274 μM) but was below the detection limit in all consortia by the time the target compound was completely degraded. The ratio of the concentration of methane, an end product of anaerobic biodegradation, in metal-amended to that in control consortia is presented in Table 2. Methane production was suppressed 71, 45, and 70%, respectively, in Cd(II)-, Cr(VI)-, and Cu(II)-amended 2CP consortia but was not strongly affected in Hg(II)-amended consortia (94%).

Phenol. A phenol consortium, derived from the 2CP consortium, was used to examine the effect of sublethal concentrations of added metals on phenol biodegradation when phenol was the initial substrate. At a sublethal concentration of Cd(II) of 0.8 ppm, or 7.1 μM, phenol concentrations decreased very slowly (9.0 μM/day) for the first 21 days but increasing degradation rates (26.0 to 80.0 μM/day) were observed for 36 days, when phenol was no longer detected. In contrast, control consortia had no acclimation period and biodegradation rates were much faster (102 μM/day) and phenol was no longer detected at 16 days (data not shown).

The addition of Cr(VI) at 1.0 ppm, or 19.2 μM, resulted in a phenol biodegradation rate of 19.8 μM/day for the first 12 days, followed by an increased rate of 80.4 μM/day until day 20, when phenol was no longer detected. Control consortia had a faster initial degradation rate (27.3 μM/day), and rates increased to 159.3 μM/day after 8 days. Phenol was below the detection limit at 12 days (data not shown).

Adding Cu(II) at 0.5 ppm, or 7.9 μM, resulted in an acclimation period of 13 days in which phenol concentrations remained unchanged. Once phenol biodegradation was initiated, its rate was 76.0 μM/day and degradation was complete by 25 days. In control consortia, phenol biodegradation started immediately, proceeding at 66.2 μM/day for 6 days and increasing to 93.6 μM/day thereafter, and phenol concentrations reached zero by 13 days (data not shown).

Adding Hg(II) at 0.7 ppm, or 3.5 μM, resulted in an acclimation period of 20 days during which phenol concentrations did not decline, but once phenol biodegradation was initiated, it proceeded at 84.3 μM/day. Phenol was biodegraded at 27.0 μM/day in control consortia for the first 8 days, after which the rate increased to 160.5 μM/day until day 12, when phenol was no longer detected.

Benzoate was not detected as an intermediate of phenol biodegradation in either control or metal-amended phenol consortia, indicating rapid benzoate turnover. Acetate was detected at higher concentrations in phenol consortia containing added metal ions (90 to 1,100 μM) than in control consortia (10 to 600 μM). The highest concentration of acetate was associated with Cu(II), and the lowest was associated with Cd(II). Acetate was subsequently degraded in all consortia. All four metal ions suppressed methane production in the phenol consortia (Table 2). Methane production was lowest (21%) in Cu(II)-amended consortia and highest (52%) in Hg(II)-amended consortia.

Benzoate. A benzoate consortium, derived from the 2CP consortium, was used to examine the effect of sublethal concentrations of added metals on benzoate biodegradation with benzoate as the initial substrate (data not shown). Sublethal concentrations of Cd(II) (1.0 ppm), Cr(VI) (1.7 ppm), Cu(II) (0.6 ppm), and Hg(II) (1.0 ppm), i.e., 8.9, 32.7, 9.4, and 5.0 μM, respectively, were added. Acclimation periods were shortest for Cr(VI) at 7 days and longest for Cd(II) at 28 days, with 17 and 20 days for Hg(II) and Cu(II), respectively. Acclimation was not observed in control consortia. Once biodegradation was initiated, its rate in the presence of added Cu(II) or Hg(II) was substantially slower (76.9 or 100.6 μM/day, respectively) than in control consortia (178.8 or 166.0 μM/day). In contrast, added Cd(II) or Cr(VI) had no effect on the benzoate biodegradation rate after the acclimation period. Acetate accumulated to relatively high concentrations (1.3 to 1.4 mM) in Cu(II)-, or Hg(II)-amended consortia, but it subsequently de-

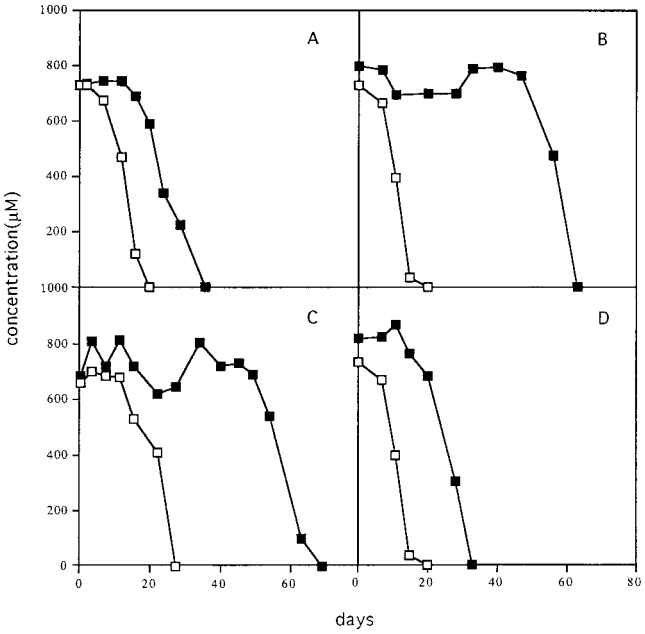

FIG. 2. Degradation of 3CB in the presence of a sublethal concentration of Cu(II) (1.0 ppm) (A), Cr(VI) (0.3 ppm) (B), Cd(II) (0.3 ppm) (C), or Hg(II) (1.5 ppm) (D). Open symbols represent control consortia, and closed symbols represent metal-amended consortia.

clined below the detection limit. There was little acetate accumulation in the presence of Cd(II) or Cr(VI). Methane production was strongly inhibited by added Hg(II), reducing the final methane yield to 3% of the control concentration, while added Cu(II) and Cd(II) reduced methane production to 63 and 76%, respectively (Table 2). Cr(VI) did not inhibit methane production from benzoate, as the methane yield was 97% of the control concentration.

3CB. With 3CB as the initial substrate, sublethal concentrations of metals were added to examine differences between effects on dechlorination of 3CB versus 2CP. Acclimation was observed in all 3CB control consortia, while none was observed in 2CP control consortia, indicating that 2CP was more readily dechlorinated than 3CB in the respective consortia. Acclimation periods for 3CB dechlorination were longest (47 days) with Cd(II) or Cr(VI) at 0.3 ppm, i.e., 2.7 and 5.8 μM, respectively (Fig. 2B and C), coinciding with the greater sensitivity of 3CB observed toward these metals in the initial study, and longer than the acclimation periods reported above for 2CP dechlorination in the presence of lower concentrations of these two metals. Acclimation periods for 3CB degradation were 11 to 12 days in the presence of Cu(II) at 1.0 ppm or Hg(II) at 1.5 ppm, i.e., 15.7 and 7.5 μM, respectively (Fig. 2A and D). A longer acclimation period (20 days) was reported for 2CP at a lower Hg(II) concentration, while no acclimation was observed for a similar concentration of Cu(II).

Once biodegradation started, the consortia amended with Cu(II), Hg(II), or Cr(VI) degraded 3CB at 57 to 59% (32 to 43 μM/day) of the control rate (55 to 78 μM/day). There was no difference between the overall degradation rates of Cd(II)-amended (37 μM/day) and control (39 μM/day) consortia, but the initial (days 12 to 23) rate (24.3 μM/day) was slower than the final (days 23 to 28) rate (80.8 μM/day). There was no accumulation of benzoate, the dechlorination product, in metal-amended or control consortia. The ring cleavage product, acetate, was detected at somewhat higher concentrations in consortia containing added Cu(II), Cr(VI), and Hg(II) than in control consortia (334 to 771 μM versus 176 to 190 μM). Added Cd(II) had no effect on acetate concentrations. Methane production was inhibited relatively strongly by Cu(II), Cd(II), and Hg(II), reducing yields to 23, 25, and 32% of the control concentrations, respectively (Table 2). Cr(VI) had a moderate effect, reducing the methane yield to 77%.

Nonbiological characteristics. The effect of adding heavy metals ions at 0.01 to 5.0 ppm on the redox potential of uninoculated anaerobic medium is presented in Fig. 3. All metal ions increased the E_h. Except for Cd(II), the major increase occurred between 0.01 and 1.0 ppm. Over this range, Hg(II) had the greatest effect, increasing the E_h from +30 to +150 mV, while Cd(II) had the least effect, raising the E_h to +45 mV. Overall, addition of Hg(II) resulted in the greatest change in E_h (+175 mV), while Cu(II) changed the E_h least (+50 mV). Cd(II) had little effect below 1.0 ppm, with a gradual increase to +120 mV as its concentration increased from 1.0 to 5.0 ppm. The pH of the medium was tested in selected cultures before and after growth in the presence and absence of the metal ions and was found to remain near 7.0 in all cases.

The anaerobic Bathocuproine assay revealed that Cu(II) at 0.1 to 5.0 ppm added to uninoculated anaerobic medium was readily reduced to Cu(I). Cr(VI) was reduced to Cr(III) in uninoculated anaerobic medium when present at ≤0.5 ppm, but amounts above this concentration remained as Cr(VI).

After degradation of the target compound and all intermediates was complete, the soluble concentration of the added metal was determined. In 2CP consortia, all metals were below the detection limits (1.2, 2.2, 0.3, and 0.2 ppb for Cu, Cd, Cr, and Hg, respectively). Cr was detected at 32.6 and 137.5 ppb in the 3CB and phenol consortia, respectively, but the other three metals were below the limits of detection. Cd, Cr, and Hg were detected in the benzoate consortium at 50.0, 113.0, and 51.6 ppb, respectively, while Cu was below the detection limit. These low concentrations did not allow differentiation of Cu(I) from Cu(II) or Cr(III) from Cr(VI) by the colorimetric assays described above.

DISCUSSION

Complete anaerobic biodegradation of 2CP (Fig. 4) involves the following steps: dechlorination of 2CP to phenol, transformation of phenol to benzoate (16, 35), and mineralization of benzoate to CO_2 and CH_4 (33) via acetate, H_2, and CO_2 by syntrophic bacterial species in association with methanogens

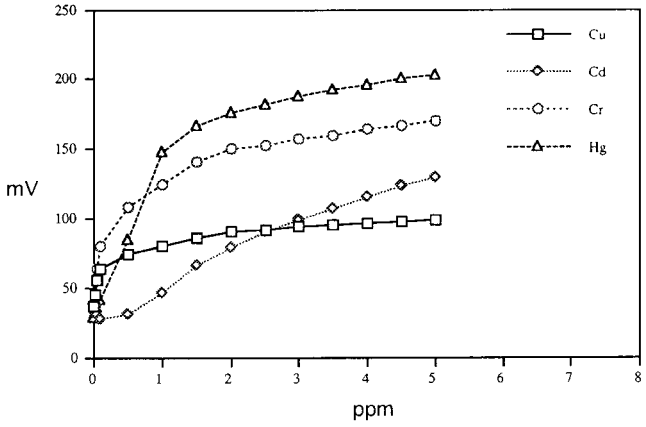

FIG. 3. Relationship between heavy metal ion concentration and redox potential in uninoculated anaerobic medium.

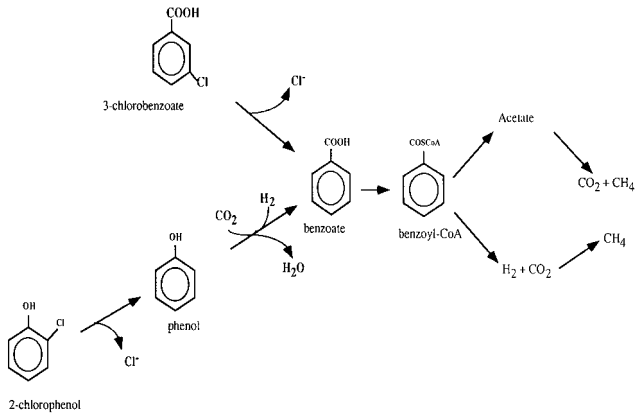

FIG. 4. Biodegradation pathway of 3CB and 2CP.

(26). 3CB is dechlorinated to benzoate and similarly degraded (6). Each step is performed by unique functional groups or species of anaerobic bacteria. Since the bioavailable concentration of an added metal ion can change with time, phenol- and benzoate-degrading consortia derived from the 2CP consortium allowed us to examine the effect of added heavy metals on metabolic intermediates of 2CP biodegradation. 2CP and 3CB consortia were used to compare the effects of selected heavy metals on two types of reductive dechlorination.

Three effects of added metal ions on dechlorination and biodegradation of 2CP and 3CB were observed, including extended acclimation periods, reduced dechlorination or biodegradation rates, and failure to dechlorinate or biodegrade the target compound. The concentration at which these effects were observed was characteristic of the metal ion added, the target compound being studied, and the consortium being used. On the basis of failure to dechlorinate the target compound, the 2CP consortium would be considered most sensitive to added Cd(II) or Cu(II). However, 2CP acclimation times increased and dechlorination rates decreased at much lower added Hg(II) concentrations, suggesting greater sensitivity. Higher concentrations of Hg(II) were associated with extended acclimation times followed by rapid degradation, suggesting adaptation to Hg(II), perhaps via removal or transformation of Hg(II) by mercury-resistant bacterial species (1). Dechlorination of 3CB was most sensitive to added Cr(VI) or Cd(II) and was more sensitive to added Cd(II) than was dechlorination of 2CP. 2CP and 3CB are dechlorinated by distinct bacterial species (3, 5, 21, 36); therefore, differences in metal sensitivity may be specific to the dechlorinating species or the dechlorinating enzymes themselves. Differences between bacterial species in the two consortia that are not directly involved in dechlorination but transform or otherwise remove the metal may also account for variations in metal sensitivity.

Considering the intermediate steps of 2CP biodegradation, phenol biodegradation was most sensitive to added Hg(II), while benzoate biodegradation was most sensitive to added Cu(II). As suggested above, either specific or general variations in the bacterial populations could account for differences in metal sensitivity between the phenol and benzoate consortia.

In some cases, addition of low concentrations of metal ions enhanced degradation rates. Synergism toward growth has been reported with combinations of low concentrations of certain toxic and nutritional metals (11, 35). Bacterial species that compete for limited reducing equivalents or nutritional factors in the consortium may be more sensitive to added metals than are species responsible for the degradation under study (2). Thus, their elimination may allow the degradation under study to proceed more rapidly than in controls. Since Cu(II) is a nutritional metal, its stimulatory effect could be the result of its presence at a more nearly optimal concentration.

Acclimation periods were observed in both control and metal-amended consortia, but extended acclimation periods were often observed above certain metal ion concentrations. In control consortia, acclimation may have resulted from the need for protein induction, enzyme synthesis, a lower redox potential, or proliferation of bacterial species responsible for production of reducing equivalence, nutritional factors, or the initial degradation step. Extended acclimation periods in metal-amended consortia may result from induction of a protein required for metal precipitation or detoxification, a genetic change, or selection of a tolerant or detoxifying bacterial species or population, resulting in precipitation and/or detoxification of added metal ions. Nonspecific binding of added metal ions to organics, inorganics, or increased biomass may also decrease bioavailability over time. Reduction of a higher initial redox potential may also be involved; however, no direct correlation between the E_h in metal-amended media (Fig. 3) and a relative increase in the acclimation period (Table 1) was observed. The longest acclimation times were associated with 2CP or 3CB dechlorination and added Hg(II). These were followed by a sudden onset of dechlorination at rates more rapid than in controls. Adaptation to Hg(II) has been reported previously (1) and could result from Hg(II) volatilization to Hg^0 by resistant bacterial species. Studies to investigate the basis for our observations are under way.

The only intermediate observed to build up at sublethal concentrations of added metals was phenol (Fig. 1) in the 2CP consortium with added Cu(II). On the basis of results obtained in the initial study, the concentration of Hg(II) added in the sublethal study should have inhibited phenol degradation in the 2CP consortium, while the Cr(VI) and Cd(II) added should have inhibited the degradation of both phenol and benzoate. Since this was not observed, it is possible that bioavailable metal ion concentrations were decreased with time by specific detoxification mechanisms or by nonspecific precipitation or binding to biomass, making them unavailable when metabolic intermediates were being produced. It is also possible that bacterial species responsible for detoxification or removal of these metal ions in the 2CP consortium were lost in deriving the phenol and benzoate consortia.

In almost all cases, the presence of added metal ions reduced methane yields, indicating that methanogenesis in our consortia was adversely affected by added metal ions. Many investigators have concluded that methanogens were most sensitive to added toxicants, while other bacterial groups were responsible for detoxification (13, 22, 27, 28, 37). Hickey et al. (9), in contrast, concluded that other trophic groups were more severely inhibited by a pulse addition of Cu(II), Zn(II), or Cd(II).

Our data have three significant implications. First, dechlorination and intermediate metabolic steps in the anaerobic mineralization of chlorinated aromatic compounds are differentially sensitive to various added heavy metal ions. Several studies have shown that heavy metals are bioavailable to anaerobic bacterial species and affect anaerobic processes, including methanogenesis and sulfate reduction in sediments (2), volatile fatty acid degradation (19), hydrogen formation and methanogenesis during anaerobic sludge digestion (8, 18), and methanogens in pure culture (10, 29). Dehalogenating anaerobic bacterial species often depend upon other anaerobes for

reducing equivalents for reductive dechlorination and upon anaerobic syntrophic bacterial species to remove the dechlorinated product. Syntrophic anaerobic bacterial species, in turn, depend upon methanogens, or other H_2-utilizing bacterial species, to remove hydrogen formed during degradation of an organic substrate, making degradation thermodynamically feasible. As a result of this interdependence, metals which affect methanogens, methanogenesis, syntrophic species, or hydrogen formation or utilization can have an indirect effect on dechlorination and degradation of halogenated aromatic compounds.

Second, some anaerobic bacterial species in our consortia may be resistant to or capable of transforming heavy metal ions. Since several anaerobic bacterial species have been shown to reduce and transform many different metals (4, 20), this aspect is currently under investigation in our laboratory.

Finally, not only must the presence of metals be determined at a potential bioremediation site as a means of predicting the outcome of anaerobic bioremediation of an organic pollutant, but there also exists the potential of using anaerobic bacterial species to bioremediate a site that is contaminated with heavy metals alone or using several anaerobic species to bioremediate both heavy metals and organic pollutants either sequentially or in combination.

ACKNOWLEDGMENTS

We thank Michael O'Keefe for performing volatile fatty acid analysis and Emil Lores for assistance in atomic absorption spectrophotometric analysis. We also acknowledge technical assistance from Heather Beck, Beat Blattmann, Maureen Downey, and Stephanie Friedman.

This research was supported by Office of Naval Research grant N00014-93-1-1222.

REFERENCES

1. **Barkay, T.** 1987. Adaptation of aquatic microbial communities to Hg^{2+} stress. Appl. Environ. Microbiol. **53:**2725–2732.
2. **Capone, D. G., D. D. Reese, and R. P. Kiene.** 1983. Effects of metals on methanogenesis, sulfate reduction, carbon dioxide evolution, and microbial biomass in anoxic salt marsh sediments. Appl. Environ. Microbiol. **45:**1586–1591.
3. **Cole, J. R., A. L. Cascarelli, W. W. Mohn, and J. M. Tiedje.** 1994. Isolation and characterization of a novel bacterium growing via reductive dehalogenation of 2-chlorophenol. Appl. Environ. Microbiol. **60:**3536–3542.
4. **Compeau, G. C., and R. Bartha.** 1985. Sulfate-reducing bacteria: principal methylators of mercury in anoxic estuarine sediment. Appl. Environ. Microbiol. **50:**498–502.
5. **DeWeerd, K. A., F. Concannon, and J. M. Suflita.** 1991. Relationship between hydrogen consumption, dehalogenation, and the reduction of sulfur oxyanions by *Desulfomonile tiedjei*. Appl. Environ. Microbiol. **57:**1929–1934.
6. **Dolfing, J., and J. M. Tiedje.** 1986. Hydrogen cycling in a three-tiered food web growing on the methanogenic conversion of 3-chlorobenzoate. FEMS Microbiol. Ecol. **38:**293–298.
7. **Gadd, G. M., and A. J. Griffiths.** 1978. Microorganisms and heavy metals. Microb. Ecol. **4:**303–317.
8. **Greenberg, A. E., L. S. Clesceri, and A. D. Eaton.** 1992. Standard methods for the examination of water and wastewater, 18th ed. American Public Health Association, Washington, D.C.
9. **Hickey, R. F., J. Vanderwielen, and M. S. Switzenbaum.** 1989. The effect of heavy metals on methane production and hydrogen and carbon monoxide levels during batch anaerobic sludge digestion. Water Res. **23:**207–218.
10. **Hobson, P. N., and B. G. Shaw.** 1976. Inhibition of methane production by *Methanobacterium formicicum*. Water Res. **10:**849–852.
11. **Hughes, M. N., and R. K. Poole.** 1989. Metal toxicity, p. 252–302. *In* M. N. Hughes and R. K. Poole (ed.), Metals and micro-organisms. Chapman & Hall, New York.
12. **Hughes, M. N., and R. K. Poole.** 1991. Metal speciation and microbial growth—the hard (and soft) facts. J. Gen. Microbiol. **137:**725–734.
13. **Johnson, L. D., and J. C. Young.** 1983. Inhibition of anaerobic digestion by organic priority pollutants. J. Water Pollut. Control Fed. **55:**1441–1449.
14. **Kawahara, F. K.** 1971. Gas chromatographic analysis of mercaptans, phenols and organic acids in surface waters with use of pentafluorobenzyl derivatives. Environ. Sci. Technol. **5:**235–239.
15. **Keith, L. H., and W. A. Telliard.** 1979. Priority pollutants. Environ. Sci. Technol. **13:**416–423.
16. **Knoll, G., and J. Winter.** 1987. Anaerobic degradation of phenol in sewage sludge: benzoate formation from phenol and CO_2 in the presence of hydrogen. Appl. Microbiol. Biotechnol. **25:**384–391.
17. **Kong, I.-C., D. A. Wubah, and W. J. Jones.** 1992. Effects of heavy metals on reductive dechlorination of chlorophenols in anoxic freshwater sediment, abstr. Q-195, p. 368. *In* Abstracts of the 92nd General Meeting of the American Society for Microbiology 1992. American Society for Microbiology, Washington, D.C.
18. **Kouzeli-Katsiri, A., N. Kartsonas, and A. Priftis.** 1988. Assessment of the toxicity of heavy metals to the anaerobic digestion of sewage sludge. Environ. Technol. Lett. **9:**261–270.
19. **Lin, C.-Y.** 1992. Effect of heavy metals on volatile fatty acid degradation in anaerobic digestion. Water Res. **26:**177–183.
20. **Lovley, D. R.** 1993. Dissimilatory metal reduction. Annu. Rev. Microbiol. **47:**263–290.
21. **Madsen, T., and D. Licht.** 1992. Isolation and characterization of an anaerobic chlorophenol-transforming bacterium. Appl. Environ. Microbiol. **58:**2874–2878.
22. **McCarthy, P. L., and C. A. McVath.** 1962. Volatile acid digestion at high loading rates. Int. J. Air Water Pollut. **6:**65–70.
23. **McInerney, M. J., and M. P. Bryant.** 1981. Basic principles of bioconversions in anaerobic digestion and methanogenesis, p. 277–295. *In* S. S. Sofer and O. R. Zaborsky (ed.), Biomass conversion processes for energy and fuels. Plenum Publishing Corp., New York.
24. **McInerney, M. J., M. P. Bryant, and N. Pfennig.** 1979. Anaerobic bacterium that degrades fatty acids in syntrophic association with methanogens. Arch. Microbiol. **122:**129–135.
25. **Mosey, F. E.** 1976. Assessment of the maximum concentration of heavy metals in crude sewage which will not inhibit the anaerobic digestion of sludge. Water Pollut. Control **75:**10–17.
26. **Mountfort, D. O., and M. P. Bryant.** 1982. Isolation and characterization of an anaerobic syntrophic benzoate-degrading bacterium from sewage sludge. Arch. Microbiol. **133:**249–256.
27. **O'Connor, O. A., R. Dewan, P. Galuzzi, and L. Y. Young.** 1990. Landfill leachate: a study of its anaerobic mineralization and toxicity to methanogenesis. Arch. Environ. Contamin. Toxicol. **19:**143–147.
28. **O'Connor, O. A., and L. Y. Young.** 1989. Toxicity and anaerobic biodegradability of substituted phenols under methanogenic conditions. Environ. Toxicol. Chem. **8:**853–862.
29. **Pankhania, I. P., and J. P. Robinson.** 1984. Heavy metal inhibition of methanogenesis by *Methanospirillum hungatei*. FEMS Microbiol. Lett. **22:**277–281.
30. **Selifonova, O., R. Burlage, and T. Barkay.** 1993. Bioluminescent sensors for detection of bioavailable Hg(II) in the environment. Appl. Environ. Microbiol. **59:**3083–3090.
31. **Sharak Genthner, B. R.** Unpublished data.
32. **Sharak Genthner, B. R., W. A. Price, and P. H. Pritchard.** 1989. Anaerobic degradation of chloroaromatic compounds in aquatic sediments under a variety of enrichment conditions. Appl. Environ. Microbiol. **55:**1466–1471.
33. **Sharak Genthner, B. R., W. A. Price, and P. H. Pritchard.** 1989. Characterization of anaerobic dechlorinating consortia derived from aquatic sediments. Appl. Environ. Microbiol. **55:**1472–1476.
34. **Sharak Genthner, B. R., G. T. Townsend, and P. J. Chapman.** 1989. Anaerobic transformation of phenol to benzoate via *para*-carboxylation: use of fluorinated analogues to elucidate the mechanism of transformation. Biochem. Biophys. Res. Commun. **162:**945–951.
35. **Sterritt, R. M., and J. N. Lester.** 1980. Interactions of heavy metals with bacteria. Sci. Total Environ. **14:**5–17.
36. **Stevens, T. O., T. G. Linkfield, and J. M. Tiedje.** 1988. Physiological characterization of strain DCB-1, a unique dehalogenating sulfidogenic bacterium. Appl. Environ. Microbiol. **54:**2938–2943.
37. **Yang, J., and R. E. Speece.** 1985. Effects of engineering controls on methane fermentation toxicity response. J. Water Pollut. Control Fed. **57:**1134–1141.