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Biodegradation of Three- and Four-Ring Polycyclic Aromatic Hydrocarbons under Aerobic and Denitrifying Conditions

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PAHs are thought to be particularly persistent in environments where anaerobic conditions exist. This study presents evidence for the biodegradation of three- and four-ringed PAHs (anthracene, phenanthrene, and pyrene) under strict anaerobic, denitrifying conditions. Three pseudomonad strains, isolated from contrasting environments, were used in this study. All three strains were known PAH degraders and denitrifiers. Degradation proceeded to nondetectable levels (<0.001 mg/L) in 12−80 h for anthracene, 12−44 h for phenanthrene, and 24−72 h for pyrene. The rates of anaerobic degradation were typically slower than under aerobic conditions in almost all cases, except for strain SAG-R which had similar removal rates for all three and four-ring PAHs. Denitrification activity was verified by monitoring nitrate utilization and nitrous oxide production. Although none of the pseudomonads were adapted to the denitrifying conditions, only the pseudomonad isolated from a noncontaminated site (strain KBM-1) consistently exhibited an adaptation period which approximated 12 h. This study supports growing evidence that the degradation of aromatic hydrocarbons coupled to denitrification may be an important factor affecting the fate of these compounds in natural and engineered systems.

Introduction

There is concern about polycyclic aromatic hydrocarbons (PAHs) due to their potential hazardous properties, recalcitrance, and prevalence in the environment. They are produced by natural and anthropogenic processes, such as by the incomplete combustion of fossil fuels and as a byproduct of coke production and petroleum refining (*1*). Due to their hydrophobicity, they partition strongly into nonaqueous phases which decreases their general availability and rate of degradation (*2, 3*). PAHs may also migrate to chemically reducing environments where only anaerobic conditions are present.

Various monoaromatic hydrocarbons degrade under anaerobic conditions (*4*−*6*). For instance, degradation of oxygenated and alkyl aromatic hydrocarbons (e.g., benzoate, hydroxybenzoic acid, phenols, and cresols) under denitrifying conditions is relatively well-established (*7*−*13*). However, information on the degradation of PAHs under denitrifying conditions is scarce. Biodegradation of naphthalene and acenaphthene under denitrifying conditions has been observed by some researchers (*14*−*16*); however, the degradation of naphthalene under denitrifying conditions has also not been observed (*17*).

Accordingly, the objectives of this study were to investigate the potential for degradation of the three- and four-ring PAHs (anthracene, phenanthrene, and pyrene) under denitrifying conditions by organisms in pure culture and to compare aerobic and anaerobic degradation rates and adaptation periods by isolated organisms.

Experimental Section

Materials. All chemicals were reagent grade or better. Naphthalene, phenanthrene, and pyrene were obtained from Sigma Chemical Co., and acenaphthene, anthracene, glycerol, and *N*,*N*-dimethylformamide were obtained from Fisher Scientific Co. All media and glassware were sterilized by autoclaving for 20 min at 120 °C and 15 psi prior to use.

Organism Isolation, Characterization, and Identification. Three microorganisms in pure culture were used in this study. These microorganisms were selected for their previously demonstrated aerobic growth on PAHs and their ability to grow anaerobically under denitrifying conditions (*18*−*19*). Two microorganisms, strains SAG-R and W-2, were isolated from contaminated sites, and the third microorganism, KBM-1, was isolated from a site with no previous direct contamination to PAHs.

SAG-R was isolated by an enrichment technique on carbazole from soil obtained from a creosote contaminated hazardous waste site (*20*). A sample of 500 mg of soil was suspended in 5 mL of minimal medium basal salts medium (BSM), vortexed for 30 s, and left to clarify for 15 min. BSM contains 4 g of K_2HPO_4, 4 g of Na_2HPO_4, 2 g of $(NH_4)_2SO_4$, 0.2 g of $MgSO_4 \cdot 7H_2O$, 1 mg of $CaCl_2 \cdot 2H_2O$, and 1 mg of $FeSO_4 \cdot 7H_2O$ per liter of distilled water and is adjusted to pH 7 with H_2SO_4. An enrichment media, consisting of 10 mL of BSM supplemented with 0.1% (w/v) carbazole solubilized in a minimal amount of *N*,*N*-dimethyl formamide (DMF), was inoculated with 100 μL of the clarified suspension. It has been shown in our laboratories that DMF, when used at concentrations less than 2.5% (v/v), is not utilized as a source of carbon and is not growth-inhibitory (*21*). The culture was then incubated at 30 °C in a gyrotory water bath shaker. After 4 days, the visually turbid enrichment culture was subcultured three times and individual carbazole-utilizing isolates were obtained in pure culture by repeated streak plating on solidified BSM supplemented with 0.01% carbazole solubilized in DMF. The isolates were subsequently maintained on NA (nutrient agar) plates and stored as slant cultures under oil at 4 °C and as frozen stock cultures at −70 °C in liquid LB (Luria Bertani) medium adjusted to 30% (w/v) glycerol. SAG-R has the novel ability to grow aerobically on pyrene, a four-ringed PAH, as a sole source of carbon and energy (*18*). Growth on pyrene was demonstrated when increases in turbidity and total cellular protein were observed within 24 h of incubation. Flasks (50 mL) containing 10 mL of BSM and 0.01% (w/v) pyrene in 100 μL of DMF were inoculated with a 100 μL cell suspension to yield an initial cell density of approximately 10^8 cells/mL. The cells had been adapted to log phase growth on glycerol (1.5% w/v) and BSM and washed 4 times prior to inoculation. Control

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incubations demonstrated that residual glycerol was not present in the washed cell suspension. Growth was monitored by visibly determining changes in culture turbidity.

W-2 was isolated at the Michigan Technological University Institute of Wood Research from the same soil samples used to isolate SAG-R. This microorganism was isolated using enrichment techniques on naphthalene similar to the ones described for SAG-R. W-2 demonstrated the ability to aerobically degrade low molecular weight PAHs. KBM-1 was isolated by a spread plate technique from sediment samples collected in the Keweenaw Bay of Lake Superior. The isolation technique was originally used for the purpose of characterizing the microbial population at the site and is described in detail by McNally et al. (*19*). Though PAHs have been detected at background concentrations in sediments of Keweenaw Bay (*22*), this site is considered noncontaminated.

Denitrifying potential was examined using fluorescence-denitrification (FN) media (*23*). Liquid FN medium and solid FN medium slants were inoculated and incubated for 24−48 h. The FN medium consisted of 10.0 g of proteose peptone number 3, 1.5 g of $MgSO_4 \cdot H_2O$, 1.5 g of K_2HPO_4, 2.0 g of KNO_3, 0.5 g of $NaNO_2$, and 15.0 g of agar (for solid medium) per liter of distilled water and adjusted to pH 7 using H_2SO_4. Denitrification was monitored by nitrogen gas formation in an inverted Durham tube in the liquid medium and at the bottom of the tube containing solid medium.

Gram stains, cytochrome oxidase tests, and other standard biological tests were conducted using conventional microbiological procedures. Identification and further characterization of the microorganisms were conducted using a commercial identification system from Biolog, Hayward, CA. The Biolog Identification System is an automated microbiological identification and characterization system used to identify microorganisms.

Preliminary examination revealed that SAG-R, W-2, and KBM-1 cells are motile, oxidase positive, Gram-negative rods. When grown on solidified LB media, SAG-R colonies strongly adhered to the media and displayed a rough texture, W-2 colonies were round and flat, and KBM-1 colonies were mucoidal, displaying a probable slime layer. SAG-R was previously identified as a *Pseudomonas stutzeri* using an Oxiferm (Roche Diagnostics, New Jersey) commercial identification test (*20*). The commercial identification system Biolog confirmed the identification of SAG-R as a *P. stutzeri*, and identified W-2 as a *Pseudomonas fluorescens* and KBM-1 as a *Pseudomonas putida* with similarity indices (SIM) after 24 h of 0.727, 0.598, and 0.876, respectively. Since a SIM of 0.500 is the baseline for a positive match, SAG-R and KBM-1 were identified with a high degree of certainty. The biochemical test results used to identify and further characterize these organisms are provided in McNally (*18*).

PAH Degradation under Aerobic and Denitrifying Conditions. To investigate the aerobic biodegradation of PAHs, 10-mL cultures were prepared using 15-mL glass centrifuge tubes with screw caps and Teflon-lined septa. The culture medium consisted of BSM supplemented with the PAHs solubilized in 100 μL of DMF in the following two mixture combinations. Strains W-2 and KBM-1 were tested with naphthalene, acenaphthene, anthracene, and pyrene, and strains SAG-R and KBM-1 were tested with naphthalene, phenanthrene, and pyrene. Initial PAH concentrations were at the water solubility level for acenaphthene (3 mg/L), anthracene (0.045 mg/L), phenanthrene (1 mg/L), and pyrene (0.13 mg/L). Naphthalene was tested at a concentration of 3 mg/L. Naphthalene and acenaphthene were added to enable the detection of nitrate utilization. They also would have utilized any trace oxygen prior to the onset of the degradation of 3- and 4-ring PAHs.

Each organism was adapted to exponential growth on 1.5% (v/v) glycerol in BSM and washed four times in BSM before being used as an inoculum. The final cell density in each experimental culture was approximately 2.5×10^8 cells/mL when measured by a Klett−Summerson photoelectric colorimeter. A relatively high cell density was chosen to ensure nongrowth conditions and to shorten the duration of the experiment. Microbial growth could not be supported at this high cell density due to the low concentration of the carbon source (PAHs). Control incubations either with no microbial inoculum or inoculated with cells and 0.1% (v/v) sodium azide as a metabolism inhibitor were prepared to monitor the abiotic loss of the PAHs. These control cultures were placed in an incubation tumbler and rotated at 15 rpm in the dark at 20 °C.

Anaerobic biodegradation of PAHs under denitrifying conditions was examined using the same microorganisms and PAH mixture combinations that were used in the aerobic degradation experiments. All media was carefully deoxygenated prior to use, and samples were prepared under a N_2/CO_2 atmosphere. Preliminary studies to determine the configuration of the anaerobic experiments showed that thick butyl rubber stoppers caused an unacceptable loss of the hydrophobic PAHs. The Teflon-lined septum/screw cap system employed in this study was rigorously tested for its ability to maintain anaerobic conditions. This was first done by preparing anaerobic samples without microbial inoculum and incubating them for 10 days, which was 5−7 days longer than any anaerobic degradation test. Samples were monitored for DO after the 10-day period and showed no oxygen, thus indicating that the experimental system was impermeable to oxgyen. In addition, anaerobic controls inoculated with microbes, but sterilized with sodium azide, never showed the presence of oxygen when sampled at the end of an experiment.

The microorganisms adapted to the denitrifying conditions after being cultivated on 1.5% (v/v) glycerol in BSM. As required for denitrification, trace elemental substances (TES) and nitrate were added to the BSM medium. TES consists of 0.07 g of $ZnCl_2$, 0.1 g of $MnCl_2 \cdot 4H_2O$, 0.062 g of H_3BO_3, 0.19 g of $CoCl_2 \cdot 6H_2O$, 0.035 g of $CuSO_4 \cdot 5H_2O$, 0.024 g of $NiCl_2 \cdot 6H_2O$, 0.036 g of $Na_2MoO_4 \cdot 2H_2O$, and 5.2 g of EDTA per liter of distilled water and adjusted to pH 7 using H_2SO_4 before adding 1 mL/L of the BSM medium. Potassium nitrate was added so that the initial nitrate concentration in each reactor was 60 mg of NO_3^-/L. The stoichiometric nitrate requirement for complete mineralization of all the PAHs assuming a nitrate end product of N_2 was approximately 24 mg of NO_3^-/L for the mixture of three PAHs and 36 mg/L for the mixture of four PAHs.

To remove oxygen, the BSM medium was sparged in a glovebox with a 95% nitrogen and 5% carbon dioxide gas mixture immediately after autoclaving. Sparging continued until an oxygen probe indicated zero DO. The 15-ml culture tubes were filled with 10 mL of BSM and the PAHs solubilized in 100 μL deoxygenated DMF before inoculation. Microbial inocula and DMF were also deoxygenated prior to use by placing them in the glovebox and monitoring the DO until nondetection. The PAHs and microorganisms were added at the same concentrations as in the aerobic degradation experiments. Prior to sealing the culture tubes with Teflon-lined septa and screw caps, acetylene inhibition was employed by purging the headspace of all culture vessels with a carbon dioxide and nitrogen gas mixture and 10% (v/v) acetylene. This method causes an accumulation of nitrous oxide by inhibiting the nitrous oxide reductase. In addition to detecting the production of nitrous oxide, the loss of nitrate was also monitored to verify denitrification. Like the aerobic experiments, the sample and control culture reactors were

placed in the incubation tumbler and rotated at 15 rpm in the dark at 20 °C.

Samples from aerobic and anaerobic experimental and control incubations were periodically removed in duplicate for analysis. Each experimental incubation was monitored for microbial contamination, and control incubations were checked for microbial growth using the spread plate technique. A DO meter with an oxygen detection limit of 0.1 mg/L was used to verify the absence of oxygen. DO was never detected in anaerobic controls which demonstrated that the experimental system was impermeable to oxgyen. After harvesting the cells by centrifugation at 1400*g* for 10 min, 4 mL of the aqueous supernatant was withdrawn and the PAHs were extracted for analysis. The extraction procedure followed a modified Bligh and Dyer procedure as described by Ames (*24*) using chloroform and methanol as the extracting solvents. The ratio of 1/2/0.8 (v/v/v) of chloroform/methanol/aqueous sample was increased by a factor of 5 to provide sufficient sample for analysis. The extraction was conducted using 40-mL sample vials with screw caps and Teflon-lined septa. After final mixing, both phases were allowed to clarify for 10 min before aliquots of the PAH−chloroform mixture were placed in GC vials for analysis.

Analysis for PAHs, Nitrate, and Nitrous Oxide. PAH concentrations were determined according to a modified EPA Method 625 using a Hewlet Packard model 5890 series gas chromatograph interfaced with a Hewlet Packard model 5970 series mass spectrometer detector (GC/MS) in single ion monitoring (SIM) mode. The column was a 30 m long, 0.25 mm i.d., J&W Scientific DB-5 capillary column. The injection and detector port temperatures were 275 °C, and the oven temperature was ramped at 25 °C/min from 65 to 140 °C and then 10 °C/min to a final temperature of 290 °C. The detection limit for all PAHs was 0.001 mg/L.

Nitrate utilization was verified using a Dionex IC-10 ion chromatograph (IC) equipped with a Dionex automated sampler and a Thermo Separation Products SP4290 integrator to determine nitrate concentration. Dionex AG9-S C and AS9-SC columns were placed in series. The 50 mM sulfuric acid regenerent's flowrate was 3.5 mL/min, and the 1.8 mM sodium carbonate/1.7 mM sodium biocarbonate eluent's flowrate was 1.15 mL/min. A 4-mL sample withdrawn from the supernatant of each reactor had to be diluted 20-fold before running the analysis.

Nitrous oxide was monitored using the GC/MS in SIM mode. The presence of an elevated concentration of nitrous oxide confirmed that denitrification had occurred. Therefore, the GC/MS conditions, set for PAH analysis, were not altered. A 10-μL sample of the headspace was withdrawn from randomly selected reactors using a gastight syringe and manually injected into the GC.

Results and Discussion

Aerobic Degradation of Three- and Four-Ringed PAHs. To ensure each microorganism had the ability to aerobically degrade three- and four-ringed PAHs within the duration of the experiment (approximately 200 h) and for comparison to the anaerobic degradation results, studies were conducted to examine the rate and extent of the aerobic degradation of anthracene, phenanthrene, and pyrene with pure cultures in batch reactors. The results of these aerobic degradation experiments are presented in Figure 1, parts a, b, and c, for the three PAHs, respectively. Aerobic degradation potential was investigated for anthracene using W-2 and KBM-1, phenanthrene using SAG-R and KBM-1, and pyrene using all three microorganisms. These three PAHs were initially present at concentrations equal to their water solubility limits, and along with naphthalene and acenaphthene, were the only available carbon sources. Figure 1, parts a and b, shows

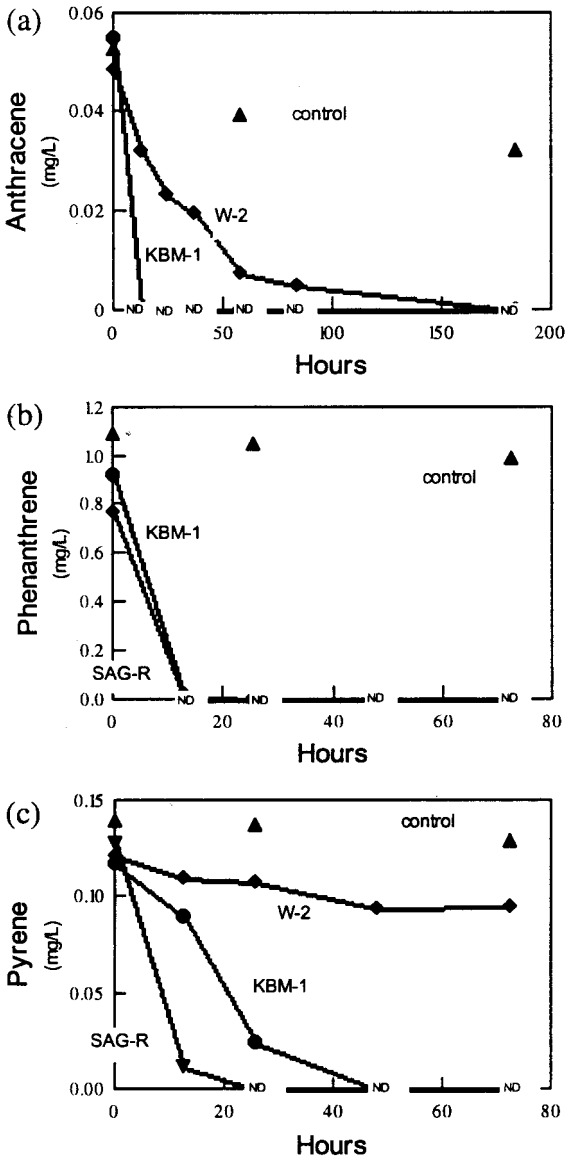

FIGURE 1. Aerobic degradation of (a) anthracene by W-2 and KBM-1, (b) phenanthrene by SAG-R and KBM-1, and (c) pyrene by SAG-R, W-2, and KBM-1 under no growth conditions. All three PAHs had an initial concentration equal to their water solubility limit. The cell concentration was approximately 2.4 × 10^8 cells/mL. (ND is nondetectable, 0.001 mg/L; 95% confidence intervals approximated the size of the symbols, and therefore, for clarity, were not shown).

that all three microorganisms degraded the three-ringed PAHs (anthracene and phenanthrene) from their water solubility limits to nondetectable levels (0.001 mg/L). Naphthalene was always degraded to nondetection levels within 6−8 h, prior to the onset of degradation of the other PAHs (data not shown). Acenaphthene took 40−50 h to reach nondetectable levels. However, W-2 required over 84 h to completely degrade anthracene, while KBM-1 took less than 12 h. Phenanthrene was degraded to a nondetectable level in approximately 12 h by SAG-R and KBM-1. Pyrene, the four-ringed PAH, was completely degraded by SAG-R within 24 h and by KBM-1 within 60 h. W-2 did not appear to degrade pyrene over the course of the 240-h experiment (data for only the first 70 h is shown). The level of the PAHs in control incubations remained constant over the 240-h duration of the test period, indicating that no significant loss of PAHs occurred due to abiotic processes. This observation was identical for both the aerobic and the anaerobic experiments. The DO concentrations of the last samples to be analyzed

indicated that aerobic conditions were maintained throughout the experiment's duration. In fact, when measured by a DO probe, the oxygen utilized after the PAHs had been degraded consistently approximated the theoretical stoichiometric demand.

As expected, organisms initially grown on glycerol as the sole carbon source did not require an adaptation period for the utilization of PAHs. It has been our experience that pseudomonads that utilize naphthalene as a carbon source display short, if any, lag periods when transferred from medium containing glycerol as the sole source of carbon, as opposed to glucose as a source of carbon, to medium containing naphthalene as the sole source of carbon after a thorough wash. This seems logical since glycerol is not as good a carbon source as glucose and probably does not promote severe catabolite repression. Thus, the enzymes of naphthalene catabolism, although always produced constitutively at some low level, are probably modulated to occur at a higher, although not fully induced, level in glycerol grown cells. However, we have not measured these enzyme levels directly. Also, we have observed that cells grown on solidified minimal medium with glycerol as the carbon source are able to simultaneously catabolize DBT, dibenzothiophene, while no DBT catabolism occurs in the presence of glucose. Thus, as a weak repressor, but reasonable carbon source, glycerol was selected for the routine growth of the isolates. Additionally, by using glycerol, the cells were able to be quickly grown to the required density for inoculation.

The immediate utilization of the PAHs also caused the difference in the PAH concentrations at the stated time 0 between the controls and the samples. This difference reflects the time required to inoculate all the samples which ranged up to 3 h. PAH sorption by the cells may also have contributed to this difference. Whitman et al. (*25*) reported an average between 2% and 5% of a soluble naphthalene concentration immediately biosorbed to a comparable cell density of a *P. fluorescens*. In this study, pyrene may initially sorb to the cells in greater amounts because it is much more hydrophobic than naphthalene. Although PAHs could still be detected in the control incubations using these same extraction procedures, they could not be detected in the incubations once they had been degraded to nondetectable levels in the supernatant. This indicates that the PAHs bound to the cells and other surfaces of the culture reactors were eventually utilized by the microorganisms. Analysis of the supernatant taken from the control incubations lacking cells indicated that approximately 10% of the pyrene was lost due to abiotic causes. Further, the majority of this abiotic loss was shown to be due to adsorption to the incubation vessel and septa.

Anaerobic Degradation of Three- and Four-Ringed PAHs under Denitrifying Conditions. Experiments were conducted to examine the rate and extent of anaerobic degradation of anthracene, phenanthrene, and pyrene under denitrifying conditions. The results of these experiments are presented in Figure 2, parts a, b, and c, for anthracene, phenanthrene, and pyrene, respectively. These results showed that all three experimental organisms degraded the three-ringed PAHs from their water solubility limit to nondetectable levels (0.001 mg/L) under denitrifying conditions. Naphthalene was again reduced from 3 mg/L to nondetectable levels in 6−8 h with all three organisms while acenaphthene took up to 80 h to reach nondetectable levels (data not shown). Except for the degradation of anthracene by W-2 and the degradation of all PAHs by SAG-R, the anaerobic degradation rates for the three- and four-ringed PAHs were slower than the aerobic degradation rates. W-2 required approximately 40 h to anaerobically degrade anthracene, while KBM-1 degraded anthracene in less than 60 h. Phenanthrene was degraded to nondetectable levels in

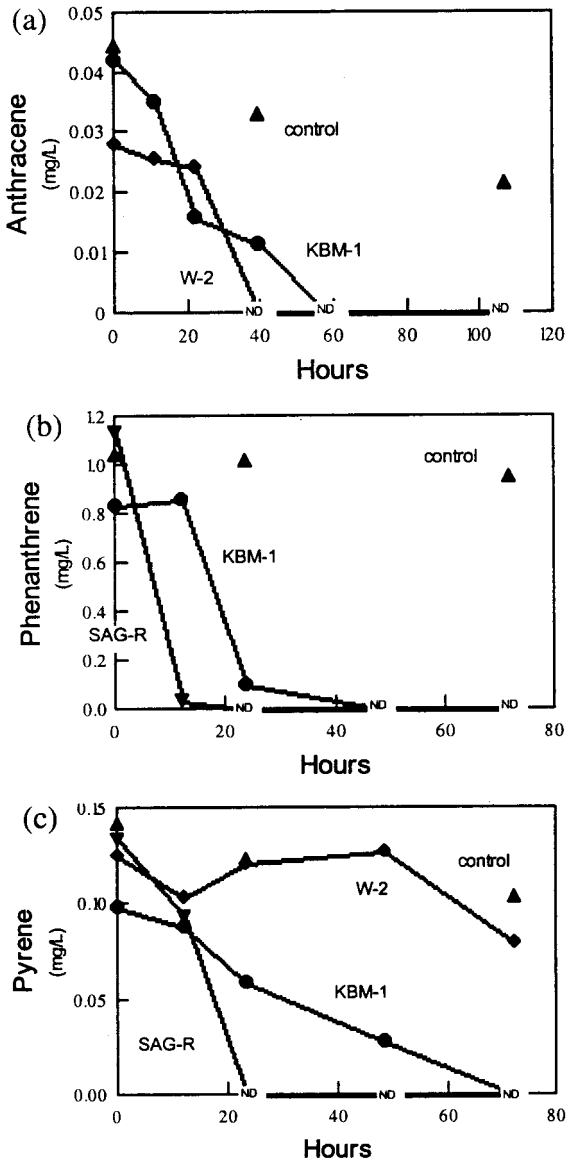

FIGURE 2. Anaerobic degradation under denitrifying conditions of (a) anthracene by W-2 and KBM-1, (b) phenanthrene by SAG-R and KBM-1, and (c) pyrene by SAG-R, W-2, and KBM-1 under no growth conditions. All three PAHs had an initial concentration equal to their water solubility limit. The cell concentration was approximately 2.4×10^8 cells/mL. (ND is nondetectable, 0.001 mg/L; 95% confidence interval approximates the size of the symbols, and therefore, for clarify, were not shown).

slightly longer than 12 h by SAG-R and 44 h by KBM-1. The four-ringed PAH, pyrene, was completely degraded by SAG-R within 24 h and by KBM-1 within 72 h. As with the aerobic experiments, W-2 did not appear to anaerobically degrade pyrene under denitrifying conditions throughout the 240-h experiment (data for only the first 70 h is shown).

In contrast to the aerobic experiments, W-2 displayed an adaptation period before degradation occurred for anthracene and KBM-1 displayed an adaptation period for phenanthrene. These adaptation periods lasted between 12 and 24 h. The remaining experiments, like the aerobic experiments, did not show evidence of an adaptation period. Generally, the cells quickly adapted to the new denitrifying conditions. Therefore, the aerobic cultivation of each microorganism on glycerol proved to be a satisfactory method to prepare cultures for these anaerobic experiments. The differences in initial PAH concentrations for the experimental and control incubations and decrease in control and blank

TABLE 1. Nitrate Utilization in Anaerobic Experiment Using Strains W-2 and KBM-1[a]

time (h)	KBM-1 (mg of NO_3^-/L)	W-2 (mg of NO_3^-/L)	control (mg of NO_3^-/L)
0	60	60	60
<3	48	42	63
12	43	41	
24	42	42	
39	42	42	61
58	39	39	
107	40	39	58

[a] The stoichiometric nitrate requirement for nitrate reduction to N_2O for the mixture of four PAHs (naphthalene, acenaphthene, anthracene, and pyrene) used in this experimental system was approximately 24 mg of NO_3^-/L. Samples represent the average of duplicates.

reactor concentrations were also observed and are attributed to the same causes discussed for the aerobic experiments.

Evidence For Denitrification. Verification that denitrifying conditions existed was established by a variety of methods. First, samples were prepared in a glovebox continuously flushed with a carbon dioxide/nitrogen gas mixture, and strict anaerobic conditions were maintained throughout the experiment. Sample preparation occurred only after oxygen could not be detected in any solution used to prepare samples (sensitivity, 0.1 mg/L which equates to 0.001 mg of O_2/sample). DO was also never detected in the supernatant withdrawn from any sample incubated under denitrification conditions, including controls which were incubated for 5−7 days longer than the time required for active samples to reach nondetectable levels. Based on the stoichiometric oxygen demand, aerobic mineralization would require 0.12 mg of O_2/sample for the mixture containing three PAHs and 0.18 mg of O_2/sample for the mixture containing four PAHs. Naphthalene alone would require 0.09 mg of O_2/sample. Because naphthalene has been shown to degrade before three- and four-ringed PAHs under aerobic conditions, any residual oxygen would have been quickly utilized prior to the onset of degradation of three- and four-ring PAHs. In addition nitrate loss in anaerobic samples commenced immediately after sample preparation.

Monitoring the loss of nitrate provided direct confirmation that denitrification occurred in these experimental systems. Table 1 shows that the nitrate concentration decreased over time in experiments with the KBM-1 and W-2 isolates. Nitrate loss was not monitored in the tests with SAG-R. The results in Table 1 show approximately 20 mg/L NO_3^-, or 80% of the stoichiometric nitrate requirement for reduction to N_2. However, the nitrate utilization in the first few hours of the test corresponds to the immediate degradation of naphthalene. Corresponding with the complete degradation of all the PAHs, the nitrate concentration for both systems appeared steady after 58 h.

The acetylene block technique was another method used to confirm that denitrification occurred. The addition of acetylene inhibits the nitrous oxide reductase which results in an accumulation of nitrous oxide, a reduced product of nitrate during denitrification. Although not quantified, the accumulation of nitrous oxide was observed and was coupled to nitrate disappearance. Nitrous oxide was never detected in control incubations. Because nitrous oxide is a product of denitrification, the presence of nitrous oxide coupled with the disappearance of nitrate supports the conclusion that PAH degradation was coupled to denitrification.

The main objective of this study was to investigate the potential for degradation of three- and four-ringed PAHs (anthracene, phenanthrene, and pyrene) under denitrifying conditions. The PAHs were added at water solubility concentrations to batch aqueous systems containing 2.5 × 10^8 cells/mL of pure cultures. The following three pseudomonads were examined for their ability to degrade PAHs under denitrifying conditions: *P. fluorescens* W-2, *P. putida* KBM-1, and *P. stutzeri* SAG-R. This study differs from others who have investigated PAH degradation under denitrification conditions because we used high cell densities of pure microbial strains well adapted on glycerol, compared to others who have typically utilized uncharacterized sediment-water slurries.

Prior to this study, very little work on denitrification, as a metabolic process to oxidize PAHs, had been conducted. Bouwer and McCarty (*17*) found no utilization of naphthalene by sewage microorganisms incubated under anoxic conditions with nitrate. Mihelcic and Luthy (*16*) were the first to show degradation of lower molecular weight PAHs under denitrifying conditions. They observed degradation of naphthalene and acenaphthene in a sediment-water slurry to nondetectable levels (<0.01 mg/L) in less than 9 weeks. This included an adaptation period of 12−36 days using soil samples not previously exposed to PAHs and denitrifying conditions. They concluded that the adaptation period was a result of the time required to attain sufficient densities of PAH-degrading microorganisms. Adaptation periods were absent with soil that had been previously exposed to PAHs. Al-Bashir et al. (*14*) conducted a similar study that demonstrated naphthalene degradation under denitrifying conditions. They showed that 90% of naphthalene, at an initial concentration close to the aqueous phase saturation level, mineralized within 50 days with an adaptation period of approximately 18 days. They observed that pre-exposure of soil to oil contamination did not improve the degradation rates and, contrary to Mihelcic and Luthy, did not reduce the adaptation periods.

Earlier work in our laboratories also confirmed the degradation of naphthalene and acenaphthene under denitrifying conditions (*18, 19*). These results showed that naphthalene and acenaphthene were reduced from 3 mg/L to nondetection (<0.001 mg/L) by all three microorganisms. The extent and rate of naphthalene and acenaphthene degradation by pure cultures under denitrifying conditions in this study approached that under aerobic conditions.

One reason so few researchers have studied the potential of contaminate degradation under denitrifying conditions is the difficulty of working with cultures under strict anaerobic conditions. Moreover, confirmation that contaminants are being degraded as a result of denitrification is sometimes difficult to assess. Most studies have monitored nitrate loss and used aromatic compounds which are much more soluble in water than the three- and four-ringed PAHs used in this study (*7−11, 14, 16, 17, 26−32*). The resulting higher concentrations of those compounds facilitate easier denitrification verification such as monitoring nitrate loss. Accordingly, this study used pure cultures of known PAH degraders and denitrifiers and monitored both nitrate loss and N_2O production to verify the occurrence of denitrification. The loss of nitrate was monitored, but nitrate utilization was only able to be measured because naphthalene and acenaphthene degradation have a high nitrate demand because of their higher water solubility. Thus, the change in nitrate concentration was significant enough to be detected using an ion chromatograph (IC). The IC was not sensitive enough to detect nitrate utilization for the individual degradation of anthracene, phenanthrene, and pyrene at their low aqueous concentrations. Additionally, the acetylene nitrous oxide reductase block was used, and the resulting accumulation of nitrous oxide was monitored chromatographically. The detection of nitrous oxide does provide conclusive proof of denitrification (*33*). However, as mentioned previously, the majority of researchers depend on monitoring the loss of nitrate to verify denitrification, and monitoring nitrate in

and by itself may not be sensitive or specific enough due to other fates of nitrate. For instance, in studies that use consortia or enriched cultures, assimilatory nitrate reduction may compete with denitrification for nitrate. However, microorganisms with this capability are normally fermenters and can grow anaerobically in the absence of nitrate. Since the microorganisms in this study are isolated pseudomonads, and are not known to ferment, nitrate loss can be predominately attributed to denitrification.

Accordingly, the results of this study offer strong first time evidence of the biodegradation of three- and four-ringed PAHs under denitrifying conditions. This is especially exciting coupled to the recent evidence of PAH (e.g., naphthalene, phenanthrene, fluoranthene, fluorene) degradation under sulfate reducing conditions (*34*, *35*). Anthracene was degraded under denitrifying conditions by both W-2 and KBM-1 from a water solubility concentration to nondetection in approximately 40−60 h. Under aerobic conditions, KBM-1 degraded anthracene in less than 12 h, while W-2 required 84 h. The comparison of the results for W-2 appears to contradict the common belief that degradation rates under denitrifying conditions are slower (*11*). There is no current explanation for this observation. Phenanthrene, initially at its water solubility limit, degraded under denitrifying conditions to below nondetection in approximately 12 h by SAG-R and 44 h by KBM-1. The time for phenanthrene to anaerobically degrade in the presence of KBM-1 was over double the time for aerobic degradation; however, SAG-R had similar rates of removal. Finally, pyrene, the four-ringed PAH initially at its water solubility limit, was degraded to nondetectable levels under denitrifying conditions by SAG-R and KBM-1 in less than 24 and 72 h, respectively. W-2 did not appear to have the capability to degrade pyrene during the course of the experiment. Pyrene was degraded aerobically by SAG-R in approximately the same amount of time as in the anaerobic experiments, while KBM-1 aerobically degraded pyrene in 60 h.

In comparing the microorganisms, SAG-R, isolated from the soil of a creosote contaminated hazardous waste site, appeared to be more efficient in its capability to degrade these PAHs aerobically and under denitrifying conditions, although KBM-1, isolated from lake sediment containing background levels of PAHs, also displayed a capability to degrade all PAHs under both conditions. These results indicate that the capability to degrade higher molecular weight PAHs aerobically and anaerobically under denitrifying conditions may exist in many environments. The microbial community's ability to degrade contaminants under denitrifying conditions also does not appear to require a history of contaminant exposure. In a field study, Hutchins et al. (*31*) observed that the rates of alkylbenzene degradation under denitrifying conditions were consistently higher in an uncontaminated aquifer. Although this occurrence was attributed to preferential substrate utilization or toxic conditions in the contaminated aquifer, the results indicate that denitrification in the uncontaminated aquifer occurs, and may play an important role in determining the fate of these contaminants. Denitrifiers also have a high growth yield when compared to other anaerobes, and genetic advances in the most competitive denitrifiers, the pseudomonads, offer the possibility of exploiting this capability. For these reasons, anaerobic bioremediation under denitrification conditions has the potential to overcome some of the limitations associated with traditional aerobic processes and technologies.

In general, anaerobic degradation rates for these three- and four-ringed PAHs under denitrifying conditions by pure cultures varied from being similar to being approximately 2-fold slower than the aerobic rates. Many researchers believe that the anaerobic degradation rates of aromatic hydrocarbons are slow and of minor ecological significance (*4*, *36*). The results in this study support growing evidence that anaerobic degradation rates under denitrifying conditions may approach those of aerobic degradation rates when cell densities are similar. A better understanding of contaminant degradation coupled to denitrification may lead to the possibility of increased contaminant utilization and increased rates of contaminant degradation. Therefore, as recently demonstrated in the field (*37*, *38*), anaerobic degradation under denitrifying conditions may strongly influence the natural attenuation and engineered remediation of contaminants once thought to be recalcitrant in soil, groundwater, and aquatic sediment systems.

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