APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 1995, p. 953–958 Vol. 61, No. 3
0099-2240/95/$04.00+0

Benzene Oxidation Coupled to Sulfate Reduction

DEREK R. LOVLEY,* JOHN D. COATES, JOAN C. WOODWARD, AND ELIZABETH J. P. PHILLIPS
U.S. Geological Survey, Reston, Virginia 22092

Received 29 September 1994/Accepted 16 December 1994

Highly reduced sediments from San Diego Bay, Calif., that were incubated under strictly anaerobic conditions metabolized benzene within 55 days when they were exposed initially to 1 μM benzene. The rate of benzene metabolism increased as benzene was added back to the benzene-adapted sediments. When a [^{14}C]benzene tracer was included with the benzene added to benzene-adapted sediments, 92% of the added radioactivity was recovered as $^{14}CO_2$. Molybdate, an inhibitor of sulfate reduction, inhibited benzene uptake and production of $^{14}CO_2$ from [^{14}C]benzene. Benzene metabolism stopped when the sediments became sulfate depleted, and benzene uptake resumed when sulfate was added again. The stoichiometry of benzene uptake and sulfate reduction was consistent with the hypothesis that sulfate was the principal electron acceptor for benzene oxidation. Isotope trapping experiments performed with [^{14}C]benzene revealed that there was no production of such potential extracellular intermediates of benzene oxidation as phenol, benzoate, *p*-hydroxybenzoate, cyclohexane, catechol, and acetate. The results demonstrate that benzene can be oxidized in the absence of O_2, with sulfate serving as the electron acceptor, and suggest that some sulfate reducers are capable of completely oxidizing benzene to carbon dioxide without the production of extracellular intermediates. Although anaerobic benzene oxidation coupled to chelated Fe(III) has been documented previously, the study reported here provides the first example of a natural sediment compound that can serve as an electron acceptor for anaerobic benzene oxidation.

Anaerobic degradation of benzene is of interest because many of the sedimentary environments that are contaminated with this toxic compound are anoxic. Other monoaromatic hydrocarbons, such as toluene and xylenes, are known to be degraded under nitrate-reducing (2, 5, 8–10, 15, 18, 30), Fe(III)-reducing (20, 21), sulfate-reducing (3, 6, 9, 14, 28), and/or methanogenic (7, 13, 32, 34) conditions. However, in only a few studies has anaerobic metabolism of benzene been documented unequivocally.

Partial mineralization of benzene to carbon dioxide and methane in the absence of molecular oxygen has been observed in enrichment cultures (13) and methanogenic river sediments (31). Studies performed with $H_2{}^{18}O$ revealed that in enrichment cultures, the oxygen from water was incorporated into benzene as a hydroxyl group with the formation of phenol (32). More than 70% of the benzene added to methanogenic aquifer material contaminated with landfill leachate disappeared within 40 weeks (34), but the fate of the benzene was not determined.

Nitrate-dependent consumption of benzene under anoxic conditions has been observed in aquifer sediments (26). However, subsequent studies, including some studies performed with material from the same aquifer in which benzene uptake was observed initially, indicated that benzene was not degraded under anaerobic conditions when nitrate was the potential electron acceptor (1, 2, 9, 15, 16, 18).

Benzene was completely mineralized to carbon dioxide in enrichment cultures in which sulfate was provided as a potential electron acceptor (6). However, the electron acceptor for benzene oxidation was not determined in that study.

Anaerobic oxidation of benzene coupled to a known electron acceptor has been documented only recently (25). Addition of the Fe(III) chelator nitrilotriacetic acid stimulated benzene oxidation in the anoxic, Fe(III) reduction zone of a petroleum-contaminated aquifer (25). The enhanced benzene degradation was associated with stimulation of microbial Fe(III) reduction. Stoichiometric studies demonstrated that the electrons derived from benzene oxidation to carbon dioxide were transferred to Fe(III). Benzene oxidation coupled to Fe(III) reduction was not observed in the absence of the chelator.

As part of a study on the decomposition of petroleum-related contaminants in harbor sediments, we investigated potential mechanisms for benzene oxidation under sulfate-reducing conditions. Our results demonstrate that benzene was oxidized to carbon dioxide in the highly reduced sediments and suggest that benzene oxidation was carried out by sulfate reducers which completely oxidize the benzene to carbon dioxide without the formation of extracellular intermediates.

MATERIALS AND METHODS

Sediments. Sediments were collected from San Diego Bay, Calif., in a pleasure boat harbor known as Shelter Island. The water depth was 10 m. Hydrocarbon contamination of the surface water was apparent visually. Although organic analyses of the sediments were not performed, we assumed that the sediments were also contaminated with organic compounds because, compared with the brown surficial sediments obtained from more pristine areas of the bay, the surficial sediments at this site were extremely black and rich in sulfides. Sediments were collected with a gravity corer that had a core liner which was 5 cm in diameter. The top 2.5 cm of each sediment core was sliced off onboard ship and was placed in canning jars under N_2. Once the jars had been filled to the top, they were sealed with lids. These sediment samples were used in the initial experiments in which the sediments were adapted to metabolize added benzene.

So that all of the benzene metabolism experiments could be performed with a uniform benzene-adapted sediment sample, the sediment from 2.5 to 10 cm in each core was also collected and transported as described above, as were surficial sediment samples obtained with an Eckman dredge. These two sets of sediment samples were pooled in the laboratory and, as described below, were inoculated with benzene-adapted sediments and used for all experiments performed after those whose results are summarized in Fig. 1.

Sediment incubation. As previously described (22), strict anaerobic conditions were used in handling the sediment samples. Sulfate-free artificial bay water was formulated on the basis of the results of an analysis of the pore water and contained (per liter) 20 g of NaCl, 1.5 g of KCl, 4.24 g of $MgCl_2 \cdot 2H_2O$, and

* Corresponding author. Mailing address: U.S. Geological Survey, 430 National Center, Reston, VA 22092. Phone: (703) 648-5825. Fax: (703) 649-5484. Electronic mail address: dlovley@stress.er.usgs.gov.

2.9 g of $CaCl_2 \cdot 2H_2O$. The artificial bay water was boiled under an N_2-CO_2 (80:20) atmosphere and then was mixed with the sediment samples (50:50, vol/vol) under anaerobic conditions in a glove bag that had an N_2-H_2-CO_2 (85:10:5) atmosphere. On the benchtop the sediment slurries were flushed with N_2-CO_2. The slurries prepared with sediments from the top 2.5 cm of the cores were dispensed (10 ml) under an N_2-CO_2 atmosphere into 25-ml serum bottles. These sediments were also amended with 10 mM sulfate as described below. The slurries prepared with the combined sediments from depths of 2.5 to 10 cm and the grab samples were dispensed (100 ml) into 160-ml serum bottles. The bottles were sealed with thick butyl rubber stoppers. Killed controls were generated by autoclaving sediments at 121°C for 1 h on 3 consecutive days.

Toluene and benzene were added to the sediments from anoxic aqueous solutions (typically, 25 to 100 μl of solutions was added to serum bottles containing sediment) in order to provide the desired concentrations. When necessary, sodium molybdate and/or sodium sulfate was added from concentrated anoxic stock solutions in order to provide a final molybdate concentration of 20 mM or the desired concentration of sulfate.

The bottles were incubated upside down at 20°C in the dark. Uptake of toluene and benzene was monitored by sampling the headspace with a syringe and needle and analyzing the samples by gas chromatography as described below.

[^{14}C]benzene studies. In radiotracer experiments, 0.22 μCi of [^{14}C]benzene (63.2 mCi/mmol, diluted in sterile anoxic water to provide ca. 1 μCi/ml) was injected with a syringe and needle. Production of $^{14}CO_2$ over time was monitored by withdrawing headspace samples (1 ml) and analyzing them for $^{14}CO_2$ by gas proportional counting as described below.

In order to study the production of potential extracellular intermediates, 10 ml of benzene-adapted sediments was incubated in anaerobic pressure tubes. The sediment samples were amended with 0.1 ml of anoxic stock solutions containing potential intermediates to increase the concentrations of the potential intermediates as follows: acetate, 1 mM; benzoate, 500 μM; *p*-hydroxybenzoate, 200 μM; phenol, 10 μM; cyclohexane, 100 μM; or catechol, 10 μM. These concentrations were chosen in order to increase the concentrations of the potential intermediates at least 10-fold compared with the expected in situ concentrations. Production of $^{14}CO_2$ from [^{14}C]benzene was monitored over time. In order to determine if radiolabel accumulated in the potential intermediates, the sediment samples were acidified with 1 ml of 5 N sulfuric acid to stop metabolism and to convert inorganic carbon to carbon dioxide. The headspace was flushed with N_2 for 20 min with mixing for 30 s with a vortex mixer every 5 min during the flushing procedure. Once the $^{14}CO_2$ had been flushed out, pore water was collected from a subsample (3 ml) of the sediment by centrifugation. The pore water was filtered (pore diameter, 0.2 μm; Acrodisc filter), and the levels of radioactivity in the potential organic intermediates were determined by radiochromatography as described below.

Stoichiometry of benzene oxidation and sulfate reduction. The amount of sulfate reduced during benzene oxidation was determined by using two large bottles containing benzene-adapted sediments which had been incubated to remove all of the benzene. The sulfate concentration was adjusted with sodium sulfate so that the initial concentration was ca. 13 mM. Subsamples (3 ml) were centrifuged to collect the pore water and were analyzed to determine the sulfate concentrations as described below. Approximately 10 μl of benzene was added directly through the stopper with a microsyringe. The weight of the added benzene was determined by weighing three consecutive 10-μl injections of benzene on a balance, injecting 10 μl of benzene into the sediment samples, and then weighing three more 10-μl injections of benzene on the balance. The value used for the amount of benzene injected into each sediment sample was the mean of the values obtained for the six samples of benzene that were weighed with the balance. The standard error of the weighed samples of benzene was less than 8%. The benzene content of the headspace was monitored over time. As soon as the added benzene was depleted (11 days), the sediments were sampled again to determine sulfate concentrations. The sediment samples were incubated for 11 days without added benzene to determine the amount of sulfate reduction in the sediment that could be attributed to electron donors other than benzene. This experiment was then repeated with the same two bottles of sediment in the same manner, except that the incubation period was 10 days because the benzene was depleted 1 day sooner.

Analytical techniques. The toluene and benzene concentrations in 100-μl headspace samples were determined with capillary gas chromatography and a flame ionization detector. The standard error of the mean of the hydrocarbon determinations was 5%. The amounts of toluene and benzene added to the sediment samples from the anoxic aqueous stock solutions were estimated from the concentrations measured in the headspace and the partitioning of toluene and benzene between the sediment and the headspace when known volumes of pure toluene and benzene were added to the sediments. The toluene and benzene concentrations were calculated per liter of pore water, and, because of slight differences in the water contents of the sediment samples and difficulties in determining actual sediment volumes in some instances, these concentrations must be considered approximate. Therefore, the data are presented below as percentages of the initial concentrations in the headspace, which are much more direct and accurate reflections of the actual toluene and benzene measurements.

Sulfate concentrations were determined by ion chromatography as previously described (24). The concentrations of dissolved sulfide were determined by filtering (pore diameter, 0.2 μm; Acrodisc filter) pore water in the anaerobic glove bag and assaying the preparation for sulfide by the methylene blue method (4). The amount of Fe(II) that could be extracted with 0.5 N HCl was determined by the ferrozine method as previously described (22).

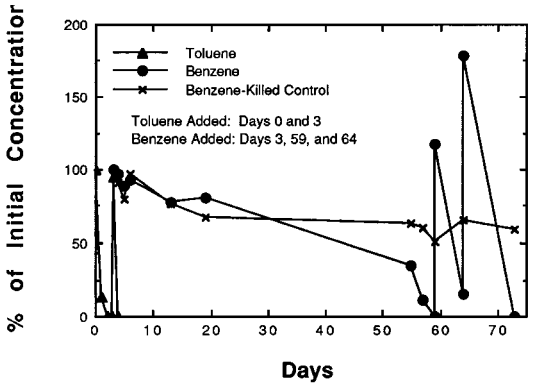

FIG. 1. Toluene and benzene uptake in San Diego Bay sediments. The results shown are the results obtained after a 14-day preincubation period during which the 20 μM toluene which was initially added was completely consumed. The initial concentrations of toluene and benzene were ca. 20 and 1 μM, respectively. The results shown are the results obtained from one representative bottle for each treatment.

Headspace samples were analyzed for $^{14}CO_2$ by separating CO_2 from other gases with a gas chromatograph equipped with a thermal conductivity detector and passing the effluent from the detector through a gas proportional counter. Total production of ^{14}C-labeled inorganic carbon was calculated from the distribution of $^{14}CO_2$ in the sediment and the headspace, as determined by adding $H^{14}CO_3$ standards to the sediment samples. The validity of this method for calculating total ^{14}C-labeled inorganic carbon contents was confirmed by the results of studies in which the total amount of ^{14}C-labeled inorganic carbon was determined by this method and then the sediment samples were acidified to convert all of the ^{14}C-labeled inorganic carbon to $^{14}CO_2$ and the $^{14}CO_2$ was flushed out, trapped, and counted as previously described (21).

As previously described (23), potential organic acid intermediates were separated and quantified by high-performance liquid chromatography (HPLC), and fractions were counted by liquid scintillation counting. The same technique was used to analyze the phenol and catechol fractions, except that these components were separated on a HPLC column designed to separate aromatic compounds, as previously described (21). Cyclohexane was not quantified, and accumulation of radiolabel in cyclohexane was not measured because a suitable method for separating and quantifying cyclohexane was not available.

RESULTS

Adaptation to benzene. Sulfate reduction was the terminal electron-accepting process in the sediments as there were high concentrations of Fe(II) but no production of Fe(II) over time and oxidation of [2-^{14}C]acetate to $^{14}CO_2$ was inhibited by molybdate (19). Toluene added at an initial concentration of ca. 20 μM completely disappeared after 14 days of incubation (data not shown). When additional toluene was added, it was rapidly metabolized (Fig. 1). When benzene (initial concentration, ca. 1 μM) was added along with the toluene, the toluene was again rapidly degraded, but there was no significant decrease in the amount of benzene for the first 19 days of incubation. However, by day 55 more than one-half of the benzene had disappeared. In contrast, there was no significant decrease in the amount of benzene in autoclaved controls between days 19 and 55. Once benzene was depleted after 59 days of incubation, benzene was again added to the live sediments, and benzene was metabolized more rapidly (Fig. 1). After benzene was added to the sediments three times, the sediments were used to inoculate a larger serum bottle (160 ml) containing 100 ml of San Diego Bay sediment slurry. This inoculated sediment continued to consume benzene without a lag period, and after benzene was added three times, it was used to inoculate 10 more large serum bottles containing sediment (inoculum, ca. 10%), which were used in the experiments described below.

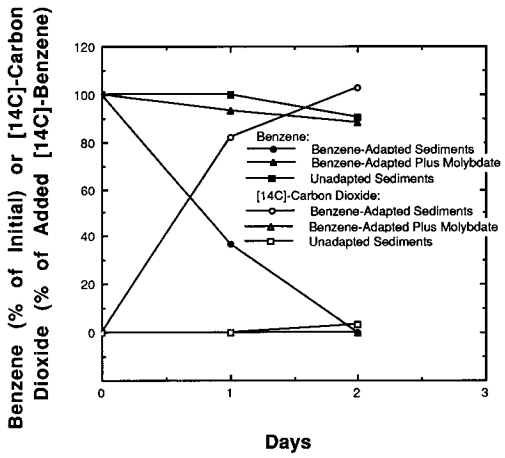

FIG. 2. Uptake of unlabeled benzene and production of $^{14}CO_2$ from [^{14}C]benzene. Benzene uptake and $^{14}CO_2$ production were measured in separate, parallel incubations. When molybdate was added, the sediments were preincubated with molybdate for 1 h before [^{14}C]benzene was added. The benzene concentration at the start of the experiment was ca. 1.5 μM. The data are the means of the values from triplicate incubations for each treatment.

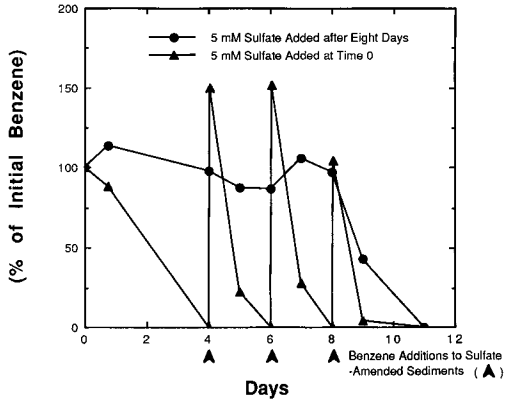

FIG. 3. Benzene uptake in sulfate-depleted sediments and sulfate-depleted sediments amended with 5 mM sulfate. Benzene was added to benzene-depleted sulfate-amended sediments at the times indicated by the arrowheads on the *x* axis. Sulfate (5 mM) was added to both types of sediments on day 8. The initial concentration of benzene was ca. 125 μM. The results shown are the results obtained from one bottle for each treatment.

Benzene oxidation to carbon dioxide linked to sulfate reduction. Aliquots (10 ml) were removed from one of the large bottles containing benzene-adapted sediments and placed into 25-ml serum bottles. These sediments contained 39 mmol of Fe(II) per kg of sediment and 40 μM free dissolved sulfide, demonstrating that there was no O_2 available for benzene oxidation. Benzene (ca. 3 μM) was added to these sediments, and once one-half the added benzene had been consumed, [^{14}C]benzene was added to one-half of the bottles in order to determine the fate of the benzene that was disappearing. There was steady production of $^{14}CO_2$ over time which coincided with the continued loss of benzene that was measured in the bottles to which [^{14}C]benzene was not added (Fig. 2). In all of the experiments in which the recovery of $^{14}CO_2$ from [^{14}C]benzene was evaluated, 92% ± 10% (n = 9) of the added [^{14}C]benzene was converted to $^{14}CO_2$. The addition of molybdate completely inhibited further benzene uptake in the benzene-adapted sediments, as well as production of $^{14}CO_2$ from [^{14}C]benzene (Fig. 2). In sediments that were not preadapted for benzene uptake, there was no benzene consumption and no production of $^{14}CO_2$ (Fig. 2).

After repeated long-term feeding of the benzene-adapted sediments in the large serum bottles with 10 to 100 μM benzene, benzene metabolism eventually stopped. An analysis of the sulfate in these sediments revealed that the sulfate concentration was less than 100 μM. The sediments from one of these sulfate-depleted bottles were then divided and placed into two serum bottles, and ca. 125 μM benzene was added. When sulfate was added to the sediments, benzene metabolism resumed immediately (Fig. 3). There was no benzene uptake in sediments to which sulfate was not initially added, but these sediments began to consume benzene once sulfate was added (Fig. 3).

After adaptation, the sediments could metabolize relatively high concentrations (ca. 1.7 mM) of added benzene (Table 1). Measurements of sulfate concentrations immediately before benzene was added and as soon as the benzene was depleted demonstrated that there was significant sulfate depletion during benzene metabolism (Table 1). The extent of sulfate reduction was much less during a subsequent incubation of the same duration without added benzene (Table 1). Addition of more benzene to the sediments stimulated sulfate reduction. When the amount of benzene-dependent sulfate reduction was calculated by subtracting the endogenous rate of sulfate reduction in the benzene-depleted sediments from the rate of sulfate reduction observed in the presence of benzene, the amount of sulfate reduction that could be attributed to benzene oxidation (Table 1) was 80% ± 5% (n = 4) of the theoretically expected amount if all of the added benzene had been completely oxidized to carbon dioxide according to the following formula: 4 benzene + 15 SO_4^{2-} + 12 $H_2O \rightarrow$ 24 HCO_3^- + 15 HS^- + 9 H^+.

Lack of extracellular intermediates. Addition of phenol, the most likely extracellular intermediate in anaerobic benzene oxidation (12), did not inhibit the production of $^{14}CO_2$ from [^{14}C]benzene, and there was no accumulation of [^{14}C]phenol over time (Fig. 4). The phenol which was added was not significantly metabolized during the incubation (Fig. 4), indicating that the lack of effect on $^{14}CO_2$ production was not due to rapid metabolism of the isotope trap. These results suggest that phenol was not an extracellular intermediate in benzene metabolism.

Addition of other potential extracellular intermediates, such as benzoate, *p*-hydroxybenzoate, cyclohexane, and acetate, also had no effect on production of $^{14}CO_2$ from [^{14}C]benzene (Fig. 5A). Cyclohexane concentrations were not monitored, but none of the other potential extracellular traps was significantly metabolized during the incubation (Fig. 5B). After 12 h of incubation, less than 2% of the radiolabel added had accumulated in any of the potential intermediates. Thus, these compounds were also not important extracellular intermediates in benzene metabolism.

The potential for production of catechol, a central intermediate in the metabolism of benzene under aerobic conditions, was also investigated in a separate experiment. Catechol was also not an extracellular intermediate in the sediment samples as added catechol had no effect on $^{14}CO_2$ production from [^{14}C]benzene in the anoxic San Diego Bay sediments (Fig. 6). Radiolabel had not accumulated in the catechol pool at the end of incubation, despite the fact that none of the 11 μM catechol present at the start of incubation was consumed.

TABLE 1. Stoichiometry of benzene uptake and sulfate reduction in benzene-adapted harbor sediments

Bottle[a]	Amt of benzene added (μmol)	Amt of sulfate reduction expected for complete oxidation of all added benzene to CO_2 (μmol)[b]	Amt of sulfate reduced in the presence of benzene (μmol)	Amt of sulfate reduced in the absence of benzene (μmol)	% Expected benzene-dependent sulfate reduction observed[c]
1A	113	422	342	25	75
1B	111	415	394	50	83
2A	113	422	405	41	86
2B	112	419	374	53	77

[a] Bottles which contained a known volume of sediment (80 to 100 ml) and in which the initial sulfate concentration was ca. 13 mM were incubated for the 11 days that it took for benzene to be depleted (bottles 1A and 2A), and then sulfate reduction was monitored for an equivalent period of time without added benzene. Then sulfate was added to the sediment samples so that the sulfate concentration was again ca. 13 mM, and a similar incubation experiment was performed, except that the incubation period was only 10 days (bottles 1B and 2B) because the benzene was depleted 1 day sooner in this experiment. The sediments contained 65% water.

[b] Calculated by assuming that 3.75 mol of sulfate was reduced per mol of benzene completely oxidized to carbon dioxide according to the equation given in the text.

[c] Calculated as follows: [(amount of sulfate reduced in the presence of benzene) − (amount of sulfate reduced in a subsequent experiment of the same length in the absence of benzene)]/(amount of sulfate reduction expected after complete oxidation of all added benzene to carbon dioxide) × 100.

DISCUSSION

The results demonstrate that, after an adaptation period, benzene was readily oxidized to carbon dioxide in anoxic San Diego Bay sediments. As described below, the results also indicate that sulfate was the electron acceptor for benzene oxidation in these sediments. Although others have hypothesized previously that sulfate might serve as the electron acceptor for benzene oxidation under anoxic conditions (6), our results provide the first documentation of this process.

Only one other study has identified an electron acceptor for anoxic benzene oxidation (25). In that study the electron acceptor was Fe(III). However, benzene oxidation was observed only when the Fe(III) in the sediments was artificially chelated with nitrilotriacetic acid (25) or EDTA (19). No benzene oxidation was observed with naturally occurring sediment Fe(III) forms. Thus, sulfate is the first electron acceptor present in its natural form that has been shown to function in anaerobic benzene oxidation.

Sulfate is an electron acceptor for benzene oxidation. It is clear that O_2 could not have been involved in the benzene oxidation described above. Strict anaerobic techniques were used throughout this study. Furthermore, the high concentrations of Fe(II) and sulfide in the sediments would have rapidly consumed any trace O_2 contamination.

More evidence that O_2 was not involved in benzene metabolism was the fact that molybdate immediately inhibited both benzene uptake and $^{14}CO_2$ production from [^{14}C]benzene. Molybdate has been shown repeatedly to be an effective, selective inhibitor of sulfate reduction in sediments (27) and is an effective inhibitor of organic carbon oxidation by sulfate reducers in San Diego Bay sediments (19). We would not expect molybdate to immediately inhibit benzene uptake if, for example, the initial activation of benzene in the sediments depended on trace contamination by oxygen. The lack of production of extracellular catechol, a central intermediate in activation of benzene with molecular O_2 (11), is also not consistent with initial activation of benzene by traces of O_2.

The fact that molybdate inhibited benzene uptake indicates that sulfate reduction was necessary for benzene metabolism, as does the sulfate requirement for benzene uptake. The cen-

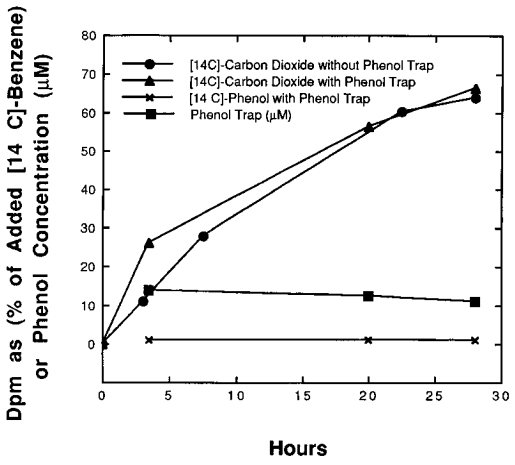

FIG. 4. Production of $^{14}CO_2$ from [^{14}C]benzene in the presence or absence of added phenol and lack of accumulation of radiolabel in the phenol pool over time. The data are the means of the values from duplicate incubations.

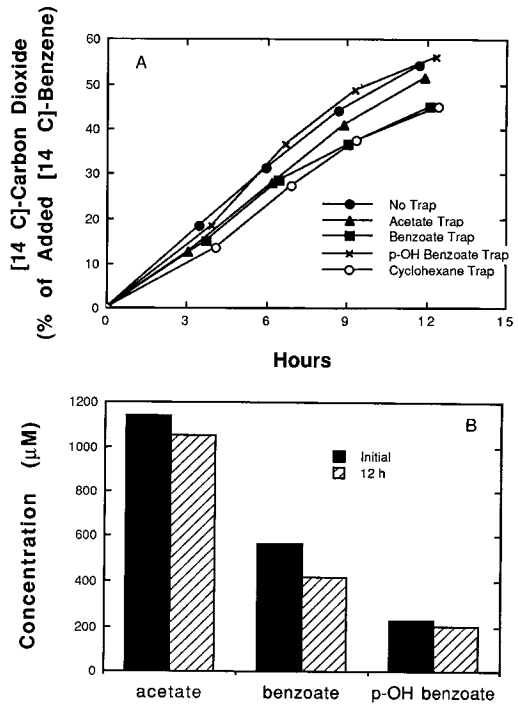

FIG. 5. Production of $^{14}CO_2$ from [^{14}C]benzene over time (A) and initial and final concentrations of added traps (B) when various potential extracellular intermediates of anaerobic benzene oxidation were added to the sediments. The data are the means of the values from duplicate incubations.

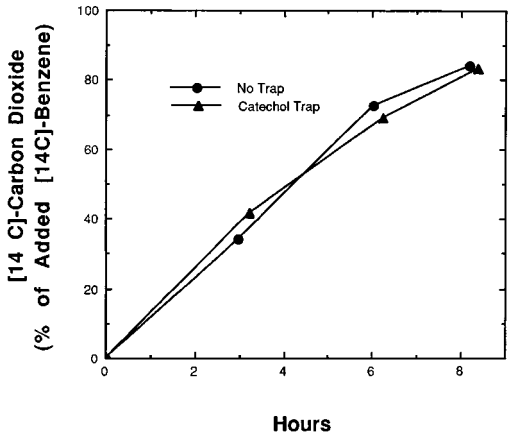

FIG. 6. Production of $^{14}CO_2$ from [^{14}C]benzene over time in the presence of catechol. The data are the means of the values from duplicate incubations.

tral role of sulfate reduction in benzene metabolism was further illustrated by the fact that sulfate was depleted during benzene metabolism. When corrections were made for endogenous sulfate reduction due to electron donors other than benzene, the extent of benzene-dependent sulfate depletion was 80% of the theoretically expected sulfate reduction if all of the added benzene had been oxidized to carbon dioxide. The studies performed with [^{14}C]benzene demonstrated that 8% ± 10% of the added benzene was not oxidized to carbon dioxide. When this is considered, it is apparent that, within the errors of the measurements, sulfate was the electron acceptor for most, if not all, of the benzene oxidation. The benzene that was not oxidized to carbon dioxide probably included benzene that was incorporated into cells, as well as possibly some small amount of benzene that was adsorbed to the butyl rubber stoppers or the sediments.

Pathway for benzene oxidation. There are several potential pathways for anaerobic metabolism of benzene, including hydroxylation to produce phenol (12), carboxylation to produce benzoate, and initial reduction of the ring to form cyclohexane. None of these potential intermediates was an important extracellular intermediate in the San Diego Bay sediments. Furthermore, acetate, an expected key intermediate if benzene were first fermented to organic acids, was not produced. These results suggest that benzene may be directly oxidized to carbon dioxide within single cells of sulfate-reducing microorganisms. This would be consistent with the finding that pure cultures of sulfate reducers which can metabolize toluene (28) and other monoaromatic compounds (33) oxidize these compounds completely to carbon dioxide. The direct oxidation of benzene to carbon dioxide under sulfate-reducing conditions contrasts with benzene metabolism under methanogenic conditions, in which extracellular production of phenol, cyclohexanone, and propionate occurs (13). If, in fact, benzene is directly oxidized to carbon dioxide within single cells of sulfate reducers, then the organisms responsible for benzene oxidation could be isolated much more easily than if a consortium of organisms were responsible for benzene oxidation.

In summary, the highly reducing conditions of the sediments, the immediate inhibition of benzene uptake by molybdate, the sulfate requirement for benzene uptake, the reduction of amounts of sulfate consistent with benzene oxidation, and the lack of production of partially oxidized intermediates rule out the possibility that O_2 was involved in benzene oxidation and suggest that sulfate reducers were directly oxidizing benzene to carbon dioxide in these sediments. The use of naturally occurring sulfate as an electron acceptor for benzene oxidation in this study contrasts with our previous studies on benzene oxidation coupled to Fe(III) reduction (25), in which the availability of Fe(III) had to be artificially modified with a chelator before benzene oxidation took place. Enrichment cultures containing benzene as the sole electron donor and sulfate as the electron acceptor have been established from the benzene-adapted sediments, but attempts to isolate a pure culture have not been successful yet. The fact that benzene can be oxidized to carbon dioxide in the absence of molecular O_2 in laboratory incubations and yet persists in most anoxic environments (17, 29) demonstrates that it will be necessary to study the factors that limit anaerobic benzene degradation in situ.

ACKNOWLEDGMENTS

We thank Bart Chadwick for help in collecting the sediment samples.

This research was supported by grant N00014-93-F-0103 from the Office of Naval Research and by the U.S. Geological Survey National Research Program.

REFERENCES

1. **Anid, P. J., P. J. J. Alvarez, and T. M. Vogel.** 1993. Biodegradation of monoaromatic hydrocarbons in aquifer columns amended with hydrogen peroxide and nitrate. Water Res. **27:**685–691.
2. **Barbaro, J. R., J. F. Barker, L. A. Lemon, and C. I. Mayfield.** 1992. Biotransformation of BTEX under anaerobic denitrifying conditions: field and laboratory observations. J. Contam. Hydrol. **11:**245–272.
3. **Beller, H. R., D. Grbic-Galic, and M. Reinhard.** 1992. Microbial degradation of toluene under sulfate-reducing conditions and the influence of iron on the process. Appl. Environ. Microbiol. **58:**786–793.
4. **Cline, J. D.** 1969. Spectrophotometric determination of hydrogen sulfide in natural waters. Limnol. Oceanogr. **14:**454–458.
5. **Dolfing, J., J. Zeyer, P. Binder-Eicher, and R. P. Schwarzenbach.** 1990. Isolation and characterization of a bacterium that mineralizes toluene in the absence of molecular oxygen. Arch. Microbiol. **134:**336–341.
6. **Edwards, E. A., and D. Grbic-Galic.** 1992. Complete mineralization of benzene by aquifer microorganisms under strictly anaerobic conditions. Appl. Environ. Microbiol. **58:**2663–2666.
7. **Edwards, E. A., and D. Grbic-Galic.** 1994. Anaerobic degradation of toluene and *o*-xylene by a methanogenic consortium. Appl. Environ. Microbiol. **60:** 313–322.
8. **Evans, P. J., D. T. Mang, K. S. Kim, and L. Y. Young.** 1991. Anaerobic degradation of toluene by a denitrifying bacterium. Appl. Environ. Microbiol. **57:**1139–1145.
9. **Flyvbjerg, J., E. Arivn, B. K. Jensen, and S. K. Olsen.** 1993. Microbial degradation of phenols and aromatic hydrocarbons in creosote-contaminated groundwater under nitrate-reducing conditions. J. Contam. Hydrol. **12:**133–150.
10. **Fries, M. R., J. Zhou, J. Chee-Sanford, and J. M. Tiedje.** 1994. Isolation, characterization, and distribution of denitrifying toluene degraders from a variety of habitats. Appl. Environ. Microbiol. **60:**2802–2810.
11. **Gottschalk, G., and H.-J. Knackmuss.** 1993. Bacteria and the biodegradation of chemicals achieved naturally, by combination, or by construction. Angew. Chem. Int. Ed. Engl. **32:**1398–1408.
12. **Grbic-Galic, D.** 1990. Anaerobic microbial transformation of nonoxygenated aromatic and alicyclic compounds in soil, subsurface, and freshwater sediments. Soil Biochem. **6:**117–189.
13. **Grbic-Galic, D., and T. Vogel.** 1987. Transformation of toluene and benzene by mixed methanogenic cultures. Appl. Environ. Microbiol. **53:**254–260.
14. **Haag, F., M. Reinhard, and P. L. McCarty.** 1991. Degradation of toluene and *p*-xylene in anaerobic microcosms: evidence for sulfate as a terminal electron acceptor. Environ. Toxicol. Chem. **10:**1379–1389.
15. **Hutchins, S. R.** 1991. Optimizing BTEX biodegradation under denitrifying conditions. Environ. Toxicol. Chem. **10:**1437–1448.
16. **Hutchins, S. R., G. W. Sewell, D. A. Kovacs, and G. A. Smith.** 1991. Biodegradation of aromatic hydrocarbons by aquifer microorganisms under denitrifying conditions. Environ. Sci. Technol. **25:**68–76.
17. **Juttner, F.** 1988. Benzene in the anoxic hypolimnion of a freshwater lake. Naturwissenschaften **75:**151–153.
18. **Kuhn, E. P., J. Zeyer, P. Eicher, and R. P. Schwarzenbach.** 1988. Anaerobic degradation of alkylated benzenes in denitrifying laboratory aquifer columns. Appl. Environ. Microbiol. **54:**490–496.
19. **Lovley, D. R.** 1994. Unpublished data.

20. **Lovley, D. R., M. J. Baedecker, D. J. Lonergan, I. M. Cozzarelli, E. J. P. Phillips, and D. I. Siegel.** 1989. Oxidation of aromatic contaminants coupled to microbial iron reduction. Nature (London) **339:**297–299.
21. **Lovley, D. R., and D. J. Lonergan.** 1990. Anaerobic oxidation of toluene, phenol, and *p*-cresol by the dissimilatory iron-reducing organism GS-15. Appl. Environ. Microbiol. **56:**1858–1864.
22. **Lovley, D. R., and E. J. P. Phillips.** 1986. Organic matter mineralization with reduction of ferric iron in anaerobic sediments. Appl. Environ. Microbiol. **51:**683–689.
23. **Lovley, D. R., and E. J. P. Phillips.** 1989. Requirement for a microbial consortium to completely oxidize glucose in Fe(III)-reducing sediments. Appl. Environ. Microbiol. **55:**3234–3236.
24. **Lovley, D. R., and E. J. P. Phillips.** 1994. Novel processes for anoxic sulfate production from elemental sulfur by sulfate-reducing bacteria. Appl. Environ. Microbiol. **60:**2394–2399.
25. **Lovley, D. R., J. C. Woodward, and F. H. Chapelle.** 1994. Stimulated anoxic biodegradation of aromatic hydrocarbons using Fe(III) ligands. Nature (London) **370:**128–131.
26. **Major, D. W., C. I. Mayfield, and J. F. Barker.** 1988. Biotransformation of benzene by denitrification in aquifer sand. Ground Water **26:**8–14.
27. **Oremland, R. S., and D. G. Capone.** 1988. Use of "specific" inhibitors in biogeochemistry and microbial ecology. Adv. Microb. Ecol. **10:**285–383.
28. **Rabus, R., R. Nordhaus, W. Ludwig, and F. Widdel.** 1993. Complete oxidation of toluene under strictly anoxic conditions by a new sulfate-reducing bacterium. Appl. Environ. Microbiol. **59:**1444–1451.
29. **Salanitro, J. P.** 1993. The role of bioattenuation in the management of aromatic hydrocarbon plumes in aquifers. Ground Water Monit. Remed. **13:**150–161.
30. **Schocher, R. J., B. Seyfried, F. Vazquez, and J. Zeyer.** 1991. Anaerobic degradation of toluene by pure cultures of dentrifying bacteria. Arch. Microbiol. **157:**7–12.
31. **Van Beelen, P., and F. Van Keulen.** 1990. The kinetics of the degradation of chloroform and benzene in anaerobic sediment from the River Rhine. Hydrobiol. Bull. **24:**13–21.
32. **Vogel, T. M., and D. Grbic-Galic.** 1986. Incorporation of oxygen from water into toluene and benzene during anaerobic fermentative transformation. Appl. Environ. Microbiol. **52:**200–202.
33. **Widdel, F., and F. Bak.** 1992. Gram-negative mesophilic sulfate-reducing bacteria, p. 3352–3378. *In* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K.-H. Schleifer (ed.), The prokaryotes, 2nd ed. Springer-Verlag, New York.
34. **Wilson, B. H., G. B. Smith, and J. F. Rees.** 1986. Biotransformations of selected alkylbenzenes and halogenated aliphatic hydrocarbons in methanogenic aquifer material: a microcosm study. Environ. Sci. Technol. **20:**997–1002.