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Geochemical and Physiological Evidence for Mixed Aerobic and Anaerobic Field Biodegradation of Coal Tar Waste by Subsurface Microbial Communities

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Abstract

We used geochemical analyses of groundwater and laboratory-incubated microcosms to investigate the physiological responses of naturally occurring microorganisms to coal-tar-waste constituents in a contaminated aquifer. Waters were sampled from wells along a natural hydrologic gradient extending from uncontaminated (1 well) into contaminated (3 wells) zones. Groundwater analyses determined the concentrations of carbon and energy sources (pollutants or total organic carbon), final electron acceptors (oxygen, nitrate, sulfate), and metabolic by-products (dissolved inorganic carbon [DIC], alkalinity, methane, ferrous iron, sulfide, Mn^{2+}). In the contaminated zone of the study site, concentrations of methane, hydrogen, alkalinity, and DIC were enhanced, while dissolved oxygen and nitrate were depleted. Field-initiated biodegradation assays using headspace-free serum bottle microcosms filled with groundwater examined metabolism of the ambient organic contaminants (naphthalene, 2-methylnaphthalene, benzothiophene, and indene) by the native microbial communities. Unamended microcosms from the contaminated zone demonstrated the simultaneous degradation of several coal-tar-waste constituents at the *in situ* temperature (10°C). Lag phases prior to the onset of biodegradation indicated the prevalence of both aerobic and anaerobic conditions *in situ*. Electron acceptor-amended microcosms from the most contaminated well waters demonstrated only aerobic naphthalene degradation. Collectively, the geochemical and microbial evidence show that biodegradation of coal-tar-waste constituents occurs via both aerobic and anaerobic terminal electron accepting processes at this site.

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Introduction

The subsurface disposal of coal tar wastes from manufactured gas plant operations has resulted in contaminated groundwater sites throughout the United States [25, 34].

Many constituents of coal tar waste are known or potential carcinogens. Fortunately, many coal-tar-waste constituents can be metabolized by microorganisms [1, 20, 38, 50]. *In situ* biodegradation of environmental contaminants is difficult to document directly [26, 30]; however, substantial progress in this discipline has been made in the past decade [27, 35, 36]. Concentration and isotopic measurements of groundwater constituents along flow paths can document microbial activity by linking consumption of carbon and energy sources (pollutants or total organic carbon) to both depletion of final electron acceptors (oxygen, nitrate, ferric iron, sulfate, etc.) and production of metabolic byproducts (carbon dioxide, methane, ferrous iron, sulfide, etc.) [6, 9, 16, 21, 36, 41]. Such data are most readily interpreted if performed on samples taken from contaminated and adjacent uncontaminated areas [14, 22]. In addition, microcosm studies can show that native microbial communities possess the ability to degrade contaminants [14, 30] and how metabolically poised the community may be [10], should a limiting nutrient become available.

The role of microorganisms in *in situ* metabolism of naphthalene at a coal-tar-waste-contaminated sandy aquifer in Glens Falls, NY, has been studied extensively [18, 19, 29–31, 34, 43, 45, 47, 48]. Yet, the broader biogeochemical impact of subsurface microorganisms on site geochemistry has remained uninvestigated. The present study was designed to document the physiological responses of naturally occurring microorganisms to coal-tar-waste-constituents and to explore the possible terminal electron acceptor limitations that may govern naphthalene metabolism at the site. Geochemical trends, biodegradation in microcosms, and microscopy-based assays provided evidence for mixed aerobic and anaerobic metabolic processes. A parallel investigation characterizing the microbial community revealed the prevalence of anaerobic microorganisms in areas of coal-tar-waste and naphthalene contamination [2].

Materials and Methods

Field Site and Groundwater Sampling

The study site is in South Glens Falls, NY (e.g., 29–31, 34, 43, 45, 47, 48). The site is a shallow unconfined aquifer (water table at ~2.5 m depth) near the western edge of the Hudson River. Coal tar waste, rich in naphthalene and other aromatic hydrocarbons, was deposited 40 years ago in this flat, sandy, forested, and hydrogeologically simple site. Four wells were positioned linearly along the groundwater flow path. Monitoring well (MW) 4 was upgradient from the original source of contamination; MW8 and MW36 were mid-plume, downgradient of the original source; and MW12 was farthest downgradient. The 1.5-m well screens were centered at 8, 5.5, 6.5, and 5.5 m depths below ground surface (bgs), respectively. Groundwater was pumped from monitoring wells using a Geopump 2 (Geotech Environmental, Denver CO) fitted with a size 15 pump head and silicone tubing (Pt-treated by manufacturer to diminish reactivity) in the peristaltic rollers. New polyethylene tubing was inserted into each well to the screening depth, connected to the pump, and groundwater samples were collected after purging for 4 well volumes at a flow rate of 300 mL/min. Groundwater from the monitoring wells was collected for geochemical analyses only in November 1999 and 2000. In December 1999 all 4 wells were sampled for microcosm and SR-DVC studies. Groundwater from MW36 was collected in September 2000 for the electron acceptor-amended microcosm study. Pristine and contaminated subsurface sediments used to determine $\delta^{13}C$ of the coal tar waste were gathered in 1989 and archived at 4°C.

Geochemical Analyses

The concentrations of oxygen, nitrate, ammonia, sulfate, sulfide, Mn^{2+}, total iron, and Fe^{2+} were measured using the HACH DR/2010 spectrophotometer and appropriate HACH (Loveland, CO) reagent kits. Alkalinity was measured using a HACH Alkalinity Test Kit (Model AL-DT) by titration to pH 4.5. Dissolved oxygen, temperature, and salinity of the groundwater were measured by placing a YSI Model 85 handheld oxygen, conductivity, salinity, and temperature probe in the bottom of a 3-L flow-through water sampling cell. pH was measured using an Accumet (AP63) portable pH Meter (Fisher Scientific, Pittsburgh, PA). Total organic carbon (TOC) was measured on duplicate HCl-fixed (0.025 N) samples using a Shimadzu TOC5000 instrument. For measurements of dissolved inorganic carbon (DIC) the method of Höhener et al. [21] was followed. Forty-mL I-Chem vials (I-Chem 100 series, Hayward, CA) were filled to the brim with groundwater. Two mL was removed and replaced with 0.4 mL 2 M NaOH and 1.6 mL 1.2 M $BaCl_2$ within 30 min of sample gathering. The vials were closed, sealed with electrical tape, and kept at 4°C. Carbonate precipitate in the DIC samples was collected by vacuum filtration onto 0.8 μm pre-size, 47 mm diameter acetate filter membranes (Micron Separations Inc., Westboro, MA); rinsed three times with 100 mL of warm, sterile deionized water; dried at 80°C; weighed; collected in sterile 2-mL glass screw-cap vials; and sent to the Environmental Isotope Laboratory at the University of Waterloo. Groundwater samples (≈30 mL) for methane determination were collected from the field site in sterile 40-mL I-Chem vials prefilled with 3 mL of poison (5% HCl, 0.25 M $HgCl_2$). Vials were weighed before and after sample addition in order to determine the amount of sample added. Triplicate samples were taken from each well and kept upside-down on ice or at 4°C until analysis. Samples (200 μL) were withdrawn from the headspace for analysis by gas chromatography as described previously [49]. The $\delta^{13}C$ values for dissolved methane in MW36 samples were determined as described elsewhere [46]. The $\delta^{13}C$ values for TOC were obtained from duplicate $HgCl_2$/HCl-fixed samples analyzed by the Environmental Isotope Laboratory, University of Water-

loo. The $\delta^{13}C$ values for the coal tar were deduced from quadruplicate samples of contaminated and uncontaminated subsurface sediment [30] analyzed by the Stable Isotope Facility in the Department of Soil, Crop, and Atmospheric Sciences, Cornell University. Calculations solved for the $\delta^{13}C$ value of coal tar after subtracting background TOC and its $\delta^{13}C$ value. The Partial pressure of H_2 in well waters was measured as previously described [7]. Elemental analyses of groundwater constituents (Ca, Mg, 7 Fe, Mn, Na, K, P, S) were carried out on duplicate samples of HCl-fixed (0.025 N) groundwater samples using an IRIS Advantage Inductively Coupled Plasma Spectrograph (Thermo Elemental, Franklin, MA) in the Department of Horticulture, Cornell University.

Microcosms

Sterile 40-mL serum bottles were filled with groundwater so that there was no headspace and capped with sterile, Teflon-lined butyl rubber septa secured with aluminum crimp seals. Samples were incubated at ambient groundwater temperature (10°C), upside-down, in the dark with no shaking. Incubation at 10°C began on site within 20 min of sampling the groundwater and was interrupted only during transport to the laboratory (approximately 4 h at ~20°C). Replicate poisoned control bottles were prepared by adding 1 mL of poison (5% HCl, 0.25 M $HgCl_2$). At various times (0, 1, 5, and 12 days), samples were sacrificed in triplicate and coal tar contaminants extracted by adding hexanes:butanol:HCl (5.4:0.6:1) (HBH). A 2.5-mL glass syringe (sterile 27 gauge, 0.5-inch needle) was used to inject 2 mL of the HBH solution into the serum bottle; a 10 mL syringe (no plunger) with a sterile 23 gauge, 1.5 inch needle caught the displaced groundwater. Bottles were shaken thoroughly and stored upside-down at 4°C. Poisoned controls were sacrificed in triplicate and compounds extracted as above at each sampling time. Blanks consisting of distilled water only and distilled water plus 1 mL of poison were also prepared beforehand, transported to the site, returned, and extracted. All extracted serum bottles were analyzed by gas chromatography/mass spectrometry (GC/MS) as detailed below. All samples from the same well were analyzed at the same time (within 12 h). A fresh standard curve was prepared and analyzed immediately preceding analysis of each batch of samples. In addition, a new septum was installed and the GC/MS was autotuned prior to each batch of samples.

Gas Chromatography/Mass Spectrometry Analysis

After 3 weeks of incubation with HBH, the organic phase in each of the periodically sacrificed serum bottles was removed from the aqueous phase and rapidly transferred to a 2 mL Target DP vial with Teflon-lined rubber septa and screw caps (Agilent Technologies, Santa Clarita, CA). For transfer all glassware that came into contact with the solutions was cooled to 4°C and a new, clean, glass Pasteur pipet was used for each sample.

A Hewlet-Packard Model 7683 AutoSampler delivered 1 μL organic phase to a Hewlett-Packard Model 6890 Series II gas chromatograph equipped with a 30 m × 0.25 mm × 0.25 μm film thickness, HP-5MS (5% phenylmethyl siloxane; Hewlett-Packard) fused silica capillary column connected to a Hewlett-Packard Model 5973 quadrupole mass-selective detector operated at an electron energy of 70 eV and a detector voltage of 1700 [48]. A splitless injection was used, with a 1 min delay before septum purge. The carrier gas was helium (linear velocity of 30 cm/s). Injector and detector temperatures were 250°C and 300°C, respectively. The ion source was at 1.0×10^{-5} torr. The temperature profile was 40°C (1 min), ramp (10° C/min) to 250°C. Standards (1, 5, 25, 50, and 75 ppm) for naphthalene, 4 benzothiophene, 2-methylnaphthalene, *p*-xylene, and indene were diluted from a stock (500 ppm; 100% methanol) into hexanes:butanol (9:1).

Extraction efficiency

Naphthalene in both aqueous and organic phases was monitored following HBH addition to sterile, aqueous naphthalene solutions in serum bottles (neat and diluted 2-fold; prepared by suspending 256 mg naphthalene in 2 L distilled, deionized water and autoclaving within a 4-L sealed flask). Initial naphthalene concentrations were determined by removing triplicate samples from stock solutions immediately before and after filling serum bottles. HBH extractions for GC/MS were incubated for 0, 0.25, 1, 4, 24, 168, 336, and 504 h, upside down, in triplicate and analyzed (as above). Following removal of the organic phase, 0.2 mL of the aqueous phase was removed from each of 3 replicate serum bottles, mixed with 1.4 mL 100% methanol (HPLC grade, Fisher Scientific, Pittsburgh, PA), filtered (0.2 μm pore size, 13 mm diameter Magna nylon membrane, MSI, Westboro, MA), and injected onto a PAH-Hypersil 150 × 4.6 mm HPLC column (Keystone Scientific, Inc., Bellefonte, PA) using a Rheodyne 7125 injector (20 μL sample loop), a Waters Model 590 Pump (Millipore, Bedford, MA), and an LC75 Spectrophotometric Detector (Perkin-Elmer, Norwalk, CT) set at 280 nm. The mobile phase was 70% methanol (1.0 mL/min). The retention time of naphthalene was 7.3 min. Peak area was integrated using a Perkin-Elmer LCI-100 Laboratory Computing Integrator. For calibration of the HPLC, 50, 5, 2, 0.5, and 0.1 ppm standards were prepared in 70% methanol.

Electron Acceptor-Amended Microcosms

Sterile 40-mL serum bottles were filled at the field study site with groundwater from MW36 so that there was no headspace and capped. Poisoned controls were prepared by adding 1 mL of poison (5% HCl, 0.25 M $HgCl_2$) to the serum bottle prior to sample addition. Samples were kept on ice for transport to the laboratory where electron acceptors were added to the microcosms within 9 h of sample collection. To promote aerobic respiration, nitrate reduction, sulfate reduction, or methanogenesis, microcosms were amended with 1.3 mL pure oxygen gas, or anaerobic preparations of 1.3 mL of 5 M $NaNO_3$, 1.2 mL 1 M Na_2SO_4 plus 0.1 mL of 5% Na_2S (reducing agent), or 0.1 mL 5%

Na_2S, respectively [49]. Time zero samples were sacrificed by HBH extraction after electron acceptor addition. Remaining samples were incubated at ambient groundwater temperature (10°C), upside down, in the dark with no shaking. At various times (2, 5, 8, 12, and 20 days), three samples and one poisoned control were sacrificed and processed for GC/MS analysis as above.

Substrate Responsive-Direct Viable Counts (SR-DVC)

SR-DVC was performed as previously described [3] and is briefly summarized here. At the field site, microorganisms in groundwater (25 mL) were concentrated onto 0.2 μm pore size, 13-mm diameter Isopore membranes (Millipore, Bedford, MA) using 10-mL plastic syringes. Membranes were washed with 20 mL of wash buffer [120 mM NaCl, 2.7 mM KCl, 10 mM potassium phosphate buffer at pH 7.6 and cell division-inhibitors (20 mg/L nalidixic acid, 10 mg/L piromidic acid, 10 mg/L pipemidic acid, and 10 mg/L cephalexin)]. Filters were placed immediately on Minimal Basal Salts [42] medium containing antibiotics (20 mg/L nalidixic acid, 10 mg/L piromidic acid, 10 mg/L pipemidic acid, 10 mg/L cephalexin, and 0.1 mg/mL cyclohexamide). Plates were incubated with or without naphthalene at outdoor and later room temperature for 48 h. After incubation, filters were fixed by immersion in 1 mL of 2% formaldehyde/PBS and stored at room temperature. Ten-mL samples were also concentrated and filters fixed at the site for acridine orange direct count analysis. Samples were stained with acridine orange and viewed by epifluorescence microscopy for analysis [3]. As previously described [3], substrate-responsive cells were $\geq$ 4 μm.

Results

Geochemical Analyses

Geochemical analyses were performed on groundwater samples from 4 wells on four sampling dates, although not all parameters were determined on each date. As demonstrated in prior studies [30, 34], naphthalene was present inside the contaminant plume (MW8, 12; with the highest concentration in MW36) and absent in the upgradient MW4 (Table 1). The background DOC concentration at MW4 (Table 1) was low (0.33 ppm) and the increases above background in the other wells were consistent with the release of coal-tar-waste constituents from sediments in the contaminant plume (Table 1). A recently installed well adjacent to MW4 exhibited a vertical oxygen profile (November 2000) ranging from 4.6 ppm just below the water table (3 m bgs) to 3.4 ppm (5 m bgs) to 0.2 ppm (7 m bgs) (T. Taylor, GEI Consultants, Inc., personal communication). Thus, finding an ambient oxygen concentration of 0.6 ppm at 8 m depth in MW4 was not surprising (Table 1). Oxygen was below detection in the near source and midgradient wells (MW 8, 36) and at the limit of detection in MW12 (Table 1). This O_2 depletion, relative to MW4, was likely due to aerobic respiration stimulated by the coal tar constituents.

In subsurface habitats where biodegradation of organic contaminants is suspected, alkalinity measurements can be insightful. As previously elucidated [21, 36, 44], when microbial utilization of organic compounds is linked to aerobic or methanogenic metabolism, no change in alkalinity is expected. However, the four remaining terminal electron accepting processes (TEAPs; reduction of nitrate, Mn oxides, Fe oxides, and sulfate) produce 1, 2, 2, and 2 equivalents, respectively, of alkalinity per mole electron acceptor consumed. The near doubling of alkalinity from background in MW4 (0.59 mM) in the mid-gradient wells MW 8 and 36 (Table 1) indicated a significant level of nonmethanogenic anaerobic metabolism of coal-tar-waste components. Microbial metabolism of the coal tar components via respiratory processes was further confirmed by the increase in DIC along the groundwater flow path: in-plume DIC levels (~3.6 mM) rose 60% above the background level in MW4 (Table 1). Interpretation of the change in alkalinity must be tempered by acknowledging that Ca- and Mg-carbonate minerals can also contribute to DIC [21, 36]. Because Ca and Mg concentrations increased somewhat across the sampling network, some coal tar-facilitated carbonate dissolution from subsurface sediments must have occurred (Table 1). Thus, both biotic and abiotic processes likely contributed to the above-described increase in DIC.

The $\delta^{13}C$ values for DIC (Table 1) can be interpreted by recognizing the signature $\delta^{13}C$ for coal tar (−27.8; based on contrasting $\delta^{13}C$ values of pristine and highly contaminated sediments samples) and by recognizing that the DIC produced from all TEAPs except methanogenesis is expected to match that of the substrate [16, 21]. Thus, if substantial masses of coal-tar-derived DIC were entering the pool, $\delta^{13}C$ values far lighter than the background (MW4 value of −15.04) would be expected. The slight decrease in the $\delta^{13}C$ value of DIC in MW8 (relative to MW4) is suggestive of methanogenesis, but the overall minor variations in $\delta^{13}C$ values across the site indicate that the variety of operating TEAPs counterbalanced one another.

Consistent with the occurrence of nonmethanogenic anaerobic TEAPs and the mid-plume rise in carbonate alkalinity, nitrate concentration was below detection in the

Table 1. Geochemical characteristics of site well waters

Characteristic[a]	Monitoring well			
	4 (upgradient)	8 (near source)	36 (midgradient)	12 (downgradient)
Naphthalene^{2}	bd[b]	0.03 ± 0.01	1.11 ± 0.2	0.02 ± 0.01
DOC^{2}	0.33 ± 0.08	0.98 ± 0.2	1.7 ± 0.3	1.0 ± 0.3
O_2^3	0.6 ± 0.2	bd	bd	0.05 ± 0.02
Alkalinity ^{2} (as mM CO_3^{-2})	0.59 ± 0.1	1.3 ± 0.2	1.2 ± 0.2	0.52 ± 0.1
DIC ^{1} (mM CO_3^2)	2.2 ± 0.1	3.8 ± 0.3	3.7 ± 0.2	3.4 ± 0.4
Ca^{2+} ^{1} (mM)	0.71 ± 0.1	1.1 ± 0.1	1.3 ± 0.1	1.3 ± 0.1
Mg^{2+} ^{1} (mM)	0.22 ± 0.06	0.45 ± 0.04	0.47 ± 0.06	0.54 ± 0.04
$\delta^{13}C$ DIC ^{1}	−15.04 ± 0.1	−12.36 ± 0.1	−15.59 ± 0.1	−14.79 + 0.1
NO_3^{-2}	0.01 ± 0.02	bd	bd	0.2 ± 0.1
Fe^{2+3}	0.03 ± 0.01	0.23 ± 0.06	0.04 ± 0.02	bd
Sulfide ^{3}	0.004 ± 0.002	0.002 ± 0.002	0.028 ± 0.006	0.001 ± 0.001
Mn^{2+} ^{2}	0.044 ± 0.01	0.067 ± 0.02	0.069 ± 0.02	0.056 ± 0.02
SO_2^{-4}	43 ± 11	32 ± 9	43 ± 14	34 ± 8
CH_4^3	bd	81 ± 17	116 ± 22	62 ± 15
$\delta^{13}C$ CH_4^1	nd[c]	nd	−55.51 ± 0.1	nd
H_2^1 (nM)	bd	>30	>30	>30
Fe^{1}	bd	0.154 ± 0.02	bd	0.044 ± 0.01
Mn^{1}	0.036 ± 0.01	0.233 ± 0.01	0.052 ± 0.01	0.785 ± 0.01
S^{1}	11.2 ± 0.1	9.5 ± 0.2	12.2 ± 0.1	11.6 ± 0.2
Na^{1}	8.2 ± 0.5	27.2 ± 0.4	43.7 ± 0.6	10.6 ± 0.6
K^{1}	0.40 ± 0.04	0.72 ± 0.08	0.70 ± 0.06	0.835 ± 0.07
P^{1}	0.041 ± 0.01	0.015 ± 0.005	0.005 ± 0.005	0.005 ± 0.005
pH ^{2}	8.6 ± 0.7	6.8 ± 0.5	7.6 ± 0.8	6.6 ± 0.6
Salinity^{4} (ppt)	0.1 ± 0.01	0.2 ± 0.01	0.2 ± 0.01	0.2 ± 0.01
NH_3^3	0.03 ± 0.02	bd	0.02 ± 0.02	bd
Temperature ^{4} (°C)	9.8 ± 0.3	10.1 ± 0.3	10.2 ± 0.3	10.0 ± 0.3

[a] Superscript indicates the number of determinations performed during site visits (November and December 1999 and September and November 2000); averages and standard deviations are shown; units are ppm (mg/L) unless otherwise noted.
[b] bd = below detection

most contaminated well waters, Fe^{2+} was found at a relatively high concentration in MW8, and sulfide was significantly enriched in MW36 (Table 1). Furthermore, Mn^{2+} concentrations were somewhat elevated (Table 1). Surprisingly, the sulfate concentration remained constant along the groundwater flow path (Table 1). The reason for lack of sulfate depletion may reflect many possibilities, including site heterogeneity and/or insufficient available organic carbon to diminish the large pool of sulfate.

The presence of significant concentrations of methane only in the contaminated well waters (Table 1) provides further evidence for the stimulation of anaerobic metabolism by the contaminants. The $\delta^{13}C$ value for methane, −55.51, measured only in MW36, is consistent with established values for biogenic methane (16) and may indicate acetoclastic-type reactions [13, 16]. The dissolved hydrogen gas concentrations (Table 1) were, as expected, below detection in aerobic MW4 and high values within the plume suggested methanogenic physiological conditions there [7]. Elemental analyses of dissolved Mn, Fe, and S along the flowpath (Table 1) were consistent with field-based assays of the same elements discussed above. Sodium and potassium concentrations (Table 1) rose substantially along the flowpath—possibly reflecting the presence of inorganic salts associated with the coal tar waste. Elemental P (Table 1) dropped markedly within the contaminant plume—possibly reflecting microbial metabolic demand for this nutrient. pH, salinity, ammonia, and temperature fluctuated only in minor degrees along the path of groundwater flow.

Biodegradation in Microcosms

Unamended microcosms were used to determine if the native microorganisms could degrade the ambient coal tar waste constituents accompanied by only the nutrients present in the groundwater. Groundwater was collected on site in serum bottles and incubated at *in situ* temperature (10°C) within 20 min of sampling. The extraction efficiency for naphthalene from water was determined by monitoring the distribution of naphthalene between both the aqueous (by HPLC) and organic phases (by GC/MS) over time following

addition of HBH. When 2.6- and 1.3-ppm naphthalene stock solutions were extracted with HBH at 4°C, the extraction efficiency rose from 30% (4 h) to 96% after 3 weeks. Therefore, all analyses of groundwater samples occurred after extraction periods of at least 3 weeks.

The amount of oxygen that entered microcosms during filling in the field was assessed by comparing the wellhead oxygen concentration to the dissolved oxygen content of samples that had been poisoned at the site. To minimize entry of atmospheric oxygen during the measurement of oxygen in poisoned samples, measurements were performed in an anaerobic glove box. Poisoned samples contained 0.85 mg/L O_2. After accounting for the oxygen reading at the well head, in the added poison, and introduced while filling serum bottles, it was concluded that 0.75 mg/L O_2 was available to microorganisms in the microcosms for respiration.

Biodegradation of naphthalene by native microorganisms at 10°C was seen in all samples where naphthalene was present (MW36, MW8, and MW12) except poisoned controls (Fig. 1 and Table 2). Abiotic losses (e.g., volatilization and/or sorption) were evident in poisoned controls and ranged from 10% to 57% of initial naphthalene present (Fig. 1). Despite abiotic losses, statistically significant differences (t-test, $n = 3$, $P < 0.025$) in naphthalene concentrations between viable and poisoned treatments were detected after 5 days of incubation. After 12 days of incubation, 43 to 72% of the initial naphthalene was biologically removed in the microcosms (Fig. 1 and Table 2). The highest average degradation rate (41 ppb/day) was in MW36, samples where the naphthalene concentration was highest (Table 2). Interestingly, the maximum rate of degradation occurred from 1 to 5 days of incubation for MW36 whereas the maximum rate for MW8 and MW12 occurred during the first day of incubation (Fig. 1). The presence of a lag phase before the onset of naphthalene degradation in MW36 samples may indicate that anaerobic physiological conditions were predominant *in situ*; physiological adjustment to oxygen introduced during sampling (see above) may have required an induction period. Conversely, the absence of a lag phase in MW8 and MW12 samples may indicate that the sediments from which these waters were drawn could possibly have included aerobic microsites.

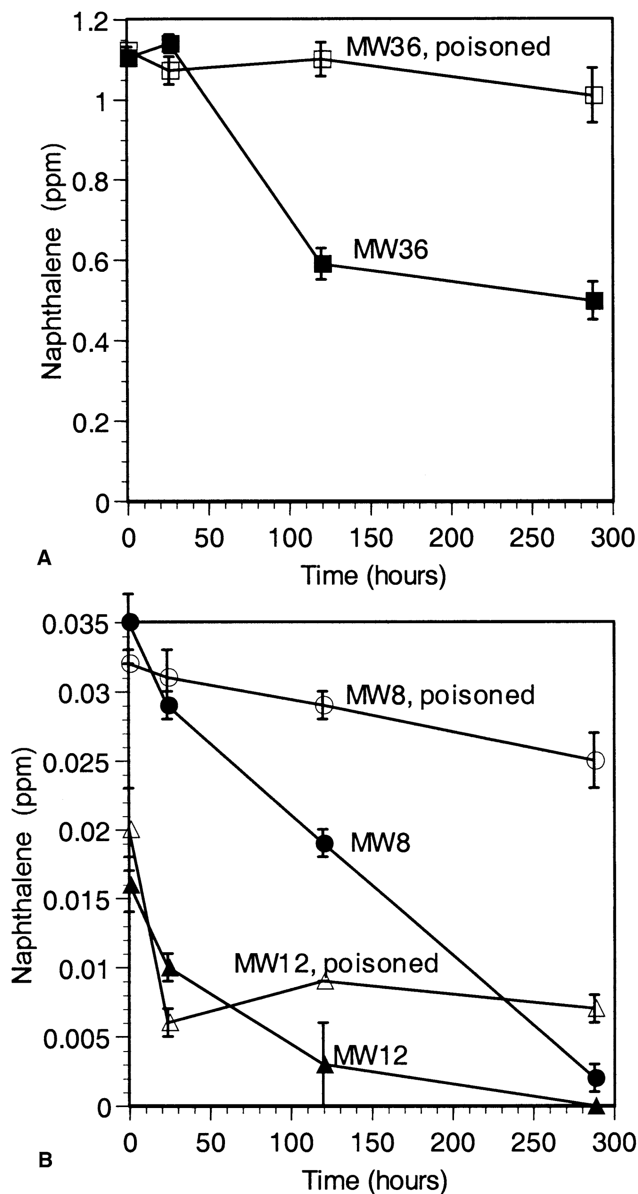

Fig. 1. Naphthalene biodegradation in groundwater microcosms from MW36 (A) and MW8 and 12 (B). Data points represent the average of three sacrificed bottles; error bars represent standard deviations.

Data analogous to those shown in Fig. 1 were obtained for four additional coal-tar-waste constituents (benzothiophene, 2-methylnaphthalene, indene, and p-xylene) present in the three contaminated well waters. A summary of initial concentration, lag phase, percent decrease, and rate of loss for each compound is presented in Table 2. All compounds were not present in all wells. Benzothiophene and 2-methylnaphthalene were only present in MW36 and MW8; indene was present in MW36, MW8, and MW12; and p-xylene was found only in MW36. The degradation patterns of these compounds were similar to the patterns of naphthalene degradation. Although indene was present in MW36, MW8, and MW12, degradation of indene occurred only in samples from MW12. Reasons for this

Table 2. Biodegradation of site-derived compounds by groundwater microorganisms in microcosms incubated at 10°C

Compound	Well	Initial concentration (ppb)	Lag phase (days)	Percent biodegradation[a]	Average rate of loss[a] (ppb/day)
Naphthalene	36	1.11	1	45	41
	8	0.03	0	72	2.2
	12	0.02	0	43	0.32
Benzothiophene	36	33	1	15	0.49
	8	1.0	0	54	0.042
2-Methylnaphthalene	36	27	1	18	0.41
	8	1.8	0	32	0.045
Indene	36	240	—	nsd	nsd
	8	6.7	—	nsd	nsd
	12	8.0	5	35	0.045
p-Xylene	36	420	—	nsd	nsd

[a] Relative to poisoned controls after 12 days of incubation
nsd = no statistically significant difference (*t*-test, $n = 3$, $P < 0.025$) compared to poisoned controls

limited biodegradation activity and the 5-day lag period by MW12 microorganisms are uncertain. No degradation of *p*-xylene was seen in any samples from MW36, the only well that contained *p*-xylene (Table 2). Abiotic losses were evident in poisoned controls and ranged from 0 to 65% of initial concentrations. Despite abiotic losses, statistically significant differences in benzothiophene, 2-methylnaphthalene, and indene concentration between samples and poisoned controls were detected (Table 2). After 12 days of incubation, 15 to 54% of the initial benzothiophene, 2-methylnaphthalene, or indene was biologically degraded by microorganisms in one or more of the wells. The highest average degradation rates for benzothiophene and 2-methylnaphthalene were in MW36 samples, but occurred only after a 1-day lag phase; no lag phases were detected for MW8 samples. Thus, similar to the naphthalene biodegradation data (Fig. 1), lag phase patterns found for biodegradation of benzothiophene and 2-methylnaphthalene suggest that anaerobic physiological reactions predominated in sediments from which MW36 waters were drawn while aerobic microsites may possibly persist at depth in MW8.

Electron Acceptor-Amended Microcosms

Figure 1 clearly reveals patterns of substrate utilization characterized by initial rapid degradation (with or without a lag phase) followed by physiologically limited conditions that cause diminished biodegradation rates. While several hypotheses are consistent with these data (e.g., cell death, predation by protozoa, diauxy, nutrient limitation), the most likely cause was depletion of the O_2 electron acceptor inadvertently introduced during sampling of the groundwater for microcosm preparation. Assuming a 50% respiration efficiency and a stoichiometry of 12 moles O_2 for every mole naphthalene respired, the 0.75 mg/L O_2 would be depleted by 0.5 ppm naphthalene. Thus, O_2 limitation was particularly germane for the highly contaminated MW 36. Two approaches were used to determine whether biodegradation in site microcosms was oxygen limited. The amount of oxygen introduced into microcosms during sampling was estimated (see above) and the effect of oxygen on the biodegradation rates of the microbial communities was compared to the effect of two other potential electron acceptors, nitrate and sulfate. Treatments designed to attain oxygen, nitrate, sulfate, and methanogenic TEAPs were implemented using MW36 microcosms. At the time of sampling, the naphthalene, oxygen, nitrate, sulfate, and sulfide concentrations of the groundwater were 1.0, 0.1, 0.1, 41, and 0.007 mg/L, respectively.

Degradation of naphthalene by native microorganisms at 10°C was seen in unamended, oxygen-amended, and nitrate-amended microcosms, except poisoned controls (Fig. 2). Abiotic losses were evident in poisoned controls and ranged from 5% to 16% of initial naphthalene present. Despite abiotic losses, statistically significant differences in naphthalene concentration between viable and poisoned treatments were detected after 8 days of incubation.

After 12 days, 43, 68, and 44% of the initial naphthalene was biologically degraded in the unamended, oxygen-amended, and nitrate-amended microcosms, respectively (Fig. 2). A 2-day lag phase occurred in all treatments with viable microorganisms. The highest average degradation

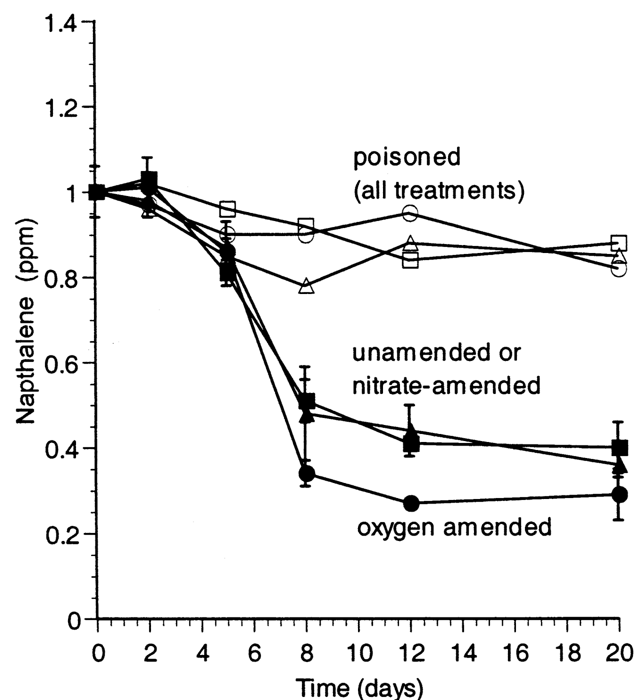

Fig. 2. Naphthalene biodegradation in groundwater microcosms from MW36 supplemented with either oxygen or nitrate. Treatments: unamended (squares), oxygen-amended (circles), and nitrate-amended (triangles). Closed symbols represent viable samples, while open symbols represent poisoned controls. Data points represent the average of three sacrificed bottles (except for poisoned controls); error bars represent standard deviations.

rate (56 ppb/day) was in oxygen-amended microcosms. Only the oxygen-amended microcosms showed enhanced naphthalene loss (rate and extent) relative to unamended and nitrate-amended microcosms (rates of naphthalene loss were both 37 ppb/day). Despite O_2-caused stimulation, naphthalene biodegradation stopped at a concentration of 0.27 ppm — very likely reflecting other nutrient deficiencies in the groundwater (e.g., N or P). Sulfate concentration did not vary in either unamended or nitrate-amended microcosms throughout the length of the experiment (data not shown).

Tests analogous to those shown in Fig. 2 were also completed examining the influence of added sulfide (to encourage methanogesis) and sulfate plus sulfide (to encourage sulfate reduction) on naphthalene biodegradation. Losses of naphthalene in the viable microcosms did not vary significantly from poisoned controls (6 to 14% over 20 days). Elimination of biodegradation activity by the O_2-scrubbing agent, sulfide, again suggested that naphthalene metabolism by the groundwater microbial community was oxygen limited in these assays.

Microscopic Assessment of Naphthalene-Degrading Potential of Native Microorganisms

Because oxygen was found to limit naphthalene metabolism in site groundwater, the aerobic degradation of naphthalene by individual microorganisms was monitored using SR-DVC. SR-DVC should reveal, in a less biased way than culturing, the number of aerobic naphthalene-degrading bacteria present at the site. Samples were collected and processed on site with immediate exposure to cell-division inhibitors and naphthalene. Quadruplicate analysis by AODC of samples from MW4, MW36, and MW12 revealed 3400 ± 210, 110,000 ± 24,000, and 110,000 ± 5,000 cells/mL, respectively. Thus, waters in the two contaminated wells counted exhibited a 30-fold increase in cell density, relative to background (MW4). To assess the ability of native microorganisms to form elongated cells in response to naphthalene vapor in the presence of cell-division-inhibiting antibiotics, elongated cells were counted in the initial community (AODC samples), after exposure to naphthalene, and in a no-substrate negative-control incubation. Interestingly, the community in MW36 (highest contaminant levels) initially contained 1000 ± 320 long cells—between 6 and 40 times more than other monitoring wells. After incubation, differences between elongated cell numbers of naphthalene and no-substrate controls were statistically significant only for MW36 samples (480 cells/mL; t-test, $n = 4$, $P < 0.025$). Therefore, only MW36 contained sufficient numbers of aerobic naphthalene-degrading bacteria to be detected in the SR-DVC assay and lack of response to the assay by microorganisms from MW8, MW12, and MW4 indicated that aerobic, naphthalene-degrading bacteria were either at low titers or required additional nutrients for growth.

Discussion

The complementary nature of field and laboratory-based biodegradation investigations is well established (e.g.,[35, 36]). Trends in groundwater concentrations of electron-acceptor reactants, reduced products, and DIC have been used to identify TEAPs associated with contaminant degradation [5, 14, 21, 22, 36]. For example, aceticlastic methanogenesis was recently confirmed as the final TEAP in petroleum hydrocarbon degradation by identifying the sequential depletion of oxidants (oxygen, nitrate, sulfate)

with a concomitant rise in methane and DIC and by detecting the dominance (up to 26%) of *Methanosaeta*, an aceticlastic methanogen, in groundwater communities with *in situ* hybridization [5]. At a diesel fuel-contaminated aquifer, diminished oxygen and nitrate concentrations in the contaminated area along with elevated DIC concentrations were used to document the degradation of petroleum hydrocarbons via aerobic respiration and denitrification; these conclusions were verified by isolating $>10^6$ aerobic and denitrifying petroleum hydrocarbon-degrading organisms per gram, identifying *Azoarcus* species among the isolates, confirming the abundance of *Azoarcus* (1% of the community) by hybridization, and demonstrating petroleum hydrocarbon degradation concomitant with electron acceptor loss in microcosms [21]. In another contaminated aquifer, microbial degradation of BTEX via sulfate reduction was assessed via changes in alkalinity, oxygen, sulfate, ferrous iron, methane, and hydrogen, and confirmed by the detection of putative metabolites in groundwater and by the degradation of BTEX in laboratory sulfate-reducing microcosms [14].

In prior investigations of the Glen Falls, NY study site (e.g., [29–31, 47]), only aerobic processes had been reported. However, in this study, the *in situ* depletion of oxygen and the production of methane, hydrogen, alkalinity, and DIC in the contaminated zone establish microbial metabolism of the coal-tar-waste constituents to be by both aerobic and anaerobic TEAPs. Unamended microcosms from MW8, 36, and 12 demonstrated the simultaneous degradation of several coal tar components under simulated *in situ* conditions and lag phases prior to the onset of biodegradation were consistent with various TEAPs *in situ*. However, electron acceptor-amended microcosms from MW36 gave no indications of anaerobic naphthalene degradation. Anaerobic naphthalene metabolism has been reported in a variety of pure and mixed culture studies [12, 33, 39, 51]. Given the 40-year age of contamination at this site, we were surprised to find no evidence of anaerobic naphthalene catabolism. Absence of evidence in the groundwater-based assays reported here should be interpreted carefully. Several factors should be considered: possible toxicity of O_2 introduced to the microcosms, "oxygen sparing" in which O_2 participates only the initial dioxygenase attack on the aromatic ring [23], or *in situ* naphthalene catabolism only on the aerobic fringes of the contaminant plume. Resolution of these competing explanations will require additional experimentation using anaerobically sampled site sediments.

Clearly, the biodegradation of many constituents of the coal tar waste supports the anaerobic TEAPs at the study site. Monocyclic aromatic hydrocarbons (MAHs), such as benzene, toluene, ethylbenzene, and xylenes, can be degraded under iron-reducing [24, 40], sulfate-reducing [11, 14, 33], and methanogenic conditions [14, 15]. There is also limited data on the anaerobic degradation of the following polycyclic aromatic hydrocarbons: naphthalene [8, 33, 39], 2-methylnaphthalene [8, 33, 51], acenaphthylene [37], acenaphthene [37], fluorene [8], phenanthrene [32, 37, 51], anthracene [32], fluoranthene [8], and pyrene [32]. These MAHs and PAHs are all constituents of the coal tar waste at this study site (amounting to 14.6% of the total mass [45]).

Use of groundwater samples to assess biogeochemical processes has limitations. Besides microbial reactions, advective transport, dispersion, and inorganic reactions can also affect concentrations of water constituents [4]. For example, methane can be transported long distances, making the exact location and extent of methanogenic zones difficult to determine. In addition, wells with broad screening depths may sample pore waters from many physiological zones [4, 14]. Moreover, the planktonic microorganisms may or may not be representative of the total (attached and planktonic) microbial community in the aquifer [17, 28]. In a study of a crude oil contaminated aquifer, the TEAPs indicated by physiologic types of bacteria generally followed the contours of dissolved O_2 and organic carbon, while planktonic bacteria represented 15% of the total population and displayed the same ratios and numbers of physiologic types of bacteria as the sediment in most samples [4].

Results from a parallel study on groundwater microbial diversity are consistent with our conclusions that a variety of aerobic and anaerobic processes other than aerobic naphthalene degradation predominate at the site [2]. Future studies, perhaps using whole sediment samples, will strive to confirm data presented here that suggest dynamic biogeochemical processes govern the fate of organic contaminants.

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