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Naphthalene and anthracene mineralization linked to oxygen, nitrate, Fe(III) and sulphate reduction in a mixed microbial population

Juliana A. Ramsay[1,*], Hao Li[1], R.S. Brown[2] & Bruce A. Ramsay[3]
[1]*Department of Chemical Engineering and* [2]*Department of Chemistry, Queen's University, Kingston, Ontario, Canada K7L 3N6 (*author for correspondence: e-mail: ramsayj@chee.queensu.ca);* [3]*Polyferm Canada Inc., RR #1, Harrowsmith, Ontario, Canada K0H 1V0*

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Abstract

A microbial consortium from a mixture of garden soil and an enrichment of a coal-tar contaminated sediment mineralized naphthalene and anthracene when oxygen, nitrate, Fe(III) (soluble and insoluble) or sulphate were provided as terminal electron acceptors (TEAs). Rates of polyaromatic hydrocarbon disappearance and mineralization were similar in the presence of oxygen and nitrate, and slower with the other TEAs. A maximum mineralization of 37.5% naphthalene and 8.5% anthracene occurred in 30 and 160 days respectively when oxygen was provided as the TEA. On the other hand, only 9.5% naphthalene and 3.2% anthracene were mineralized in 42 and 160 days respectively with FeOOH. Mineralization occurred only when a TEA was provided and ceased when the naphthalene concentration decreased to non-detectable levels (less than 0.008 μmoles/L), as measured by fluorescence spectroscopy. CH_4 was not detected in the headspace of any microcosm. These results showed that mineralization of polyaromatic hydrocarbons such as naphthalene and anthracene can be linked to wide range of TEAs demonstrating that intrinsic polyaromatic hydrocarbon bioremediation is possible if any of these TEAs were available.

Introduction

Polyaromatic hydrocarbons (PAHs) are listed as priority pollutants by the US EPA (Keith & Telliard 1979). Some are toxic, mutagenic, and carcinogenic to humans (Cerniglia 1992) and are known to persist in the environment. The aerobic degradation of polyaromatic hydrocarbons has been well studied (Crawford 1991; Cerniglia 1992, 1993). The degradation of polyaromatic hydrocarbons can also occur under nitrate-reducing (Mihelcic & Luthy 1988; Al-Bashir et al. 1990; MacRae & Hall 1998; Rockne et al 2000), sulfate-reducing (Bedessem et al. 1997; Coates et al. 1997; Zhang & Young 1997; Meckenstock et al. 2000), methanogenic (Parker 1995; Genthner et al. 1997) and Fe(III)-reducing (Anderson & Lovley 1999) conditions. Fe(III) is of particular interest as it is the most abundant electron acceptor in many aquifers (Lovley & Coates 2000) and consequently has the potential to play an important role in the degradation of any organic compounds which might be present. However, at pH values where biodegradation might be expected to occur, Fe(III) is quite insoluble and hence is poorly bioavailable. For example, only 10^{-19} M of soluble Fe(III) is present in a saturated $Fe(OH)_3$ solution at neutral pH (Seeliger et al. 1998).

Although site remediation regulations usually stipulate maximum PAH levels as monitored by chromatographic analysis of groundwater or soil extracts (US EPA 1982), such measurements are slow, expensive, and imprecise. Hence, frequent analysis of even the aqueous phase PAHs is rare in remediation studies. In addition to terminal electron acceptor (TEA) bioavailability, the PAH themselves must be accessible for microbial metabolism. An analysis of PAHs in the soil may not reflect how much is bioavailable. PAHs are hydrophobic and will sorb strongly to the soil matrix especially if its organic matter content is high. The

readily bioavailable portion may only be what is soluble in the aqueous phase. Fluorescence spectroscopy may allow direct, rapid and economical measurement of low concentrations of aqueous phase PHAs.

Fe(III) reduction has been shown to be coupled to the mineralization of toluene, phenol and *p*-cresol in *Geobacter metallireducens* (Lovley & Lonergan 1990), and of other organic matter in aquatic environments (Lovley 1991). Although naphthalene degradation has been demonstrated under Fe(III)-reducing conditions in a petroleum-contaminated aquifer (Anderson & Lovley 1999), a direct link between the degradation of polyaromatic hydrocarbons such as naphthalene and Fe(III) reduction has not been proven.

Although intrinsic bioremediation of certain sites may involve multiple TEAs, few studies (Genthner et al. 1997) have examined the ability of the indigenous, mixed, microbial population to utilize a range of terminal electron acceptors in the degradation of PAHs. This study examines whether a mixed microbial culture, derived from a mixture of garden soil and a sediment from a former coal gasification site, is capable of using a variety of TEAs and, whether Fe(III) in particular can be linked to the degradation of PAHs such as naphthalene and anthracene. In addition, fluorescence spectroscopy is used to monitor disappearance of low concentrations of naphthalene from the aqueous phase.

Material and methods

Source of inoculum and composition of mineral salts medium

The inoculum used in these experiments came from a one-step enrichment culture of a coal-tar-contaminated sediment obtained from a former coal gasification plant (Kingston, Ontario) and an uncontaminated garden soil. The one-step enrichment was performed in a four-Liter culture bottle (10 cm in dia. $\times$ 50 cm) containing 20% (wt/vol) of the contaminated sediment, 3.5 L of a modified mineral salts medium (MSM) (Lovley & Phillips 1986), 1 g/L amorphous Fe(III) oxyhydroxide and 20 mg/L each of naphthalene, phenanthrene and anthracene at room temperature for 18 months (Robertson 1998). Analysis of the coal-tar contaminated sediment showed that it contained a mixture of PAHs (255 mg/kg of dry sediment). The slurry was purged with high purity nitrogen prior to being capped and mixed on a Modular Cell Production Roller Apparatus (Wheaton Instruments) for about five weeks and then allowed to settle. The color of the sediment at the water interface gradually changed from a deep black to grey. After mixing, the sediment at the water interface would become black again. Prior to use, the redox potential at the top of the water column was 50 mV and -6 to -16 mV at the sediment-water interface while air-saturated water had a value of 200 mV.

The MSM contained (per Liter): 8.75 g $NaHCO_3$, 0.875 g NH_4Cl, 2.1 g $NaH_2PO_4{\cdot}H_2O$, and 0.35 g KCl, 35 mL of a vitamin solution and 35 mL of a trace element solution (TES). The vitamin solution consisted of (in milligrams per Liter): 2.0 biotin, 2.0 folic acid, 10.0 pyridoxine HCl, 5.0 riboflavin, 5.0 thiamine HCl, 5.0 nicotinic acid, 5.0 pantothenic acid, 0.1 B–12, 5.0 p-aminobenzoic acid, and 5.0 thioctic acid. The TES contained (in grams per Liter): 1.5 $C_6H_6NO_6Na_3$ (sodium nitrilotriacetate), 3.0 $MgSO_4$, 0.5 $MnSO_4{\cdot}H_2O$, 1.0 NaCl, 0.1 $FeSO_4{\cdot}7H_2O$, 0.1 $CaCl_2{\cdot}2H_2O$, 0.1 $CoCl_2{\cdot}6H_2O$, 0.13 $ZnCl_2$, 0.01 $CuSO_4{\cdot}5H_2O$, 0.01 $AlK(SO_4)_2{\cdot}12H_2O$, 0.01 H_3BO_3, 0.025 Na_2MoO_4, 0.024 $NiCl_2{\cdot}6H_2O$, and 0.025 $Na2WO_4{\cdot}2H_2O$. Amorphous Fe(III) oxyhydroxide (FeOOH) was prepared by neutralizing a 0.4 M $FeCl_3{\cdot}6H_2O$ solution to pH 7.0 with 10 M NaOH (Lovley & Phillips 1986). The precipitate was recovered by centrifugation and the Cl- removed by repeated washing with distilled water.

Microcosm mineralization with various TEAs

Duplicate abiotic and biotic microcosms were prepared in 125 mL serum bottles. For naphthalene mineralization, 10 g clean garden soil, 10 mL of the enrichment culture, 30 mL of MSM, and 50 mL of a solution containing three times the stoichiometric amount of each terminal electron acceptor required to mineralize 80 μmoles/L of naphthalene were added to a final volume of 100 mL. Nitrate (2.34 mM) was added as $NaNO_3$, soluble Fe(III) (11.25 mM) as ferric citrate, insoluble FeOOH was prepared as described above, and sulphate (1.41 mM) as Na_2SO_4. Oxygen-free nitrogen gas passed through a column of hot reduced copper turnings was used to purge the microcosms for 20 min (Hungate 1969). [U-^{14}C] naphthalene (17.8 μCi μmol^{-1}, Sigma, St. Louis, MO), greater than 98% pure, dissolved in methanol and nonradiolabeled naphthalene were added to have an initial naphthalene concentration of about 80 μmoles/L and an initial activity of about

100,000 cpm. ^{14}C-Naphthalene microcosms were sealed with Mininert^{TM} teflon valves (Alltech Associates Inc., Deerfield, Ill.) and kept in an anaerobic glove box (Nexus One, Vacuum Atmospheres Co., Hawthorn, Calif.), with an anaerobic naphthalene in methanol stock solution under a nitrogen atmosphere. Periodic additions of labeled and unlabeled naphthalene to a concentration of about 120 μmoles/L and an activity of about 100,000 cpm were made. Each microcosm contained a 5-mL test tube containing 1 mL of 1N KOH to trap volatile ^{14}C activity (Greer et al. 1993) which was measured with a Beckman LS 6500 Scintillation Counter using the Optiphase "HiSafe 3" liquid scintillation cocktail (Wallac Scintillation Products, Turku, Finland). Identical microcosms in duplicates were prepared with nonradiolabeled naphthalene and mercuric chloride (3.89% wt/vol) was added to the abiotic controls.

For anthracene microcosms, 4 mL of 1.12 mmoles/L anthracene in ethanol was mixed with 10 g of clean garden soil in serum bottles and the ethanol allowed to volatilize overnight in the chemical hood. After the MSM and TEA were added as above, the serum bottles were purged for 20 min with oxygen-free nitrogen then capped with a butyl rubber stopper and an aluminium crimp. [U-^{14}C] Anthracene (20.6 μCi μmol^{-1}, Sigma, St. Louis, MO), greater than 99% pure, dissolved in toluene and 10 mL of the enrichment culture were added. Radiolabeled purity of both naphthalene and anthracene is based on duplicate HPLC analysis provided by the supplier.

Aerobic microcosms were prepared as above but were not purged with oxygen-free nitrogen and were kept outside the anaerobic glove box. All Fe(III) microcosms were incubated in the dark.

Naphthalene analysis by fluorescence spectrometry

Prior to sampling, nonradiolabeled microcosms were shaken vigorously for 10 s and the sediment allowed to settle for 2 h. A volume of 0.5 mL from the upper aqueous phase was added to a vial containing 2.5 mL of ethanol and 2.0 mL of deionized water. All samples were stored in the freezer until analyzed. Samples were sonicated for 10 min, prior to analysis. Naphthalene concentrations were measured using a fluorescence spectrophotometer (Model QM1, Photon Technologies International, London, ON) with the excitation source coming from a 75 watt xenon lamp. A synchronous fix scan was run for each sample with scan parameters at the appropriate excitation (265 nm) and emission (312 nm) wavelengths. Using a calibration curve, peak areas for each sample were used to calculate concentration. Between samples, the cuvette was rinsed with ethanol and dried with nitrogen gas.

Analyses

The following analyses were performed on the identically prepared, nonradiolabeled microcosms. Nitrate and sulphate were analysed by ion chromatography using a Dionex DX-300 with a conductivity detector using solutions of $NaNO_3$ and Na_2SO_4 as standards. Samples were filtered through a 0.45 μm polycarbonate filter, then injected into Ionpac AS9-SC guard and analytical columns with a 1.7 mM sodium bicarbonate and 1.8 mM sodium carbonate solution as mobile phase. Fe(II) concentration was measured by the ferrozine assay using standards prepared with ferrous ethylene-diammonium sulphate (Lovley & Phillips 1986) and Fe(III) concentration was determined as the difference in Fe(II) concentration before and after the conversion of all the Fe(III) to Fe(II). Fe(III) was converted to Fe(II) using 0.25 M hydroxylamine hydrochloride in 0.25 M HCl (Lovley & Phillips 1987). One-mL samples of the headspace gas were analysed for methane using a Varian Star 3400 equipped with a 30 m $\times$ 0.53mm DB1 column with a 5 mm thick film (JW Scientific, Folsom, Calif.) and a flame ionization detector. With an injector temperature of 50 °C, detector temperature of 150 °C, a column temperature of 50 °C and the helium carrier gas at a flow rate of 50 mL/min, the retention time of pure methane was 0.15 min.

Results

Naphthalene disappearance with no TEA or under abiotic conditions

During the first 25 days, aqueous phase naphthalene concentration slowly decreased at a similar rate and extent in the biotic microcosms without TEA (Figure 1- I(A)) and in all abiotic microcosms with TEA (Figure 1-II(A) to VI(A)), probably due to sorption of naphthalene to the soil particles in the microcosm as well as to the inner surfaces of the serum bottle. There should have been no sorption to the teflon stopper. This was followed by an even slower rate of disappearance possibly due to further sorption or leakage during sampling. When fresh naphthalene aliquots were added, there was little to no further abiotic naphthalene

Table 1. Rates of naphthalene disappearance and mineralization (calculated from the data shown in Figure 1) for the first three naphthalene additions to microcosms containing different TEAs. 50 μmoles/L of naphthalene were added in aliquot 1 and 125 μmoles/L in aliquots 2 and 3

TEA	Disappearance rate (μmole/L/day) after addition of Aliquot			Mineralization rate (%/day) after addition of Aliquot		
	1st	2nd	3rd	1st	2nd	3rd
O_2	2.44	4.00	3.25	0.41	1.25	0.9
NO_3^-	2.35	3.04	4.31	0.34	0.76	1.12
Fe^{3+}	1.36	2.81	2.28	0.27	0.37	0.23
FeOOH	1.07	2.44	5.00	0.15	0.30	0.25
SO_4^{2-}	1.73	2.43	2.50	0.27	0.52	0.25

loss (Figure 1-II(A) to VI(A)). There was no radioactivity in the CO_2 traps of any of the abiotic controls containing a TEA. However, there was a small amount of mineralization (2.2%) during the first month in the biotic microcosms without TEA. All results shown in Figure 1 are the averages of duplicate microcosms with mineralization data varying from 1 to 3%.

O_2

The aerobic microcosms were not purged with oxygen-free nitrogen and the headspace contained air. Based on the oxygen content in the headspace, it was calculated that there was at least 15 times as much oxygen as needed for complete mineralization of naphthalene and seven times that needed for complete anthracene mineralization. No other TEA was provided. The first 50 μmoles/L of naphthalene disappeared in about 20 days at a rate of 2.4 μmoles/L per day (Table 1). Each of the three subsequent aliquots of 125 μmoles/L of naphthalene totally disappeared in 30–40 days at slightly decreasing rates with each naphthalene addition (i.e., 4.0, 3.3 and 3.2 μmoles/L per day respectively). About 35% of the ^{14}C-naphthalene added at each spike was mineralized to CO_2 (Figure 1-II(B)). Three weeks after the first addition of naphthalene, its aqueous phase concentration decreased to a level not detectable by fluorescence spectroscopy (less than 0.008 μmoles/L). Mineralization ceased at this point, demonstrating that naphthalene mineralization could not occur when the aqueous phase naphthalene concentration dropped to undetectable levels.

NO_3^-

With nitrate provided as the sole TEA, the first 50 μmoles/L of naphthalene disappeared in about 20 days at 2.4 μmoles/L per day as with oxygen (Table 1). With the next two aliquots at higher initial concentrations of 125 μmoles/L of naphthalene, naphthalene disappeared completely and at a faster rate with each subsequent addition (i.e., in 40 days or 3.0 μmoles/L per day with the second addition and 30 days or 4.3 μmoles/L per day with the third). The initial rate after the fourth naphthalene addition was faster than the third but then slowed, probably due to nitrate limitation. This trend was also seen in the mineralization rate (Figure 1-III(B)) with an average extent of mineralization of about 30%. At the time of the third and fourth naphthalene addition, 1.66 and 0.89 mmoles/L of nitrate respectively were measured in the microcosm. Only 0.14 mmoles/L of nitrate was detected at the final point shown in Figure 1-III(A). For every mole of nitrate which disappeared, a stoichiometric amount of nitrite was produced.

Soluble Fe^{3+} and insoluble FeOOH

The disappearance profiles of the first 50 μmoles/L of naphthalene were similar (Figure 1-IV(A) and V(A)) whether soluble Fe^{3+} (provided as ferric citrate) or insoluble FeOOH was provided as the sole TEA. The naphthalene disappeared at a steady rate for 25–35 days (1.4 μmoles/L per day) (Table 1) but slowed to about 0.16 μmole/L per day when the aqueous phase naphthalene concentration dropped below about 2 μmoles/L. The rate of disappearance and mineralization decreased slightly but progressively with the second and third naphthalene additions in the presence of soluble Fe^{3+}. However, in the presence of FeOOH, there was a significant increase in the rate of naphthalene disappearance from the second to the third addition. In both cases the average extent of mineralization was around 12%. For the third naphthalene addition, about 0.86 and 0.73 mmoles/L of Fe(III) were consumed in the microcosms with soluble Fe(III) and with the insoluble FeOOH respectively. There was no change in the Fe(III) concentration in the abiotic controls. In a separate experiment, there was no inhibition of naphthalene oxidation to $^{14}CO_2$ when 20 mM molybdate was added to one of the duplicate microcosms mineralizing naphthalene with FeOOH as the TEA. This indicates that Fe(III) reduction was the primary terminal electron accepting mechanism and significant sulphate reduction was not taking place.

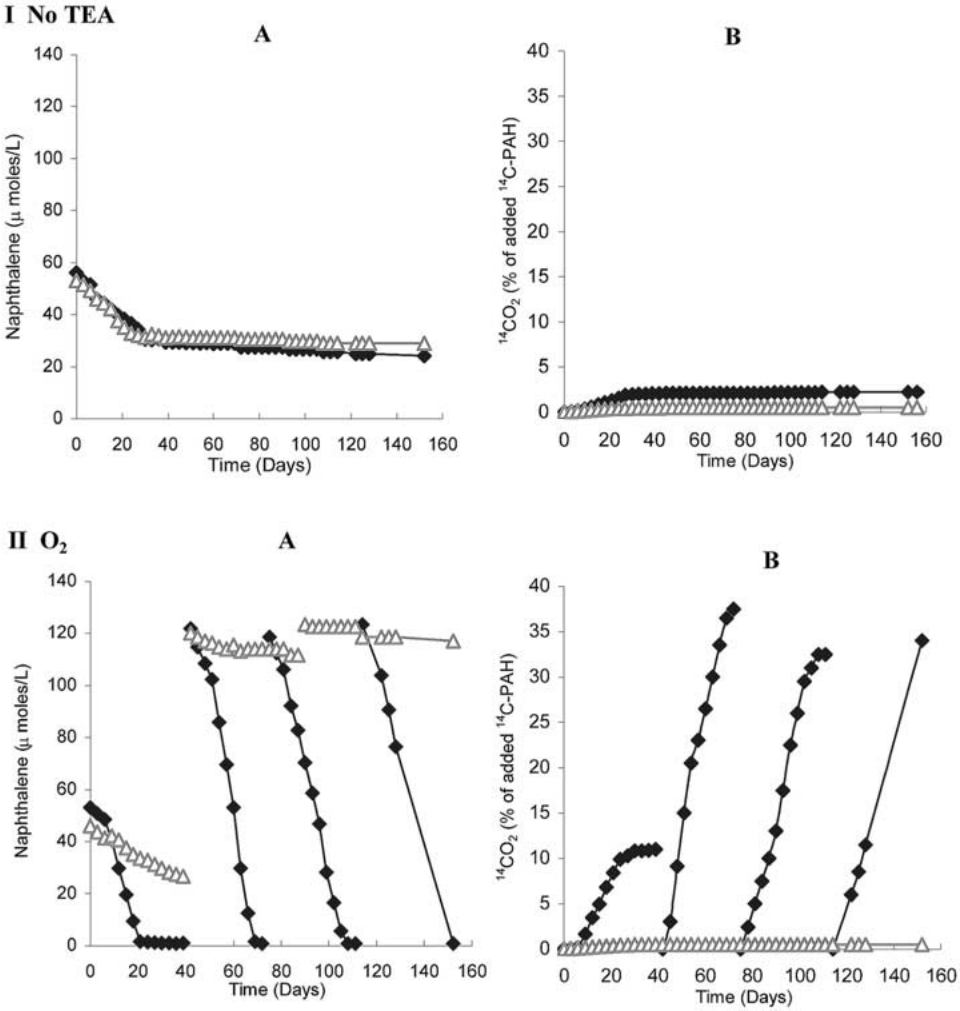

Figure 1. Naphthalene disappearance (A) and mineralization (B) in the presence of either (I) no terminal electron acceptor (TEA), (II) oxygen, (III) nitrate, (IV) soluble Fe(III) provided as ferric citrate, (V) insoluble Fe(III) as FeOOH or (VI) sulphate as TEAs in biotic (♦) and abiotic (△) microcosms.

SO_4^{2+}

The rate and extent of the disappearance of the first naphthalene addition in the presence of SO_4^{2-} (Figure 1-VI(A)) was similar to that observed for soluble and insoluble iron. The rate of naphthalene disappearance of the second addition was 2.4 μmoles/L per day (Table 1). The third addition was slightly slower than the second. The highest extent of mineralization was 30% with the second naphthalene aliquot. The slower mineralization rate of the third naphthalene addition was probably not due to sulphate limitation as there was 1.07 mmoles/L of sulphate measured at the last time point on the graph.

Methanogenic activity

There was no detectable CH_4 in the headspace of any of the anaerobic microcosms at the end of the experiment.

Anthracene mineralization

There was no mineralization in the abiotic controls when anthracene was provided as the carbon source with or without any TEA by day 160 (Table 2). Mineralization occurred only when a TEA was provided and began only after about 80 days of incubation. Anthracene mineralization was much slower than that of naphthalene. The rate of mineralization was fast-

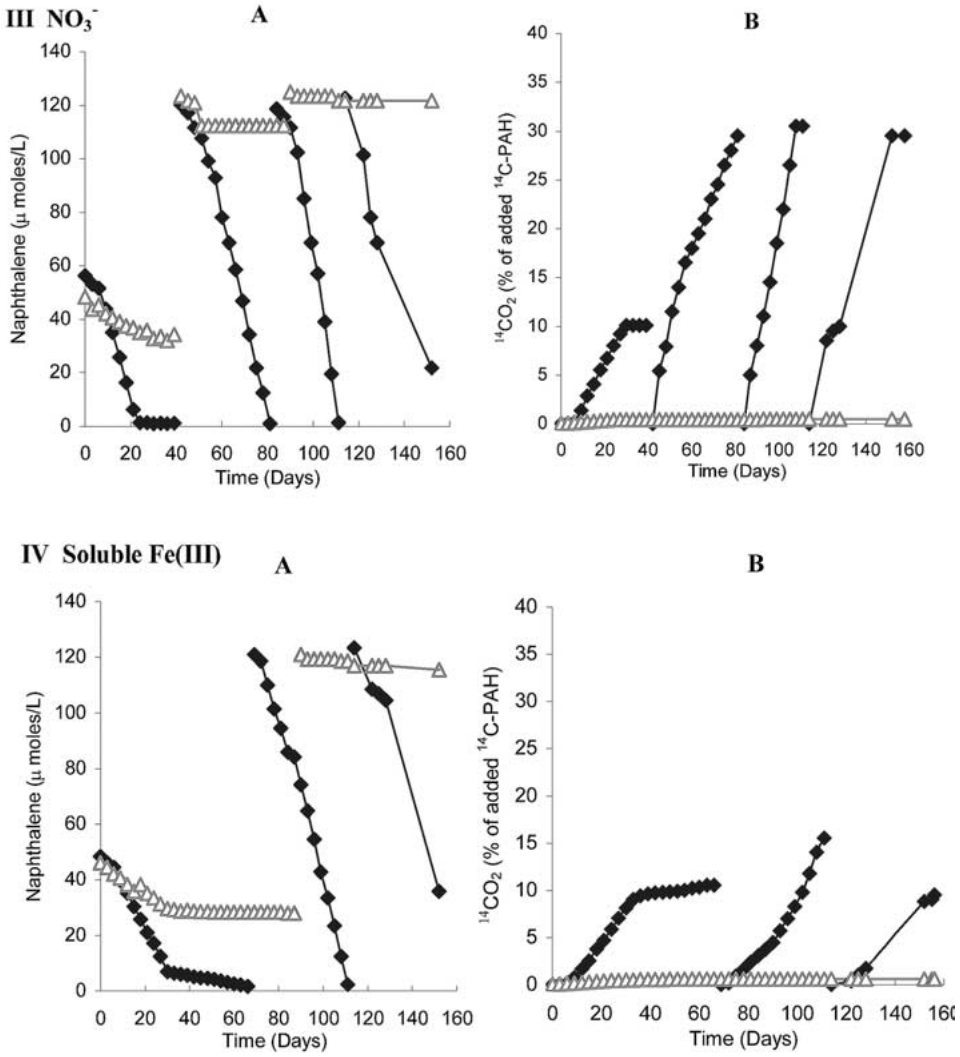

Figure 1. Continued.

est with oxygen then nitrate, sulphate, soluble iron (III) and slowest with insoluble FeOOH. The amount of $^{14}CO_2$ detected when Fe(III) and sulphate were present was always greater than 3% while the radiolabelled anthracene was at least 99% pure.

Discussion

Although 80 μmoles/L of naphthalene was initially added to all biotic and abiotic microcosms, only about 50 μmoles/L was actually measured. Most of this naphthalene loss was likely due to sorption on soil particles and onto the walls of the microcosm. It has been shown that in the absence of soil up to 10% of

Table 2. Anthracene mineralization in the presence of different TEAs at day 160

TEA	% Mineralizatio at day 160	
	Abiotic	Biotic
No TEA	0	0
O_2	0	8.5
NO_3^-	0	5.8
Fe^{3+}	0	3.7
FeOOH	0	3.2
SO_4^{2-}	0	4.9

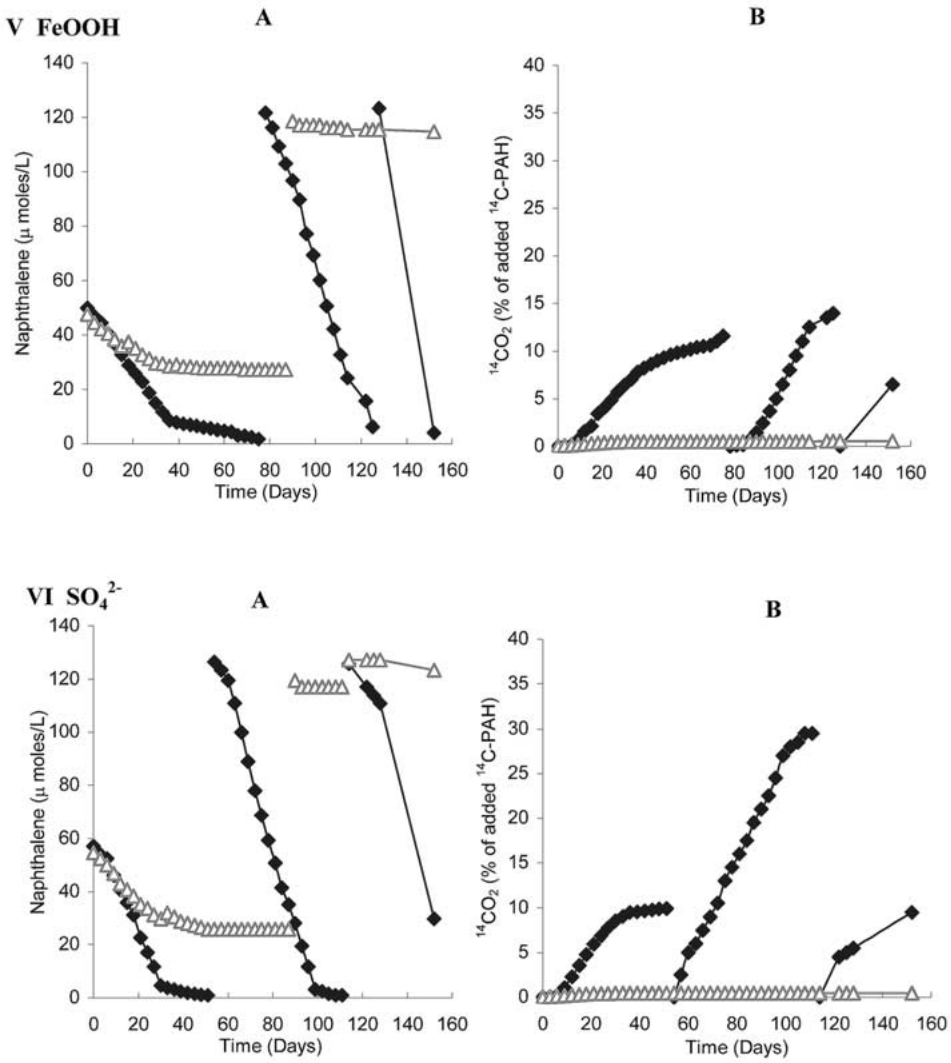

Figure 1. Continued.

the PAH added will adsorb to the incubation vessel (McNally et al. 1998) and up to 5% to the cells (Whitman et al. 1995) and is usually seen as an immediate loss. In this case with about 20% soil in the slurry, an initial 30–40% loss due to sorption is reasonable. An additional 15–20 μmoles/L of naphthalene was lost over the first 25–30 days in the abiotic microcosms. Since abiotic losses of these magnitudes did not occur after further naphthalene additions, it is likely that the slurry and the microcosm walls had become saturated with naphthalene within this time period.

Without a TEA, there was very little difference in naphthalene disappearance between the biotic and abiotic microcosms. In addition, since there was only 2.2% mineralization in the biotic microcosms lacking TEA (Figure 1-I) and since the purity of the radiolabeled naphthalene was 98%, it can be concluded that mineralization required the addition of a TEA and that no significant amount of TEA came from the inoculum or soil. HPLC-ion chromatography showed that around 0.02 mmoles/L of sulphate had been consumed in the biotic microcosms. This trace amount most likely came from the soil and no other TEA was detected. Anthracene was also mineralized only when a TEA was added (Table 2). These results also demonstrate that the microcosms prepared under anaerobic conditions contained no oxygen nor did oxygen leak into them during sampling. Furthermore,

since naphthalene and anthracene mineralization occurred only when a TEA was provided (O_2, NO_3^-, soluble Fe(III), FeOOH or SO_4^-) and did not occur in identical microcosms without a TEA (Figure 1-I to VI), the TEA provided must have been the primary terminal electron acceptor in the PAH oxidation. This is further supported by the fact that CH_4 was not detected in the headspace of any microcosm, and that in the Fe(III) microcosms, the addition of 20 mM molybdate, an inhibitor of sulphate reduction, did not inhibit mineralization.

Aliquots of naphthalene were added whenever its concentration approached zero (Figure 1). For each of the first three aliquots, the TEAs could be divided into two groups based on comparable rates of naphthalene disappearance and mineralization (Table 1). The first group with the higher rates consisted of O_2 and NO_3^- and the second was Fe(III), FeOOH, and sulphate. There was always 100% disappearance of naphthalene but only a maximum of 30–34% mineralization with O_2 and NO_3^- and around 10% mineralization with the second group of TEAs. Under nitrate-reducing conditions, there was an almost stoichiometric conversion of nitrate to nitrite. This level of nitrite does not appear to be inhibitory in this study as there was no apparent change in the rate of degradation under these conditions (Figire 1-III).

The fact that naphthalene degraded without a detectable lag phase when O_2, NO_3^-, soluble Fe(III), FeOOH or SO_4^- were provided as TEAs indicates that the microbial population was capable of degrading naphthalene under widely varying conditions. This demonstrates that if any or all of these TEAs were present, the PAHs could be intrinsically biodegraded. Most studies demonstrate the ability of a microbial consortium to use a single TEA to degrade a specific contaminant. Very few (Genthner et al. 1997) have shown that such a consortium can utilize a range of TEAs to degrade these contaminants as shown in this study.

The results also demonstrate that both naphthalene and anthracene mineralization can be linked to Fe(III) reduction and that the microorganisms could use the very poorly soluble FeOOH as a TEA just as efficiently as soluble Fe(III) or SO_4^-. Regardless of the TEA, it appeared that only dissolved naphthalene was accessible for metabolism since the end point of naphthalene mineralization corresponded to its disappearance from the aqueous phase as measured by fluorescence spectroscopy. Since fluorescence spectrometry can measure very low naphthalene concentrations, it may be a very useful tool to monitor its bioavailability as measured by the aqueous phase concentration.

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