

# Anaerobic Hydrocarbon Degradation in Petroleum-Contaminated Harbor Sediments under Sulfate-Reducing and Artificially Imposed Iron-Reducing Conditions

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The potential use of iron(III) oxide to stimulate in-situ hydrocarbon degradation in anaerobic petroleum-contaminated harbor sediments was investigated. Previous studies have indicated that Fe(III)-reducing bacteria (FeRB) can oxidize some electron donors more effectively than sulfate-reducing bacteria (SRB). In contrast to previous results in freshwater sediments, the addition of Fe(III) to marine sediments from San Diego Bay, CA did not switch the terminal electron-accepting process (TEAP) from sulfate reduction to Fe(III) reduction. Addition of Fe(III) also did not stimulate anaerobic hydrocarbon oxidation. Exposure of the sediment to air [to reoxidize Fe(II) to Fe(III)] followed by anaerobic incubation of the sediments, resulted in Fe(III) reduction as the TEAP, but contaminant degradation was not stimulated and in some instances was inhibited. The difference in the ability of FeRB to compete with the SRB in the different sediment treatments was related to relative population sizes. Although the addition of Fe(III) did not stimulate hydrocarbon degradation, the results presented here as well as other recent studies demonstrate that there may be significant anaerobic hydrocarbon degradation under sulfate-reducing conditions in harbor sediments.

## Introduction

A potential option for stimulating the in-situ degradation of organic contaminants in anaerobic sedimentary environments is to add electron acceptors to provide microorganisms with a more energetically favorable mechanism for hydrocarbon oxidation. This approach is most common

in the bioremediation of petroleum-contaminated aquifers, in which O<sub>2</sub> is added to stimulate the degradation of aromatic hydrocarbons (1).

The low solubility and high volatility of O<sub>2</sub> limits its usefulness as an electron acceptor that could be added in order to stimulate the in-situ degradation of aromatic hydrocarbons in petroleum-contaminated marine sediments, such as those found at the bottom of many harbors. Nitrate, which has been proposed as an alternative to O<sub>2</sub> for bioremediation of petroleum-contaminated aquifers (2), would also be difficult to effectively add to bottom sediments.

It is becoming increasingly apparent that Fe(III) reduction is an important process for the degradation of naturally occurring organic matter and organic contaminants in a wide variety of sedimentary environments (3, 4). The predominate form of microbially reducible Fe(III) in most soils and sediments is poorly crystalline iron(III) oxides (3). These iron(III) oxides are highly insoluble and could be readily added to bottom sediments and, once added, could be expected to remain there.

Previous studies have suggested that Fe(III)-reducing bacteria (FeRB) can more effectively metabolize some electron donors than sulfate-reducing bacteria (SRB). When Fe(III) was added to freshwater aquatic sediments in which sulfate reduction was the terminal electron-accepting process (TEAP), electron flow was diverted from sulfate reduction to Fe(III) reduction (5). This was because FeRB in the sediments were able to metabolize important electron donors such as acetate and H<sub>2</sub> down to lower concentrations than SRB and to maintain the concentrations of acetate and H<sub>2</sub> so low that the SRB were not able to utilize them (5, 6).

In petroleum-contaminated marine harbor sediments, sulfate reduction is often the predominant TEAP because O<sub>2</sub>, nitrate, and Fe(III) have been depleted due to the high rates of microbial metabolism. We hypothesized that the addition of iron(III) oxides to such sediments would stimulate the degradation of petroleum-related hydrocarbons because, as with acetate or H<sub>2</sub>, FeRB would be able to metabolize hydrocarbons more effectively than SRB. However, the results presented here demonstrate that this approach will probably not be effective because (1) in contrast to previous results with freshwater sediments, the addition of Fe(III) to sulfate-reducing marine sediments does not necessarily switch the TEAP to Fe(III) reduction and (2) as suggested from recent studies with pure cultures (7-9) and sediments (10, 11), SRB are quite effective in metabolizing a number of hydrocarbons in marine sediments.

## Materials and Methods

**Sediments and Sediment Incubation.** Sediments used in this study were collected from the Shelter Island site in the north of San Diego Bay as outlined before (10, 11). Sediments were visually black with a strong sulfide odor. Canning jars were filled with grab samples taken from a

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depth of 10 m and sealed under a headspace of N<sub>2</sub> gas. Samples were transported directly back to the laboratory and used immediately for the outlined experiments. All sediments were handled using strict anaerobic procedures described previously (12). Sediment studies were carried out in 35-mL serum vials with 30 g of slurried sediment (1:1, v/v) prepared with seawater collected from just above the surficial sediment layers in San Diego Bay. Molybdate (20 mM) was added from a sterile anaerobic stock as necessary. Air-oxidized sediment was prepared by bubbling 1.0 L of slurried sediment with lab air through a bubbling stone while continuously mixing on a magnetic stirrer over a 5-day period. Volume losses due to evaporation were made up with water. After oxidation, the sediment was flushed out with N<sub>2</sub>-CO<sub>2</sub> (95/5) and dispensed.

TEAP determinations for the sediments were done using <sup>14</sup>C-labeled acetate (56.8 mCi/mmol) as previously outlined (10). The ability of the sediments to oxidize aromatic hydrocarbons was determined by amending sediment samples with [<sup>14</sup>C]toluene (53.2 mCi/mmol), [<sup>14</sup>C]benzene (63.2 mCi/mmol), or [<sup>14</sup>C]naphthalene (8.9 mCi/mmol) and following the production of [<sup>14</sup>C]CO<sub>2</sub> by gas proportional counting as described previously (10, 11). Radiolabeled toluene and benzene were added to give a total of 1 μCi <sup>14</sup>C from stocks prepared with sterile anoxic water to provide 1 μCi/mL (10). Radiolabeled naphthalene was prepared as a methanolic solution to provide a final activity of 60 μCi/mL, and 25-μL quantities were added to clean dry serum bottles. The methanol was allowed to evaporate, and 30 g of sediment slurry was added to the bottles under N<sub>2</sub>-CO<sub>2</sub> gas stream.

**Most Probable Number Counts.** Numbers of dissimilatory FeRB and SRB were determined by three-tube most probable number counts with 10 mM acetate as the electron donor. The Fe(III)-reducing medium contained 150 mmol/L amorphous iron(III) oxide as the electron acceptor as outlined previously (13). The sulfate-reducing medium contained 4.0 g/L Na<sub>2</sub>SO<sub>4</sub> as the electron acceptor. In addition, both media contained (per liter) 1.36 g of sodium acetate, 20 g of NaCl, 3.0 g of MgCl<sub>2</sub>·H<sub>2</sub>O, 0.15 g of CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.25 g of NH<sub>4</sub>Cl, 0.20 g of KH<sub>2</sub>PO<sub>4</sub>, 0.50 g of KCl, 1.0 mL of trace metal solution outlined by Widdel and Bak (14), 1.0 mL of selenite-tungstate solution (0.4 g/L NaOH, 6 mg/L Na<sub>2</sub>SeO<sub>3</sub>·5H<sub>2</sub>O, 8 mg/L Na<sub>2</sub>WO<sub>4</sub>·2H<sub>2</sub>O), and 1.0 mL of resazurin solution (0.1 g/100 mL). The medium was boiled, degassed, and cooled under N<sub>2</sub>-CO<sub>2</sub> (80/20). Aliquots (9 mL) were dispensed into pressure tubes and sealed under N<sub>2</sub>-CO<sub>2</sub> with thick butyl rubber stoppers. After being autoclaved, 0.2 mL of an anoxic filter sterilized vitamin/NaHCO<sub>3</sub> solution was added to each tube. The vitamin/NaHCO<sub>3</sub> solution contained (per liter) 5 mg of pyridoxine hydrochloride, 2.5 mg of riboflavin, 2.5 mg of calcium pantothenate, 2.5 mg of *p*-aminobenzoic acid, 2.5 mg of thioctic acid, 2.5 mg of nicotinic acid, 1 mg of biotin, 1 mg of folic acid, 2.5 mg of vitamin B<sub>12</sub>, and 125 g of NaHCO<sub>3</sub>. Just prior to inoculation 0.1 mL of an anoxic autoclaved reductant solution was added to each tube [Na<sub>2</sub>S·9H<sub>2</sub>O (4.8 g/100 mL) or FeCl<sub>2</sub>·4H<sub>2</sub>O (5.0 g/100 mL) for the SRB and FeRB, respectively]. This gave final reductant concentrations of 2 and 2.5 mM for the SRB and FeRB media respectively. All incubations were at 20 °C for 90 days. Positive tubes were determined by assaying for sulfide or Fe(II) in the sulfate-reducing and Fe(III)-reducing tubes, respectively.

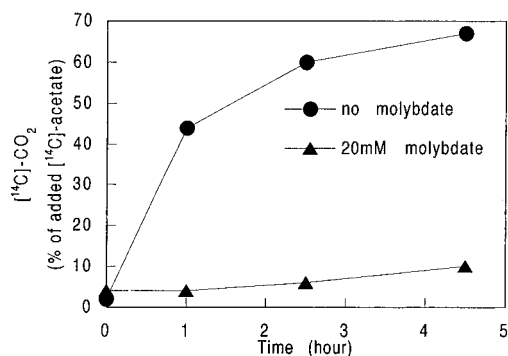


FIGURE 1. Molybdate inhibition of the oxidation of [<sup>14</sup>C]acetate by Shelter Island sediments. The results are the means of triplicate incubations.

**Cultures.** *Desulfuromonas palmitatis* was isolated from San Diego Bay sediments and maintained on previously described medium (15) with acetate (10 mM) as the electron donor and iron(III) citrate (50 mM) as the electron acceptor. Sediment microcosms were amended with 1.0 mL of an active cell suspension. The cell suspension was prepared by centrifuging and twice washing 400 mL of an acetate/iron(III) citrate grown culture with an anoxic bicarbonate buffer under N<sub>2</sub>-CO<sub>2</sub> (80/20). The buffer contained (per liter) 2.50 g of NaHCO<sub>3</sub>, 20 g of NaCl, and 4.24 g of MgCl<sub>2</sub>·6H<sub>2</sub>O. The final cell pellet was resuspended in 10 mL of the bicarbonate buffer.

**Analytical Methods.** Acid-soluble Fe(II) in the sediment was determined by digesting 0.1 g of sediment slurry for 1 h in 5 mL of HCl (0.5 N). A 100-mL sample of the sediment digests was then analyzed for Fe(II) content by the ferrozine assay as previously described (12). Total iron determinations were made on the same sediment digests by adding 200 μL of a 6.25 N hydroxylamine stock and further digesting for 1 h prior to Fe(II) analyses by the ferrozine assay. Sulfate and nitrate concentrations were determined by ion chromatography as previously described (11, 16). Soluble sulfide concentrations were determined by the methylene blue assay as previously outlined (17). [<sup>14</sup>C]CO<sub>2</sub> concentrations in 1.0-mL headspace samples were analyzed by gas proportional counting detection after separation by gas chromatography as previously outlined (10).

## Results and Discussion

**Sediment Selection and Characterization.** Shelter Island, a pleasure boat dock in the north of San Diego Bay, has been heavily contaminated with hydrocarbons for the last 30 years due to the activity of motorboat enthusiasts. Previous studies in our lab have indicated an active hydrocarbon-oxidizing, sulfate-reducing microbial population in the sediment (10, 11). Sediments collected from this site were highly reduced containing in excess of 60 mM/kg Fe(II). Sediments readily oxidized [<sup>14</sup>C]acetate to [<sup>14</sup>C]CO<sub>2</sub> (Figure 1) under strictly anaerobic conditions. The addition of molybdate (20 mM), a specific inhibitor of sulfate-reducing bacteria (18), resulted in the complete inhibition of [<sup>14</sup>C]CO<sub>2</sub> evolution (Figure 1), indicating that TEAP in the sediment samples was sulfate reduction.

**Effect of Added Fe(III) on TEAP and Hydrocarbon Oxidation.** As might be expected from a previous study that demonstrated rapid anaerobic uptake of toluene added to sediments from the same site (10), [*ring*-<sup>14</sup>C]toluene was readily oxidized to [<sup>14</sup>C]CO<sub>2</sub> in unamended Shelter Island

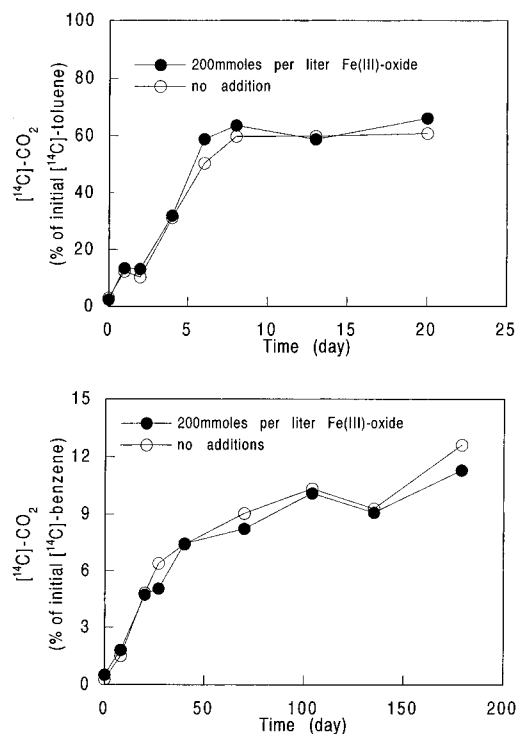


FIGURE 2. Oxidation of [<sup>14</sup>C]toluene (a) and [<sup>14</sup>C]benzene (b) by sediments from the Shelter Island site in the presence and absence of 200 mmol/L iron(III) oxide. The results are the means of triplicate incubations.

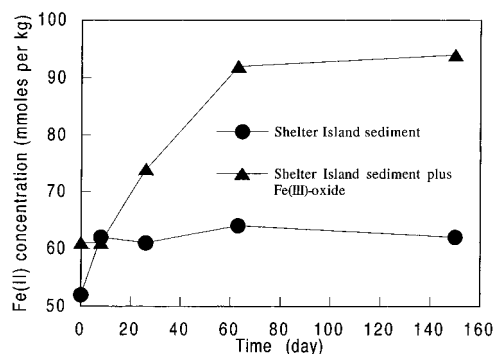


FIGURE 3. HCl-extractable Fe(II) content of the iron(III) oxide amended and unamended Shelter Island sediments during the course of the experimental incubations. The results are the means of triplicate incubations.

sediments (Figure 2). The addition of iron(III) oxide had no influence on the rate of toluene oxidation (Figure 2a).

Previous studies demonstrated that benzene was oxidized in these sediments with sulfate serving as the electron acceptor (10). As expected from previous results on the loss of unlabeled benzene over time (10), the rate of benzene oxidation was much slower than that for toluene (Figure 2b). As with toluene, the addition of Fe(III) had little effect on benzene degradation (Figure 2b). In similar studies, addition of Fe(III) also had no influence on the rate of hexadecane oxidation (19).

The lack of effect of added Fe(III) was not due to rapid consumption of all of the added Fe(III). Fe(III) persisted in the sediments and continued to be reduced to Fe(II) during the course of the incubations (Figure 3). Even after 21 days of incubation, over 30% of the added Fe(III) could still be recovered from the Fe(III)-amended sediments. In contrast to previous studies in which the addition of Fe(III) to sulfate-reducing sediments switched the TEAP from

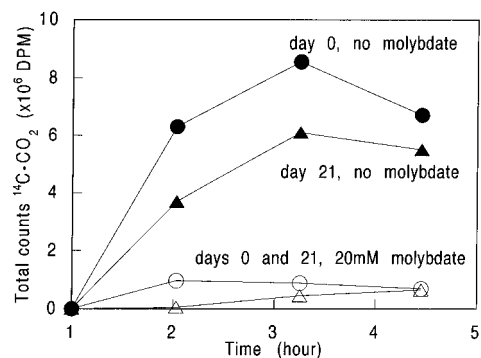


FIGURE 4. Molybdate inhibition of [<sup>14</sup>C]acetate oxidation in Shelter Island sediments after preincubation in the presence of 200 mmol/L iron(III) oxide. Circles are samples with no preincubation; triangles are samples that were preincubated for 21 days.

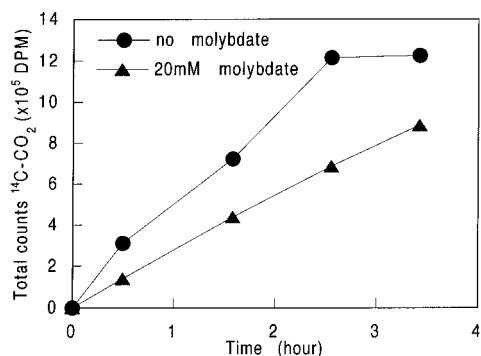


FIGURE 5. Oxidation of [<sup>14</sup>C]acetate by Shelter Island sediment amended with the marine Fe(III)-reducer *Desulfuromonas palmitatis*.

sulfate reduction to Fe(III) reduction, sulfate reduction remained the TEAP in Fe(III)-amended San Diego Bay sediments as evidenced by the fact that molybdate continued to inhibit [2-<sup>14</sup>C]acetate oxidation (Figure 4). Furthermore, the H<sub>2</sub> partial pressure in the Fe(III)-amended sediment ranged from 1 to 2 × 10<sup>-6</sup> atm, which is typical for a sulfate-reducing environment and an order of magnitude higher than the H<sub>2</sub> found in sediments in which Fe(III) reduction is the TEAP (5, 20).

MPN estimates of acetate-oxidizing SRB and FeRB suggested that the inability of the FeRB to outcompete sulfate reducers when Fe(III) was added to the sediment was due to the low numbers of FeRB in the sediments. There was only 9.33 ± 4.17 × 10<sup>2</sup> acetate-oxidizing FeRB/g of sediment, which was 3 orders of magnitude less than the 9.33 ± 4.17 × 10<sup>5</sup> of acetate-oxidizing SRB.

To determine if increasing the size of the acetate-oxidizing FeRB population would permit the FeRB to outcompete the SRB for acetate, a cell suspension of the marine acetate-oxidizing FeRB, *D. palmitatis*, was added to the sediment. This organism was previously isolated from San Diego Bay sediments (15). With the addition of 4.72 × 10<sup>6</sup> cells of *D. palmitatis* per gram to Fe(III)-amended sediment, the molybdate inhibition of [2-<sup>14</sup>C]acetate oxidation was overcome (Figure 5). These results further suggest that the natural population of FeRB in the Shelter Island sediments was just too low to significantly compete for electron donors in the sediment and, when the FeRB numbers were increased to levels similar to the SRB, Fe(III) reduction became the predominant TEAP.

Laboratory studies with a toluene-oxidizing sulfate-reducing enrichment culture have suggested that the addition of Fe(III) need not necessarily change the TEAP

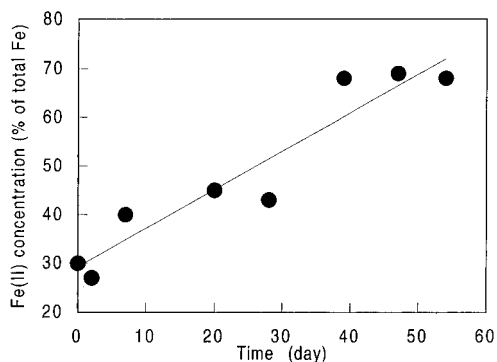


FIGURE 6. Production of Fe(II) in the air-oxidized sediments during incubation. Results are the means of triplicate determinations.

from sulfate reduction to Fe(III) reduction in order to stimulate contaminant degradation (21, 22). In that instance, the stimulation of toluene degradation was suggested to be the result of Fe(II) removing toxic sulfide from solution. However, those results were obtained with media in which there was no other significant sink for sulfide. In the San Diego Bay sediments, which naturally contain high concentrations of Fe(II) (61 mM/kg of sediment), exogenous Fe(III) is probably not necessary to maintain sulfide at low levels. In fact, the Shelter Island pore water soluble sulfide concentration was only 1.24  $\mu$ mol/L whereas the acid volatile sulfide concentration of the sediment was 20 mM/kg of sediment.

**Effect of O<sub>2</sub> Exposure on Anaerobic TEAPs and Hydrocarbon Degradation.** The major source of Fe(III) in many marine and estuarine sediments is reoxidation of Fe(II) by O<sub>2</sub> as the mixing of anaerobic zones with oxygenated water (23, 24). Such mixing may be the result of bioturbation or physical resuspension. Petroleum-contaminated sediments dredged from contaminated harbors may also be extensively mixed with air causing the oxidation of Fe(II) to Fe(III) in aerobic bioremediation strategies.

In order to generate Fe(III) from air oxidation of Fe(II), sediments were mixed under air until greater than 75% of the total iron had been converted to Fe(III). No significant pH variation was observed in the oxidized sediment (data not shown). When these sediments were incubated under anaerobic conditions, there was a steady production of Fe(II) over time as the result of Fe(III) reduction (Figure 6). In contrast to the sediments that had not been air oxidized (Figure 4), there was significant oxidation of [2-<sup>14</sup>C]acetate in anaerobic incubations of the air-oxidized sediments in the presence of 20 mM MoO<sub>4</sub> (data not shown). Nitrate was not detected in these sediments (detection limit 200 nM), and the presence of Fe(II) in the sediment demonstrates that there was no microbially reducible Mn(IV) because Fe(II) rapidly reduces Mn(IV) in sediments (25). Thus, the results suggest that Fe(III) reduction was the predominant TEAP in the sediments.

The finding that Fe(III) reduction was an important process when Fe(III) was generated via air oxidation but that the addition of poorly crystalline iron(III) oxide did not stimulate Fe(III) reduction is unlikely to be due to differences in the availability of the two types of Fe(III) for microbial reduction. The synthetic poorly crystalline iron(III) oxide is readily reducible by a wide variety of FeRB (13, 15, 26–29).

A more likely explanation is that FeRB were better able to compete with SRB in the air-oxidized sediments. The

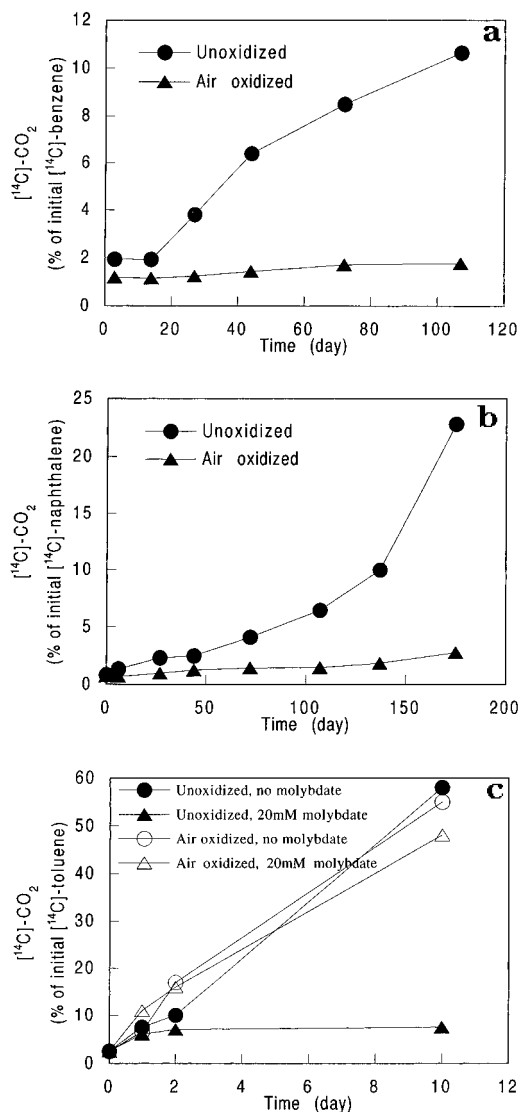


FIGURE 7. Oxidation of <sup>14</sup>C-labeled (a) benzene, (b) naphthalene, and (c) toluene in the air-oxidized and unoxidized Shelter Island sediments. The results are the means of triplicate incubations.

MPN estimate of acetate-oxidizing SRB in the air oxidized sediment was  $2.33 \pm 1.33 \times 10^1$  cells/g of sediment, or over 4 orders of magnitude lower than in the unoxidized sediments. The MPN estimate of acetate-oxidizing FeRB was  $4.27 \pm 2.14 \times 10^1$  cells/g, which was only 20-fold lower than in the unoxidized sediment. Thus, air oxidation preferentially eliminated SRB from the sediments. The greater relative importance of FeRB in the air-oxidized sediments would be expected to improve their potential for outcompeting SRB for electron donors.

The development of Fe(III)-reducing conditions in the air-oxidized sediment did not enhance contaminant degradation over that observed in the unoxidized sediment. There was no benzene oxidation in the air-oxidized sediments incubated under anaerobic conditions whereas in a subset of the sediments that had not been oxidized, benzene oxidation occurred a rate similar to that observed previously (Figure 7A). The lack of benzene oxidation under Fe(III)-reducing conditions is consistent with previous studies with aquifer sediments that indicated that benzene was only oxidized with the reduction of Fe(III) if Fe(III) chelators were added to enhance the availability of Fe(III) for reduction (30, 31).

Similarly, anaerobic naphthalene oxidation was observed in the unoxidized sediment after an extended lag period of 70 days and not in the air-oxidized sediment (Figure 7b). Previous studies on PAH oxidation using San Diego Bay sediment (11) indicated that naphthalene was not oxidized in the Shelter Island sediments; however, these studies were done over a time frame of 60 days, which is shorter than the lag phase for naphthalene oxidation observed here.

Rates of anaerobic toluene oxidation in the sediments that had been air-oxidized were comparable to those in the sediments that had not been oxidized (Figure 7c). However, toluene oxidation appeared to proceed through different mechanisms in the two types of sediments. In the sediments that had not been oxidized, the addition of molybdate completely inhibited toluene oxidation whereas molybdate had no effect on the air-oxidized sediments. These results suggest that SRB were oxidizing toluene in the unoxidized sediments, but that FeRB were responsible for the toluene oxidation in the air-oxidized sediments.

**Implications for Bioremediation.** The results suggest that the addition of insoluble iron(III) oxides to petroleum-contaminated sediments in San Diego Bay is not likely to enhance the degradation of the contaminants. Although it was expected that the addition of high concentrations of poorly crystalline iron(III) oxide to the sediment would switch the TEAP from sulfate reduction to Fe(III) reduction, this did not happen, presumably due to the low number of FeRB in these sediments. Even though FeRB have higher affinities for acetate and  $H_2$  than SRB (5, 20, 26, 27, 32), this may not be enough of a competitive advantage when SRB are numerically predominant. It can be expected that once SRB are established and maintaining low concentrations of acetate and  $H_2$  in sediments, that, even if Fe(III) is made available, the growth rates of FeRB will be quite low. Thus, FeRB may not be able to grow to sufficient numbers to outcompete SRB before all of the added Fe(III) is consumed via chemical reduction by the sulfide produced by the SRB. Another study has also recently found that the addition of iron(III) oxide to marine sediments did not significantly inhibit sulfate reduction (33). The difference between these results and the previous finding that the addition of Fe(III) switched the TEAP from sulfate reduction to Fe(III) reduction in freshwater sediments might be due to higher relative proportions of FeRB in freshwater sediments.

It is not clear that even if the TEAP could be switched from sulfate reduction to Fe(III) reduction in contaminated harbor sediment, that degradation of hydrocarbon contaminants would be faster under the Fe(III)-reducing conditions. As shown here, switching the TEAP to Fe(III) by air oxidizing the sediments did not stimulate toluene oxidation and actually inhibited benzene and naphthalene oxidation. Recent studies have indicated that, in the presence of the appropriate SRB, a wide range of petroleum-related contaminants can be anaerobically oxidized including alkanes (7, 9, 19), monoaromatic hydrocarbons (8, 10), and even polycyclic aromatic hydrocarbons (11). Although some monoaromatic hydrocarbons such as toluene are rapidly degraded under Fe(III)-reducing conditions (34, 35), others such as benzene are generally only rapidly degraded when a chelator is added to provide Fe(III) in soluble form (30, 31, 36). It is yet to be demonstrated that alkanes and polycyclic aromatic hydrocarbons can be oxidized under Fe(III)-reducing conditions.

Petroleum-contaminated sediments that are dredged from harbors may have to be remediated to remove the

hydrocarbons prior to disposal. Considering the probable high cost of maintaining aerobic conditions during such treatments, and the fact that some of the hydrocarbon contaminants can be degraded anaerobically, an alternative approach might be to combine an anaerobic treatment phase with the aerobic remediation. The results presented here suggest that aerobic bioremediation followed by anaerobic treatment would not be a good strategy as air oxidation may inhibit some of the subsequent anaerobic biodegradation capacity. The fact that many hydrocarbons can be degraded under sulfate-reducing conditions suggests that a more effective approach may be to allow anaerobic degradation to proceed as long as possible and then treat the sediments under aerobic conditions to complete the degradation of the most recalcitrant contaminants.

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