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Anaerobic Degradation of Polycyclic Aromatic Hydrocarbons and Alkanes in Petroleum-Contaminated Marine Harbor Sediments

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Although polycyclic aromatic hydrocarbons (PAHs) have usually been found to persist under strict anaerobic conditions, in a previous study an unusual site was found in San Diego Bay in which two PAHs, naphthalene and phenanthrene, were oxidized to carbon dioxide under sulfate-reducing conditions. Further investigations with these sediments revealed that methylnaphthalene, fluorene, and fluoranthene were also anaerobically oxidized to carbon dioxide in these sediments, while pyrene and benzo[*a*]pyrene were not. Studies with naphthalene indicated that PAH oxidation was sulfate dependent. Incubating the sediments with additional naphthalene for 1 month resulted in a significant increase in the oxidation of [^{14}C]naphthalene. In sediments from a less heavily contaminated site in San Diego Bay where PAHs were not readily degraded, naphthalene degradation could be stimulated through inoculation with active PAH-degrading sediments from the more heavily contaminated site. Sediments from the less heavily contaminated site that had been adapted for rapid anaerobic degradation of high concentrations of benzene did not oxidize naphthalene, suggesting that the benzene- and naphthalene-degrading populations were different. When fuels containing complex mixtures of alkanes were added to sediments from the two sites, there was significant degradation of the alkanes. [^{14}C]hexadecane was also anaerobically oxidized to $^{14}CO_2$ in these sediments. Molybdate, a specific inhibitor of sulfate reduction, inhibited hexadecane oxidation. These results demonstrate that a wide variety of hydrocarbon contaminants can be degraded under sulfate-reducing conditions in hydrocarbon-contaminated sediments, and they suggest that it may be possible to use sulfate reduction rather than aerobic respiration as a treatment strategy for hydrocarbon-contaminated dredged sediments.

Petroleum hydrocarbon contamination of harbor sediments from shipping activity, fuel oil spills, and runoffs is becoming a great concern due to the toxicity and recalcitrance of many of the fuel components. Polycyclic aromatic hydrocarbons (PAHs) are of most concern due to their toxicity, low volatility, resistance to microbial degradation, and high affinity for sediments (25). Although it is well known that PAHs can be degraded aerobically (references 7 and 8 and references therein), many studies have indicated that PAHs are not degraded under strict anaerobic conditions (3, 4, 16–18, 26, 27). Studies have demonstrated the degradation of PAHs in the absence of oxygen with nitrate as the apparent electron acceptor (2, 14, 20, 26, 27). However, Mn(IV) reduction, Fe(III) reduction, and sulfate reduction are the primary terminal electron-accepting processes in most marine sediments, and nitrate is only a minor electron acceptor (6, 32). Thus, the microbial metabolism of hydrocarbon contaminants under anaerobic conditions can be effective for remediation of harbor sediments only if the hydrocarbon oxidizers are dissimilatory sulfate, Fe(III), or Mn(IV) reducers. Due to the abundance of sulfate in marine environments, in many instances bioremediation of hydrocarbon contaminants would be most effective under sulfate-reducing conditions.

Several recent studies have demonstrated that monoaromatic hydrocarbons such as benzene, toluene, and xylene (BTEX) and hexadecane can be biodegraded in the absence of oxygen (1, 2, 5, 10, 11, 13, 15, 19–22, 24, 30, 33). There are now several pure culture examples of nitrate-reducing, Fe(III)-reducing, and sulfate-reducing bacteria that are capable of completely oxidizing some of these hydrocarbon contaminants to carbon dioxide (1, 12, 23, 30, 33). However, there are only two reports in the literature (31, 33) where nitrate- or sulfate-reducing isolates were investigated for their ability to degrade hydrocarbon components in a complex hydrocarbon mix under anaerobic conditions. In both of these studies, only a minor fraction of the crude-oil components were oxidized (31, 33). This raises the question of whether or not most hydrocarbon components will be degraded under anaerobic conditions in marine sediments.

In previous studies we found that while PAHs were not initially degraded under anaerobic conditions in typical soil and sediment samples, they were anaerobically degraded without a lag in harbor sediments that had been chronically contaminated with high concentrations of PAHs (11). As reported here, further investigation of this phenomenon demonstrated that the microorganisms in these sediments can anaerobically oxidize a wide range of hydrocarbons and also that the capacity to degrade PAHs at this site but not at others is most likely the result of the previous long-term exposure to PAHs.

MATERIALS AND METHODS

Sediments and sediment incubations. Sediments were collected as grab samples from the previously described (10, 11, 22) Shelter Island and Naval Station sites of San Diego Bay, San Diego, Calif. These sites were selected due to their differences in the extent and time frame of hydrocarbon contamination. The

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Shelter Island site has been contaminated (4 mg of PAH per kg of sediment) with runoffs and waste from pleasure boat activities for 30 years. In contrast, the Naval Station site has been chronically contaminated with hydrocarbons (33 mg of PAH per kg of sediment) through heavy shipping and naval activity for over 60 years. All experiments, unless otherwise stated, were prepared with freshly collected sediments that had not been stored for more than 7 days at room temperature. Seawater for preparing sediment slurries was collected from just above the sediment surficial layers and was stored at 4°C in the dark prior to use. Previous studies demonstrated that these sediments are highly reducing (10, 11) and that sulfate reduction is the predominant microbial terminal electron-accepting process (10, 11, 22). Sediment samples (30 g of a 1:1 [vol/vol] sediment-seawater slurry) in 35-ml serum bottles were incubated under strict anaerobic conditions under a gas phase of N_2–CO_2 (95:5) as previously described (10). All sediment incubations were carried out at 25°C in the dark. Heat-killed controls were prepared similarly to the test sediments and autoclaved at 121°C for 1 h on three consecutive days.

The degradation of hydrocarbons was determined by amending triplicate sediment samples prepared as described above with 200 μl of marine diesel fuel or JP-5 jet fuel per kg of wet sediment or 1.0 μCi of [1-^{14}C]hexadecane (2.2 mCi/mmol), [1-^{14}C]naphthalene (8.9 mCi/mmol), [8-^{14}C]methylnaphthalene (7.5 mCi/mmol), [9-^{14}C]phenanthrene (8.3 mCi/mmol), [9-^{14}C]fluorene (16.2 mCi/mmol), [3-^{14}C]fluoranthene (45 mCi/mmol), [4,5,9,10-^{14}C]pyrene (32.3 mCi/mmol), or [7-^{14}C]benzo[*a*]pyrene (26.6 mCi/mmol). All ^{14}C-labeled hydrocarbons were supplied by Sigma Chemical Corp., St. Louis, Mo. They were prepared as methanolic stocks (40 μCi per ml), and 25 μl was dispensed onto small squares cut from glass fiber filters (Whatman GF/D glass microfiber filters). The methanol was allowed to evaporate for 3 min, and the hydrocarbon-bearing filter squares were added to the sediment slurries under an N_2–CO_2 gas phase. Once the test vial was sealed and shaken, the microfiber filters disintegrated and the ^{14}C-hydrocarbons were distributed throughout the test vial. The results are presented in terms of $^{14}CO_2$ production as a percentage of the initial amount of ^{14}C label added. The standard errors of the means of the data presented were less than 20%.

In order to determine if naphthalene oxidation was sulfate dependent, a sulfate-depleted sediment slurry was prepared by centrifuging 200 g of freshly collected Naval Station sediment at 3,000 × *g* for 5 min under a gas phase of N_2. The supernatant was poured off, and the sediment pellet was resuspended in an equal volume of anaerobic saline buffer (20 g of NaCl/liter, 4.24 g of $MgCl_2 \cdot 6H_2O$/liter, 2.9 g of $CaCl_2$/liter, 1.5 g of KCl/liter) under N_2. This washing procedure was repeated twice. The final sulfate concentration was determined by ion chromatography to be 1.2 mM. Aliquots (20 g) were dispensed into six 35-ml serum vials. Three of these vials were immediately reamended with sulfate to a final concentration of 10 mM. All vials were amended with 1 μCi of [^{14}C]naphthalene as previously outlined and sealed under N_2–CO_2.

In order to determine if increased exposure to naphthalene enhanced naphthalene degradation, Naval Station sediments (1 liter) were incubated at 25°C in a 2.0-liter glass bottle under a gas phase of N_2–CO_2 (95:5) and were amended with 100 μM naphthalene. After 30 days' incubation, more than 50 μM naphthalene was consumed. Subsamples (30 g) were placed in 35-ml serum bottles and amended with 1.0 μCi of [^{14}C]naphthalene. $^{14}CO_2$ production was compared with that of similarly treated sediments that were not amended with the 100 μM naphthalene.

Analytical methods. $^{14}CO_2$ concentrations in 1-ml-headspace samples, taken in N_2-flushed syringes, were determined by gas chromatography (GC) with gas proportional counting detection as previously described (22). Sulfate concentrations were determined by ion chromatography as outlined previously (11). Naphthalene concentrations in the adapted sediments were determined by isocratic high-pressure liquid chromatographic analysis of an acetonitrile extract of subsamples of the sediment using a 3.3-cm C_{18} column and UV detection at 210 nm. The mobile phase was 60:40 (vol/vol) acetonitrile-water with a flow rate of 0.5 ml/min. The acetonitrile extract was prepared by adding 3 ml of acetonitrile to 3 g (wet weight) of sediment. After vigorous mixing the suspension was allowed to settle for 5 min. Subsamples (1.0 ml) of the extract were diluted in 4 ml of an aqueous acetonitrile solution prepared similarly to the high-pressure liquid chromatography eluant. The diluted extracts were filtered through 0.2-μm-pore-size acrodiscs (Gelman Scientific) and analyzed for naphthalene concentration.

In order to measure the hydrocarbon components in marine diesel fuel and JP-5 jet fuel, 10 g of sediment was mixed with 30 ml of methylene chloride and sonicated for 4 min. The sample suspension was centrifuged at 3,000 × *g* for 3 min, and the supernatant was decanted through an anhydrous sodium sulfate trap to remove moisture. The methylene chloride extract was directly injected into a Varian 3300 GC equipped with a 1075 splitless injector and either a DB-1 or a DB-1HT fused silica capillary column (30 m by 0.25 mm [inner diameter]) and temperature programmed from 40°C (1.5 min) to 300°C at 4°C/min. The fuel mixtures were also analyzed by GC-mass spectrometry in order to identify the major components. A Finnigan TSQ 70 GC-mass spectrometry system was used for the analyses performed under conditions similar to those for the GC analyses described above.

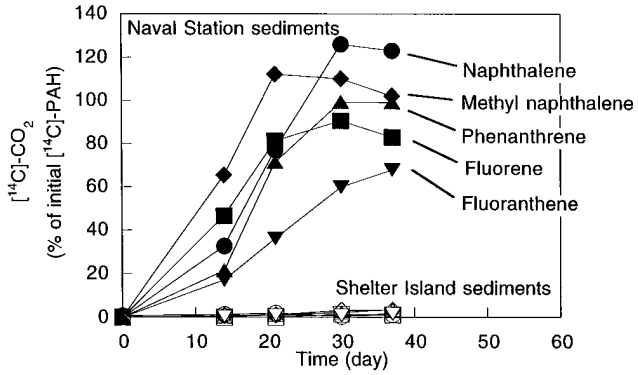

FIG. 1. Oxidation of ^{14}C-labeled PAHs in Naval Station and Shelter Island sediments from San Diego Bay. The results are the means of triplicate determinations.

RESULTS

Anaerobic PAH degradation. Five of the ^{14}C-labeled PAHs evaluated (naphthalene, phenanthrene, methylnaphthalene, fluorene, and fluoranthene) were readily oxidized to $^{14}CO_2$ in sediments from the Naval Station site (Fig. 1). Depending upon the PAH, 60 to 120% of the added radiolabel was recovered as $^{14}CO_2$ within 37 days. Minor amounts of $^{14}CH_4$ were produced during the oxidation of the PAHs by the sediments. In the cases of [^{14}C]naphthalene and [^{14}C]phenanthrene, this amounted to 0.6% or less of the added ^{14}C label and was entirely produced during the first 15 days of incubation, although $^{14}CO_2$ production continued for the following 40 days (data not shown). No $^{14}CO_2$ or $^{14}CH_4$ was produced from ^{14}C-labeled pyrene or benzo[*a*]pyrene. Studies with [^{14}C]naphthalene demonstrated that sulfate was required for PAH oxidation in the Naval Station sediments. When sulfate was removed from sediments by washing with sulfate-free buffer, oxidation of [^{14}C]naphthalene was inhibited (Fig. 2). Production of $^{14}CO_2$ from [^{14}C]naphthalene was restored upon the readdition of sulfate to a final concentration of 10 mM on day 9 (Fig. 2).

In contrast to what was found for the sediments from the Naval Station site, there was no production of $^{14}CO_2$ or $^{14}CH_4$ from any of the ^{14}C-labeled PAHs in the Shelter Island sedi-

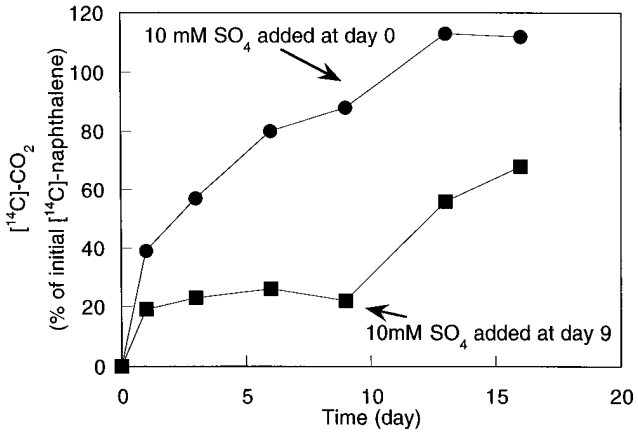

FIG. 2. $^{14}CO_2$ production from [^{14}C]naphthalene in Naval Station sediments washed with sulfate-free buffer. Sulfate (10 mM) was readded at day 0 to the control vials and at day 9 to the test vials. The results are the means of duplicate incubations.

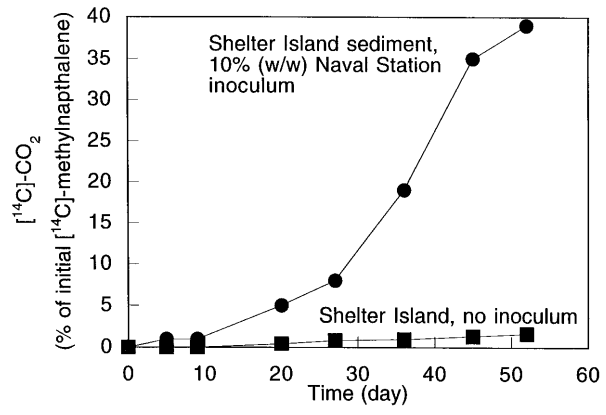

FIG. 3. $^{14}CO_2$ production from [^{14}C]methylnaphthalene in Shelter Island sediments left untreated or inoculated (10%, wt/wt) with Naval Station sediments. The results are the means of triplicate incubations.

ments over 37 days (Fig. 1). However, if Shelter Island sediments were incubated for extended periods (>80 days), $^{14}CO_2$ was produced from [^{14}C]phenanthrene or [^{14}C]naphthalene. This long lag in the oxidation of PAHs in Shelter Island sediments could be overcome if the Shelter Island sediments were amended with a 10% (vol/vol) inoculum of sediments from the Naval Station site, as demonstrated with [^{14}C]methylnaphthalene in Fig. 3.

When Naval Station sediments were amended with 100 μM naphthalene, more than 50 μM naphthalene was removed after 30 days. When subsamples, taken at 30 days, were amended with [^{14}C]naphthalene, there was rapid production of $^{14}CO_2$ (Fig. 4). Within 6 days, 75% of the added radiolabel was recovered as $^{14}CO_2$ (Fig. 4). In contrast, only 20% of [^{14}C] naphthalene was recovered as $^{14}CO_2$ after 6 days in sediments that had not been exposed to additional naphthalene prior to the 30-day incubation. When [^{14}C]naphthalene was added to Shelter Island sediments that had been adapted for rapid benzene degradation by repeatedly being fed benzene over time (22), there was no $^{14}CO_2$ produced from [^{14}C]naphthalene. In contrast, [^{14}C]benzene was completely oxidized to $^{14}CO_2$ overnight (data not shown).

Anaerobic alkane degradation. Alkanes are another important fuel component that may contaminate sediments. In order

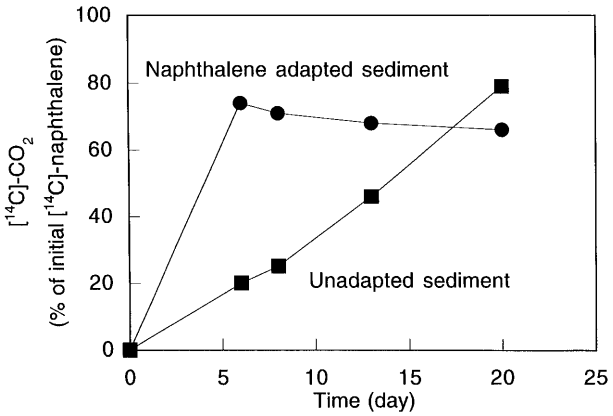

FIG. 4. $^{14}CO_2$ production from [^{14}C]naphthalene in naphthalene-adapted and unadapted Naval Station sediments. The results are the means of triplicate incubations.

to determine if alkanes might also be anaerobically degraded in San Diego Bay sediments, marine diesel fuel or JP-5 jet fuel, both of which contain complex mixtures of alkanes, were added to the sediments. After 80 days of anaerobic incubation, the concentrations of C11 to C24 alkanes in the live Naval Station sediments amended with marine diesel fuel were much lower than those in heat-killed controls (Fig. 5a). Alkanes also appeared to be degraded in sediments from the Shelter Island site, but to a lesser extent than those in the Naval Station sediments (Fig. 5b). There was a similar removal of alkanes from JP-5 jet fuel added to the two sediments (9).

In order to determine whether the loss of alkanes in the sediments could possibly be attributed to anaerobic oxidation, the metabolism of [^{14}C]hexadecane was examined. When sediments were amended with 1.0 μCi of [^{14}C]hexadecane, $^{14}CO_2$ was produced over time, with a final recovery of 75% of the added [^{14}C]hexadecane as $^{14}CO_2$ (Fig. 6). As with the other hydrocarbons oxidized in these sediments, the addition of 20 mM MoO_4 inhibited production of $^{14}CO_2$ from [^{14}C]hexadecane (Fig. 6, inset). There was no further increase in $^{14}CO_2$ production observed in the MoO_4-amended sample after 1 year of incubation.

DISCUSSION

These studies demonstrate that microorganisms in sediments of San Diego harbor can degrade a wide variety of important hydrocarbon contaminants that were hitherto considered recalcitrant under highly reduced, anaerobic conditions. Previous studies in our laboratory at the U.S. Geological Survey had demonstrated that benzene could be oxidized to CO_2 in San Diego Bay sediments and that this metabolism was linked to sulfate reduction (10, 11, 22). The two PAHs, naphthalene and phenanthrene, were also found to be mineralized to CO_2 in these sediments, and specific-inhibitor studies with MoO_4 (28) suggested that this oxidation may also be linked to sulfate reduction (10, 11). The findings reported here extend the number of PAHs known to be biodegradable under anaerobic conditions and demonstrate that *n*-alkanes are also anaerobically degraded in these sediments. These studies also suggest that preexposure to high concentrations of PAHs is an important factor for developing a microbial community that can rapidly degrade PAHs under anaerobic conditions.

Anaerobic PAH oxidation. The oxidation of PAHs in San Diego Bay sediments takes place under strict anaerobic, sulfate-reducing conditions, and sulfate reducers are involved in the PAH oxidation. The sediments are highly reducing, with high concentrations of ferrous iron and sulfide (10, 11). Sulfate reduction is the terminal electron-accepting process in the sediments (10, 11, 22). Inhibition of sulfate reducers with molybdate inhibits oxidation of the PAHs (11). Furthermore, sulfate is required for PAH oxidation, as exemplified in this study. These results do not prove that sulfate reducers are directly metabolizing the PAHs, as similar results might be observed if other organisms converted the PAHs to intermediate products that were then oxidized by the sulfate reducers. The source of the very minor amounts of methane during the metabolism of PAHs is unknown. No $^{14}CH_4$ was produced by these sediments when they were amended with [^{14}C]acetate (data not shown), [^{14}C]toluene (10, 22), [^{14}C]benzene (10, 22), or [^{14}C]hexadecane (this study). The mechanism of $^{14}CH_4$ production during the oxidation of the ^{14}C-labeled PAHs might be similar to the mini-methane system which results in the production of small amounts of methane from pyruvate in *Desulfovibrio* and *Desulfotomaculum* species (29).

Until the studies with the Naval Station site sediments at San

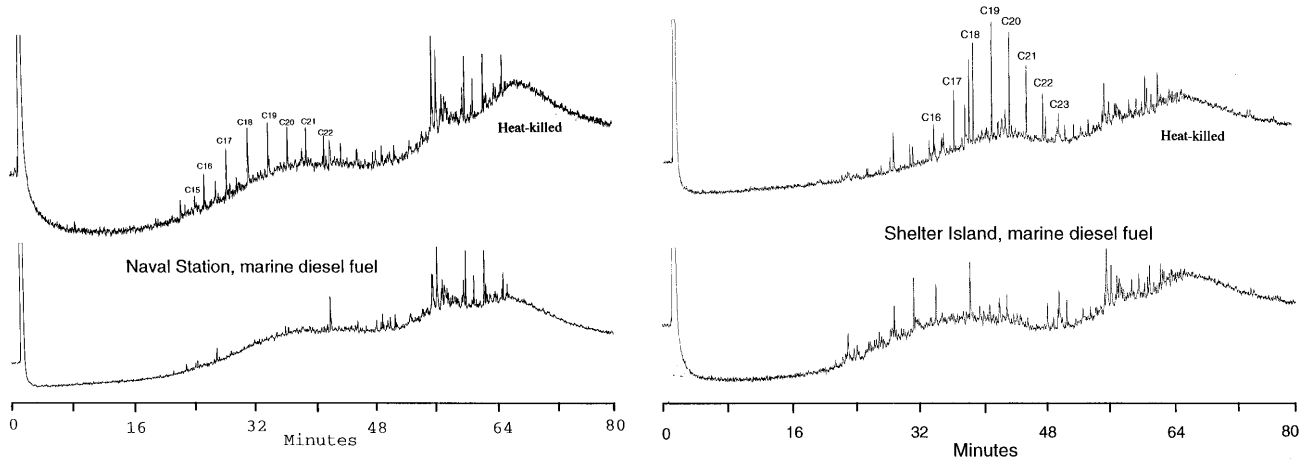

FIG. 5. Oxidation of marine diesel fuel in Naval Station (left) and Shelter Island (right) sediments relative to that in heat-killed controls.

Diego Bay, PAHs were thought to persist under sulfate-reducing conditions (3, 20, 26). However, previous studies had investigated the potential for PAH oxidation coupled to sulfate reduction in soils or sediments which had not had long-term exposure to both PAHs and sulfate. The studies with sediments from San Diego Bay suggest that long-term exposure to PAHs may be required for an active anaerobic PAH-degrading community to develop. PAHs are rapidly degraded in Naval Station sediments that have been contaminated with high levels of PAHs. PAHs are not initially degraded in the less-contaminated Shelter Island sediments but are eventually degraded after long adaptation periods. Even in the Naval Station sediments, preincubation with high levels of naphthalene greatly stimulated subsequent PAH oxidation. The fact that inoculation with Naval Station sediments greatly stimulated PAH metabolism in the Shelter Island sediments, and the finding that PAHs were eventually degraded in Shelter Island sediments after preexposure to PAHs, suggest that low numbers of the appropriate microorganisms rather than adverse environmental conditions limited PAH oxidation in the Shelter Island sediments.

Anaerobic alkane degradation. Although there is no pure culture model for PAH oxidation under sulfate-reducing conditions, microorganisms which can oxidize alkanes with the reduction of sulfate have been described (1, 33). However, significant potential for anaerobic alkane degradation in petroleum-contaminated marine sediments has not been previously described. The studies with the complex fuel mixtures suggest that all the major alkane components of diesel marine fuel and JP-5 jet fuel can be degraded in anaerobic sediment under sulfate-reducing conditions. The studies with [^{14}C]hexadecane indicated that the consumption of the alkanes can be attributed to anaerobic oxidation of alkanes to CO_2. The inhibition of hexadecane oxidation with molybdate suggests that sulfate reducers are responsible for the hexadecane oxidation. The finding of significant anaerobic oxidation of hexadecane in San Diego Bay sediments contrasts with the findings of a previous study of anaerobic lake sediments in which less than 6% of the added [^{14}C]hexadecane was recovered as $^{14}CO_2$ (34). Furthermore, in that study a nearly equivalent amount of $^{14}CO_2$ was recovered in formaldehyde-killed controls.

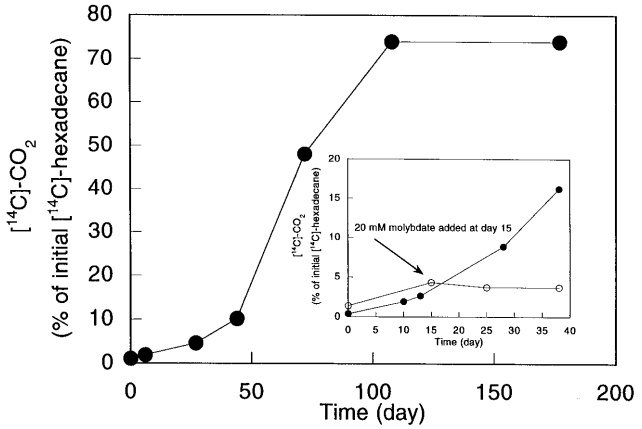

FIG. 6. Oxidation of [^{14}C]hexadecane to $^{14}CO_2$ in Shelter Island sediments and inhibition of $^{14}CO_2$ production by the addition of 20 mM MoO_4. The results are the means of triplicate incubations.

Implications for bioremediation. The finding that PAHs and alkanes can be degraded under sulfate-reducing conditions in some petroleum-contaminated marine sediments has important implications for the self-purifying capacity of polluted harbor environments. The results presented here suggest that although hydrocarbon compounds entering pristine anaerobic marine sediments may not be degraded immediately, microbial populations can develop over time to metabolize these compounds. The fact that this metabolism can be linked to sulfate reduction is beneficial because sulfate is one of the most abundant electron acceptors in marine environments (32). Thus, if inputs of hydrocarbon pollution can be controlled, much of the hydrocarbon contamination may eventually be eliminated.

The results suggest that anaerobic degradation of hydrocarbon contaminants may also be a useful strategy for the ex situ treatment of dredged sediments which must be remediated prior to disposal. Anaerobic treatment could be much less expensive than the more commonly considered aerobic approach, which is costly and energy intensive due to the need for vigorous agitation and aeration in order to introduce sufficient quantities of O_2. The finding that anaerobic hydrocarbon degradation can be stimulated via inoculation with adapted sediments may aid in the initiation of anaerobic treatment strategies.

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