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Microbial Degradation of Acenaphthene and Napthalene Under Denitrification Conditions in Soil-Water Systems

Annual Report, October 1987

**J.R. Mihelcic
R.G. Luthy**

October 1987

Work Performed Under Contract No.: DE-AC18-84FC10619

For
U.S. Department of Energy
Office of Fossil Energy
Grand Forks Project Office
Grand Forks, North Dakota

By
Department of Civil Engineering
Carnegie Mellon University
Pittsburgh, Pennsylvania

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Abstract

This study examined the microbial degradation of acenaphthene and naphthalene under denitrification conditions at soil-to-water ratios of 1:25 and 1:50 with soil containing approximately 10^5 denitrifying organisms per gram of soil. Under nitrate-excess conditions, both acenaphthene and naphthalene were degraded microbially from initial aqueous-phase concentrations of about one and several mg/l, respectively, to nondetectable levels (< 0.01 mg/l) in time periods less than 9 weeks. Acclimation periods of 12 to 36 days were observed prior to the onset of microbial degradation in tests with soil not previously exposed to PAH, while acclimation periods were absent in tests with soil reserved from prior PAH degradation tests. It was judged that the apparent acclimation period resulted from the time for a small population of organisms capable of PAH degradation to attain sufficient densities to exhibit detectable PAH reduction, rather than being a result of enzyme induction, mutation, or use of preferential substrate. About 0.9 percent of the naturally occurring soil organic carbon could be mineralized under denitrification conditions, and this accounted for the greater proportion of the nitrate depletion. The mineralization of the labile fraction of the soil organic carbon via microbial denitrification occurred without an observed acclimation period, and was rapid compared to PAH degradation. Under nitrate-limiting conditions the PAH compounds were stable owing to the depletion of nitrate via the more rapid process of soil organic carbon mineralization. Soil sorption tests showed at the initiation of a test that the total mass of PAH compound resided in comparable proportions between solute in the aqueous phase and solute sorbed on the solid phase. The microbial degradation of the PAH compound depends on the interrelationships between: the desorption kinetics and the reversibility of desorption of sorbed compound from the soil, the concentration of PAH-degrading microorganisms, and the competing reaction for nitrate utilization via mineralization of the labile fraction of naturally occurring soil organic carbon.

Introduction

Polycyclic aromatic hydrocarbon (PAH) compounds are a generally hazardous class of organic compounds which may be produced as the result of combustion or pyrolysis of fossil fuels or other organic matter. PAH contamination of groundwater and soil environments from anthropogenic sources has been documented in water and sediment samples collected in the vicinity of coal conversion facilities,

petroleum plants, and waste disposal sites (14,20,33). Because many PAH compounds are toxic and/or carcinogenic, there is interest in understanding the physicochemical processes and microbial degradation reactions that affect the transport and fate of these compounds in groundwater and soil-sediment systems. In groundwater and soil environments, microbial transformations may largely determine the persistence of PAH compounds, and thus affect their degree of hazard to the environment (21). In general, organic-contaminated soil-water systems are anoxic, and thus subsurface microbial degradation of PAH compounds depends on the availability of suitable nutrients and soil microorganisms which can degrade the compounds through utilization of electron acceptors other than molecular oxygen.

A companion paper (27) reported on the microbial degradation of PAH compounds under various redox conditions in soil-water systems. It was demonstrated that naphthalene and acenaphthene were degraded under denitrification conditions, but were otherwise stable under strict anaerobic conditions. Those results indicated that PAH compounds may persist in anoxic soil-water systems in the absence of nitrate or a suitable substituent group on the aromatic ring. Denitrification entails biologically mediated processes whereby oxidized forms of nitrogen may be reduced while organic carbon is oxidized. The source of organic carbon may be contributed from natural material, as in the case of a labile fraction of soil organic carbon, or from an organic contaminant, as in the case of PAH compounds. Thus, upon introduction into a soil-water environment, an organic contaminant may, or may not, become the dominant source of organic carbon for use in microbial denitrification reactions, depending on the characteristics of the organic carbon content of the soil, and the amount and the nature of the organic contaminant.

Microbial degradation may also depend on the PAH compound sorption-desorption reactions with soil and on the role of naturally occurring soil organic carbon, as a sorbent. Aqueous concentrations of hydrophobic organic compounds such as PAH are highly dependent on soil sorption-desorption equilibria (13). The sorption of hydrophobic organic solutes onto soils and sediments has been well documented and shown to be dependent on the organic carbon content of the soil (17,22,23,26). Typical mineral soils may contain on the order of 0.5 to 5 percent by dry weight organic carbon.

The role of soil organic carbon as an electron donor in the denitrification process has been widely recognized (30). The amount of soil organic carbon participating in these reactions has been correlated with denitrification rates, for which an increase in denitrification rate is observed with increasing soil organic carbon content (8,11,30,38). Approximately 0.6 to 1.4 percent of the total naturally occurring organic carbon content of soil may be mineralized by denitrifying soil microorganisms (30); thus, much of

the organic carbon content of soils is resistant to mineralization. Several researchers have reported a greater rate of denitrification upon addition of labile organic carbon to soil (10,29). Organic amendments may influence microbial degradation of xenobiotic compounds in soil; for example, exposure to amino acids has been shown to enhance aerobic degradation of *m*-cresol, *m*-aminophenol, and *p*-chlorophenol, while experimental results with naphthalene were inconclusive (35).

Much of the available information on microbial degradation of aromatic compounds under nitrate-reducing conditions has been directed towards phenolic or benzoate derivatives (6,7,16,34,41). Few investigations on microbial denitrification have addressed degradation of aromatic compounds having no oxygen substituent groups, although recently *p*-, *m*-, and *o*-xylene have been shown to be degraded under denitrification conditions (25, Zeyer, E.P. Kuhn, and R.P. Schwarzenbach, Appl. Environ. Microbiol., in press). In contrast, dichlorobenzenes have been found to be stable under denitrification conditions (9,25). The biotransformation of aromatic compounds having no oxygen on the ring or ring substituent is particularly important from the viewpoint of soil and groundwater contamination. Unlike phenolics, benzoates, and related compounds which may be degraded in anaerobic as well as in denitrifying environments, it is currently believed that aromatic compounds which lack oxygen as a substituent are stable or degrade at negligible rates in anaerobic systems (5,27).

The objectives of this study were to evaluate the microbial degradation of naphthalene and acenaphthene under denitrification conditions in soil-water systems, and to understand the nitrate demand exerted by both the PAH compound and available soil organic carbon. This was accomplished by enumerating the denitrifier population, assessing the nitrate demand exerted by naturally occurring soil organic carbon, evaluating naphthalene and acenaphthene sorption onto the soil, and comparing PAH compound degradation under both nitrate-limiting and nitrate-excess conditions.

Materials and Methods

The soil, reagents, equipment, and procedures used in this study are described in a companion paper (27). The water used for cleaning glassware and experimentation was produced by a MegaPure High Capacity Deionizer (Coming Glass Works, Coming, New York), followed by distillation and passage through four ultrapure (mixed-bed) cartridges (No. D8902, Barnstead, Boston, Mass.).

Sorption Isotherm Tests: The soil used in these and other tests was undisturbed, subhumid grassland soil from the Barnes-Hamerly Association, Cass County, North Dakota. The soil was collected

from the A soil horizon, and then air-dried and screened to pass a U.S. standard sieve No. 12, 2.0 mm (27). Sorption isotherms of naphthalene and acenaphthene onto the soil were determined to estimate solute partitioning to the solid phase with an aqueous phase consisting of PAH, mineral medium, and 0.01 N CaCl_2 . The soil was autoclaved for one hour at a temperature of 121°C and a steam pressure of 20 psi. The sorption isotherm tests were performed in a batch mode at room temperature with 50 ml glass centrifuge tubes fitted with Teflon-lined septums and open-port screw caps. The samples contained varying amounts of soil and an initial aqueous-phase solute concentration ranging from approximately 0.2 to 10 mg/l, depending on the solute's solubility in water. The amount of soil was varied from 1.5 to 25.0 grams, weighed to 10^{-4} grams, per 50 ml to produce a range of aqueous-phase solute concentrations after equilibration. The soil was placed in a centrifuge tube and then an aqueous mixture containing mineral medium and solute was added with the volume recorded to 10^{-2} ml. Samples were filled to zero headspace, sealed with Teflon-lined septums and screw caps, and covered with aluminum foil to prevent photodegradation. Blanks without soil were run concurrently.

After sample preparation, the sealed centrifuge tubes were transferred to a wrist action shaker and shaken for an equilibrium period of 24 hours. Previous studies had indicated that the sorption process was not significantly time dependent after approximately four hours and that sorption equilibrium was attained in less than one day. After equilibration, the tubes were centrifuged for thirty minutes and analysis of the separated aqueous phase was performed by high pressure liquid chromatographic (HPLC) methods by sampling directly through the septum.

Soil Organic Carbon Utilization: Batch experiments were used to determine the evolution of CO_2 from a nitrate-rich soil-water mixture. A 500 ml Erlenmeyer flask was fitted with a side port at the top for purging the system with nitrogen gas, and the bottom for withdrawing a sample of the suspension. Soil and water were added to the flask at a soil-to-water weight ratio of 1:25 so that the initial amount of soil was 14.07 grams by dry weight. A soil-to-water ratio of 1:25 was utilized as there were no difficulties with mixing. This was the same ratio used in the naphthalene microbial degradation tests. Companion soil organic carbon utilization tests performed at soil-to-water ratios of 1:3 and 1:10 resulted in lower values of cumulative CO_2 evolution, due to difficulty in maintaining the system completely mixed.

The soil suspension was magnetically stirred in darkness at room temperature. The system was purged with nitrogen gas for three hours prior to nitrate addition and purged continuously thereafter for the duration of the experiment. Sodium nitrate was added such that the initial nitrate concentration was

approximately 800 mg/l NO_3^- as nitrate. The flask was immediately sealed and connected to a CO_2 trap which contained 100 ml of 0.025 N KOH solution. An aliquot of soil suspension was removed periodically from the bottom side port, centrifuged, filtered, and analyzed for nitrate by colorimetric procedures. The caustic solution in the CO_2 trap was replaced daily and titrated with standard acid. Blank flasks without soil were run concurrently to monitor the purity of the nitrogen gas and to verify nitrate reduction due to microbiological processes.

Most Probable Number Tests: The denitrifying cell population was enumerated by a most probable number method (MPN) described by Alexander (1). Two different bacterial growth media were used for comparison (1,4). Positive tubes were indicated by a color change, due to an increase in alkalinity, in conjunction with the formation of gaseous nitrogen.

PAH Degradation Under Denitrification Conditions: One liter of stock PAH and mineral medium solutions were prepared separately. Stock PAH solution consisted of a saturated PAH solution in deionized water. A mineral medium-phosphate buffer solution was prepared which consisted of NaNO_3 and the following (mg/l): 17.0 KH_2PO_4 , 43.50 K_2HPO_4 , 66.8 $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 0.50 $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 3.4 NH_4Cl and sufficient CaCl_2 so that a final ionic strength of 0.01 N CaCl_2 was obtained in each individual sample.

The mineral medium solution was degassed by vacuum for thirty minutes and then purged with helium for 30 minutes. The saturated PAH solution was filtered through an extra thick glass fiber filter (Gelman Sciences Inc., Ann Arbor, Mich.) and then both solutions were combined and purged with helium for one hour. The pH of the final solution was adjusted to 7.0 by the addition of 1 N HCl or 1 N NaOH as needed. The absence of dissolved oxygen was verified by titrametric procedures (3).

Aliquots were transferred to 50 ml glass centrifuge tubes which typically contained one or two grams by dry weight of Barnes-Hamerly soil for acenaphthene and naphthalene experiments respectively. The samples were sealed immediately with a Teflon-lined septum and open-port screw cap, and covered with aluminum foil. No headspace was visible in samples. Samples were shaken three hours per day on a wrist action shaker and maintained at room temperature. Periodically individual samples were withdrawn, centrifuged at 875 x g, and sampled directly through the septum for analysis of aqueous PAH compound concentration by HPLC methods with fluorescence detection. Nitrate, nitrite, and sulfate were analyzed by either colorimetric or ion chromatographic (IC) methods after analysis for PAH compound. Individual

samples were withdrawn for analysis during the course of an experiment and then removed from service after being analyzed, except for some samples in the special experiments in which additional nitrate was introduced into the system following depletion of nitrate.

Sterilized blanks containing no soil, and controls containing sterilized soil were run concurrently. Sterilization consisted of autoclaving samples three consecutive days for one hour at 121°C and steam pressure of 20 psi. Heat sterilization was supplemented in later experiments by the addition of HgCl_2 at a concentration of 200 mg/l. Heat sterilization was effective in inhibiting microbial degradation of PAH compounds, while addition of HgCl_2 with autoclaving was required to completely inhibit nitrate reduction.

Initially, analysis for nitrate was performed spectrophotometrically by reduction of nitrate to nitrite, followed by diazotization and coupling of nitrite with N-[1-naphthyl]-ethylenediamine (Bausch and Lomb, Rochester, NY). Nitrite was analyzed similarly but with the absence of the preceding reduction step. In all later tests, aqueous phase nitrate, nitrite, and sulfate concentrations were analyzed by IC methods utilizing a Dionex IC 14 equipped with an AS3 separator column and No. 35350 anion fiber suppressor column (Dionex, Sunnyvale, Cal.). An eluent of 0.0015M NaHCO_3 and 0.0012 M Na_2CO_3 was used. Prior to analysis by IC methods, organic constituents were removed by passing 15 ml of aqueous sample through a C_{18} cartridge (Waters Associates Inc., Milford, Mass.). Samples were then stored in a freezer prior to I.C. analysis. Ammonia was analyzed by a colorimetric automated phenate method (15). A detection limit of 0.01 mg/l was observed for all anions. Nitrate and other compound analyses are reported as compound, e.g., mg/l as NO_3^- .

Tests with Acclimated Soil: Experiments were performed to determine if the acclimation period was affected by use of soil which had been exposed previously to naphthalene. Individual centrifuged samples were reserved and stored under refrigeration at the conclusion of a set of degradation tests following attainment of nondetectable aqueous-phase concentration of naphthalene. These samples were used in a subsequent test by adding aqueous solution containing deoxygenated PAH and mineral medium, and resuspending the solid phase. The aqueous solution consisted of a mixture of naphthalene stock solution combined with aqueous phase decanted from former test samples having mineral nutrients, NaNO_3 , and electrolyte described previously.

Results

Experiments were conducted to evaluate the microbial degradation of naphthalene and acenaphthene under denitrification conditions in soil-water systems. The study included experiments to examine the partitioning of acenaphthene and naphthalene between the solid and the aqueous phase, the role of soil organic carbon in nitrate reduction, and the significance of nitrate-limiting conditions on microbial degradation of PAH compound via nitrate reduction.

Solute Sorption: Figure 1 shows a linear sorption isotherm for naphthalene and Barnes-Hamerly soil. Various laboratory studies have shown for sparingly soluble, hydrophobic organic solutes that sorption onto soils can be described by a linear isotherm (13,23):

$$S = K_p C \quad (1)$$

where S is the amount of solute sorbed, mg solute per gram of soil, C is the equilibrium aqueous solute concentration, mg/l, and K_p is the linear sorption partition coefficient, l/g or l/kg. For the sorption of acenaphthene and naphthalene onto Barnes-Hamerly soil, K_p was 52 ($r^2=96.4\%$) and 12 ($r^2=99.1\%$) l/kg respectively.

The sorption of hydrophobic organic solutes onto soils can be correlated with the organic carbon content of a given soil. The role of soil organic matter in sorption of hydrophobic aromatic solutes onto soil has been studied extensively. Its role is similar to that of an organic solvent in solvent extraction, and thus the partitioning of a relatively nonpolar compound between soil organic matter and water should correlate well with the solute's partitioning between water and an immiscible organic solvent (13). A normalized soil sorption coefficient can be defined as:

$$K_{oc} = K_p / OC \quad (2)$$

where OC is the fractional mass of organic carbon in a soil. Various researchers have presented relationships between values of K_{oc} and a solute's octanol/water partition coefficient, K_{ow} , for nonpolar aromatic compounds (22,23,26,32). For sorption of relatively hydrophobic organic solutes, the relationship between K_{oc} and K_{ow} is usually given by a linear regression in the form:

$$\log K_{oc} = a \log K_{ow} + b \quad (3)$$

where a and b are regression coefficients. The correlations between K_{oc} and K_{ow} result in a somewhat divergent set of relationships which may be attributed to various factors including kinetic or steric effects, as well as hydrophobic contributions to sorption.

Measured values of $\log K_{ow}$ for naphthalene are about 3.36 (19,22). $\log K_{ow}$ for acenaphthene may

be estimated as 4.03 on the basis of molecular structure using atomic group hydrophobic fragment constants (13,44). Following Equations 2 and 3, it is expected that the ratio of the values of K_{ow} for acenaphthene and naphthalene, 4.7, should be similar to the ratio of the experimental values of K_p for the two solutes, 4.3, as evidenced here. The organic carbon content of the soil was measured by the Walkley-Black Method (2) and found to be 2.9 ± 0.28 , which resulted in a predicted value of K_{oc} which agrees within a factor of about 1.5 of that given by Equation 3 with regression coefficients suggested by Schwarzenbach and Westall (32).

MPN Tests: Most probable number tests were performed to enumerate nitrate-reducing microorganisms in soil samples prior to use in the degradation experiments. The average value for five individual MPN tests by the method of Alexander (1) was $1.2 \times 10^5 \pm 6 \times 10^4$ denitrifying organisms per gram of soil. MPN tests, conducted with a second growth medium (4), also yielded an average value of 1.2×10^5 denitrifying organisms per gram of soil.

Microbial Utilization of Soil Organic Carbon: Figure 2 shows results of experiments to assess the mineralization of soil organic carbon by denitrifying microorganisms. Figure 2 presents the average results for three individual tests conducted simultaneously for cumulative CO_2 evolution over twenty days at a soil-to-water ratio of 1:25. These data show that the denitrification process was initiated immediately. Soil organic carbon utilization was assumed to follow first-order kinetics (30), and thus carbon mineralization can be described as:

$$dC/dt = -k_c C \quad (4)$$

where C is the available carbon remaining in the soil, meqv of CO_2 , k_c is a first-order carbon mineralization coefficient, hr^{-1} , and t is time, hr. Integration of Equation 4 yields:

$$C = C_{max} \exp(-k_c t) \quad (5)$$

where C_{max} is defined as the maximum available soil organic carbon, meqv CO_2 . Since C equals C_{max} minus the evolved CO_2 , substitution of this expression into Equation 5 and rearranging yields an equation describing the cumulative evolution of CO_2 .

$$CO_2 = C_{max} [1 - \exp(-k_c t)] \quad (6)$$

The data in Figure 2 were used to estimate k_c and C_{max} as 0.0049 hr^{-1} and 0.63 meqv ($0.27 \text{ mg C/gram of soil}$) respectively ($r^2=88\%$). The fitted parameters, k_c and C_{max} , were found using a nonlinear, parameter estimation program (McCroskey, P.S., and G.J. McRae, PAREST, A Computer Program for Estimation of Parameters in Algebraic and Ordinary Differential Equation Models, Carnegie Mellon

University, Pittsburgh, Pa., 1987).

PAH Degradation: The data points shown in Figures 3 to 9 for blanks, controls, and test samples, represent the average values of analysis of two or three individual samples. The individual samples were discontinued from the experiment after analyses. The data for the sample blanks show no change through the duration of any of the experiments for either PAH compound or nitrate, for durations up to 84 days. This demonstrates that there was no loss of PAH compound or nitrate via the Teflon-lined septum, nor PAH compound degradation owing to photolysis. Sterile controls were also stable through the duration of the experiments which confirms the absence of PAH compound loss across the Teflon-lined septum, as well as lack of significant abiotic degradation reaction with the soil.

The experiments to examine the microbial degradation of naphthalene and acenaphthene under denitrification conditions encompassed a time span of nearly one year. The results of the experiments shown in Figures 3 to 9 were performed with soil samples withdrawn from a single large batch, and thus the soil aged over the approximate one year duration of the microbial degradation tests. The chronology of the experiments progressed from results shown in Figures 3 and 4, to 5, and to 6 through 9. The data show that the acclimation period associated with the onset of naphthalene degradation increased with soil age from approximately 12 days (Figure 3) to about 24 days (Figure 6). Similar results were evident for acenaphthene in which the acclimation period increased with soil age from approximately 12 days (27) to about 36 days (Figure 8). In all cases nitrate reduction via mineralization of soil organic carbon was observed to be initiated immediately with no acclimation period even with aged soil.

Figure 3 shows naphthalene degradation under nitrate-reducing conditions for two samples sets in concurrent experiments, each with a soil-to-water ratio of 1:50. The results show aqueous-phase naphthalene concentration decreasing from approximately 4 and 3 mg/l to nondetectable levels (ND) in 47 and 33 days for sample sets 1 and 2 respectively. An acclimation period of approximately two weeks was observed prior to evidence of significant degradation of naphthalene. The decrease in nitrate concentration over the duration of the experiment was in the range of 40 and 18 mg/l NO_3^- , for sample set 1 and 2 respectively as determined by colorimetric procedures. The residual nitrate concentration in the test samples at the conclusion of the experiment was 110 and 66 mg/l NO_3^- ; thus, there was excess nitrate in the samples through the duration of the microbial degradation reactions. The results shown in Figure 3 were supplemented by monitoring selected samples for ammonia to assess nitrate assimilation to ammonium. Aqueous-phase ammonia concentration in the test samples exhibiting naphthalene

degradation was similar to that in the blanks and controls (i.e. < 1.0 mg/l).

Acenaphthene degradation under denitrification conditions is shown in Figure 4 for two concurrent sample sets. In these tests the initial nitrate concentration was adjusted to provide for less than half the nitrate demand observed for the sample sets in Figure 3. The initial nitrate concentration was 11 and 9 mg/l NO_3^- for sample sets one and two respectively. In both sample sets, nitrate was depleted to nondetectable levels in approximately two weeks; thus, thereafter the microbial denitrification degradation reactions were restricted owing to the lack of nitrate. Initial aqueous acenaphthene concentration was approximately 0.60 and 0.40 mg/l for sample test one and two respectively. Sample set one showed no significant degradation of acenaphthene over a time span of 12 weeks while sample set two showed a small decrease in acenaphthene concentration to approximately 0.2 mg/l over a time period of about 40 days, after which the acenaphthene concentration remained constant for the final month of the experiment. In comparison with results shown in Figure 3 for naphthalene, and results presented in a companion paper for acenaphthene (27), there was no substantial degradation of acenaphthene under nitrate-limiting conditions.

Additional experiments were conducted to assess the role of nitrate on microbial degradation of naphthalene. In these and subsequent tests, nitrate, nitrite, and sulfate analyses were performed by IC methods. The analysis for aqueous sulfate concentration was used to determine if the observed PAH compound degradation was related to sulfate reduction. Sulfate was not added to samples intentionally, but was present due its natural occurrence in the soil. In the following experiments with naphthalene, two grams of Barnes-Hamerly soil were utilized in 50 ml samples which resulted in an initial aqueous sulfate concentration in the range of 4 to 7 mg/l. Sulfate concentration did not change from the initial levels through the course of the experiments. Nitrite was nondetectable (< 0.01 mg/l) in almost all of the samples except during the first and second day when nitrite was observed in many samples at concentrations less than 1 mg/l. Selected samples were monitored throughout the experiment for pH, and the pH values in the completed samples ranged from 6.8 to 7.2.

Figure 5 shows aqueous naphthalene and nitrate concentration with time for an experiment in which a small amount of nitrate was added to the test samples initially. After the initial nitrate was depleted to nondetectable levels (< 0.01 mg/l), additional nitrate was introduced to the samples by injection through the septum with a syringe equipped with a removable gas chromatography needle. Approximately 8 μl of 3.0 M nitrate solution was added to the samples which increased the aqueous nitrate concentration to

28.5 mg/l NO_3^- . As a precaution against possible naphthalene volatilization losses due to the puncturing of the Teflon-lined septum, the septum was sealed with a drop of silicone glue, although independent screening tests had shown no loss of naphthalene over a 10 day period from test blanks which had been punctured 8 times with the syringe needle.

The results in Figure 5 show that the initial nitrate concentration of approximately 6.0 mg/l was depleted to nondetectable levels in about 30 days, and that the nitrate reintroduced into the samples on day 38 was depleted to nondetectable levels by day 65. Naphthalene degradation showed an acclimation period of approximately 25 days after which naphthalene was reduced from about 5 mg/l to 1.5 mg/l. Naphthalene concentration remained constant from day 30 to 38 owing to the depletion of nitrate. Following injection of nitrate on day 38, the microbial degradation of naphthalene was resumed and naphthalene attained nondetectable levels three weeks after the reintroduction of nitrate into the samples.

Figures 6 and 7 show a set of confirmation tests for microbial degradation of naphthalene under nitrate-excess and nitrate-limiting conditions, respectively, with 2 grams soil per 50 ml. Figure 6 shows that sufficient nitrate was available through the duration of the test and nitrate was decreased from approximately 95 to 40 mg/l NO_3^- . Fifty-five mg/l of nitrate was depleted during the first two weeks of the test due to the oxidation of the mineralizable portion of soil organic carbon, followed by a 16 mg/l decrease in nitrate concentration after approximately one month of the experiment. The latter decrease in nitrate coincided with aqueous naphthalene concentration decreasing from approximately 4.4 mg/l to nondetectable levels in 3 to 4 weeks following a 30 day acclimation period. In contrast, Figure 7 shows that under nitrate-limiting conditions aqueous naphthalene concentration remained constant at 6 mg/l for a period of 11 weeks. In this test the initial nitrate concentration was 9 mg/l and nitrate was reduced to nondetectable levels in 20 days. The sample blanks and controls remained at constant levels for the duration of the test which again confirmed the stability of the samples with respect to volatilization or abiotic degradation. Furthermore, the stability of the test samples in Figure 7 confirmed no oxygen intrusion across the septum.

Figures 8 and 9 show an analogous set of confirmatory tests with acenaphthene and one gram of soil per 50 ml under nitrate-excess and nitrate-limiting conditions respectively. Figure 8 shows that acenaphthene degraded from an initial aqueous-phase concentration of approximately 0.60 mg/l to near nondetectable levels in 80 days following an acclimation period of approximately 42 days. Nitrate

concentration decreased from approximately 55 mg/l to 15 mg/l over the duration of the test with most of the nitrate depletion occurring in the first two weeks. Figure 9 shows aqueous-phase acenaphthene concentration under nitrate-limiting conditions. In this test the initial nitrate concentration was 58 mg/l and this was reduced to nondetectable levels in approximately 22 days. There was no degradation of acenaphthene over a time period of 83 days and the aqueous phase acenaphthene concentration remained at approximately 0.60 mg/l throughout the test. As observed in previous experiments, stability of blanks, controls, and test samples confirm the integrity of individual samples and the absence of volatilization and/or oxygen intrusion.

A concluding set of tests were performed to determine if the acclimation period associated with naphthalene degradation could be eliminated if acclimated soil was used in the experiment in lieu of field soil. Samples showing naphthalene degradation from the tests in Figure 6 were used in this experiment. The results are shown in Figure 10 in which aqueous-phase naphthalene concentration decreased from approximately 3.0 mg/l to nondetectable levels in approximately 16 days. No acclimation period was observed in this experiment, and nitrate concentration decreased from approximately 37 to 17 mg/l NO_3^- .

Discussion

This study evaluated the microbial degradation of naphthalene and acenaphthene in soil-water systems under denitrification conditions. Acenaphthene and naphthalene were degraded microbially to nondetectable levels under denitrification conditions in systems for which nitrate was not a limiting constituent. An evaluation of the manner in which the PAH compounds may be degraded by soil microorganisms under nitrate-reducing conditions requires understanding of the relevant fractions of organic carbon in the soil-water system. With regard to the tests described here, carbon may exist as four entities: 1.) soil organic carbon available for mineralization, 2.) soil organic carbon refractory to mineralization, 3.) PAH compound in the aqueous phase, and 4.) PAH compound in the sorbed phase. The nature of these entities influenced the results of this study.

Sorption and Degradation: Sorption isotherms demonstrated the partitioning of the PAH solute between sorbed and aqueous phases. As expected, acenaphthene exhibited a greater tendency to sorb than naphthalene, and the extent of solute sorption at equilibrium was correlated with the organic carbon content of the soil and the solute's octanol water partition coefficient. It is anticipated that solute sorption is important for understanding the mechanistic processes which may govern the microbial degradation of

sorbable, hydrophobic organic solutes such as PAH compounds. Intracellular degradation of PAH compounds depends in part on the availability of the organic compound to the microorganisms. If an organic compound were irreversibly sorbed to soil organic matter it may be isolated and protected from intracellular degradation. Alternatively, if the organic compound were reversibly sorbed to soil organic matter, the solute would desorb from the soil organic carbon in response to a decrease in the aqueous phase concentration. Thus solute desorption and diffusion through the aqueous phase would increase the accessibility of the solute to microorganisms. It may be hypothesized that surface diffusion and surface scavenging of sorbed organic solute by sorbed microorganisms may occur; however, the current perception is that soil microbial degradation reactions are inhibited or significantly slowed for a sorbed phase solute than for an aqueous-phase solute.

The role of organic solute sorption on microbial degradation is not well understood, although available evidence suggests that sorption decreases the total amount of the organic solute available to microorganisms for degradation (28). Steen et al. (40) have presented results for degradation of chlorpropham and di-n-butyl phthalate in soil for which it was assumed that sorption rendered the sorbed solute unavailable for degradation. Ogram et al. (28) evaluated the effects of sorption of both bacteria and a herbicide (2,4-dichlorophenoxy acetic acid; 2,4-D) on microbial degradation in soil. It was concluded that sorbed 2,4-D was completely protected from microbial degradation. If these findings are applicable to soil-water systems with the solutes naphthalene and acenaphthene, the reversibility and kinetics of desorption of the PAH compounds from soil will affect the rate of degradation of the PAH compounds in a soil-water environment. This issue is currently being investigated by experimental and modeling activities in our laboratory to assess the kinetics and reversibility of PAH compound desorption in conjunction with the kinetics of microbial degradation. Initial observations with the solute naphthalene have indicated that the soil sorption-desorption process is reversible, a finding which is supported by the work of Gschwend and Wu with chlorobenzenes (18). Therefore, for these lower molecular weight PAH compounds, the total amount of PAH in the soil-water system, i.e. aqueous phase plus sorbed phase, may be potentially available to the soil microorganisms.

Mineralization of Soil Organic Carbon: Aside from the PAH compound, a portion of the soil organic carbon is also utilized by soil denitrifying microorganisms. Many of the degradation tests reported here were performed at a soil-to-water weight ratio of 1:25 and it was found in separate experiments that the maximum amount of mineralizable soil organic carbon (C_{max}) under denitrification conditions was 0.27

mg C per gram of Barnes-Hamerly soil. The soil used in this study had a organic carbon content of 2.9 percent. Thus 0.9 percent of the total organic carbon was available for mineralization by the soil denitrifiers. This agrees with other work which has shown that $C_{\max}$ may range from about 0.6 to 1.4 percent of the total organic carbon for various mineral and organic soils (30). Thus, much of the naturally occurring organic matter in soil is resistant to microbial decomposition under denitrification conditions, and therefore the mineralization of soil organic carbon via denitrification should have minimal effect on the bulk of the soil carbon phase associated with PAH compound sorption.

The total mass contributions of potentially degradable PAH compound and labile soil organic carbon are comparable in magnitude in the tests reported here. In an experiment with an initial aqueous solute concentration of naphthalene equal to 7.0 mg/l, two grams of soil per 50 ml, and an experimentally determined K_p of 12 l/kg, the mass of degradable naturally occurring organic carbon is about 0.54 mg while the total amount of degradable naphthalene is about 0.52 mg. In the case of the solute acenaphthene, with one gram of soil per 50 ml, an initial equilibrium concentration of 1 mg/l, and an experimentally determined K_p of 52 l/kg, the amount of degradable naturally occurring soil organic carbon is approximately 0.27 mg with the total amount of acenaphthene being about 0.15 mg. There is insufficient evidence to judge conclusively the extent with which the PAH compound may be mineralized to CO_2 via microbial denitrification. Nonetheless, as discussed below, the nitrate demand per mg of PAH compound may be comparable to the nitrate demand per mg soil organic carbon for naphthalene degradation under nitrate-excess conditions.

Nitrate Demand: The tests with unacclimated soil showed that the mineralizable portion of the soil organic carbon is utilized quickly in comparison to the PAH compound. At approximately twenty days, this supply of soil organic carbon is depleted, assuming slow conversion of residual refractory soil organic carbon to mineralizable soil organic carbon. If the assumption is made that soil organic carbon can be described by a carbohydrate equivalent (30), the mineralization of soil organic carbon under nitrate-reducing conditions can be described as follows:

In this case, 0.8 moles of NO_3^- are reduced per mole of CO_2 produced. This relationship may be used to estimate the nitrate demand from soil organic carbon assuming that the labile fraction of the soil organic carbon is completely mineralized, as given by $C_{\max}$. It is predicted that approximately 45 mg/l NO_3^- should be reduced during the initial phase of the test owing to the demand exerted by the soil organic

carbon contributed by 2 grams of soil. This relationship was generally followed in the experiments. For example, from the data in Figure 6 at a soil-to-water ratio of 1:25, nitrate was depleted from approximately 95 mg/l to 40 mg/l during the first three weeks of the test.

Although there is insufficient data to clearly assess the extent of mineralization of the PAH compounds, the results in Figures 6 and 10 suggest qualitatively that much of the naphthalene was mineralized under nitrate-excess conditions. The data in Figure 6 show following the mineralization of soil organic carbon that nitrate was depleted during the period of naphthalene degradation from about 40 to 24 mg/l, or by about 0.065 meqv assuming reduction to molecular nitrogen:

Aqueous-phase naphthalene was depleted from about 4.5 mg/l to nondetectable levels, for a total of 2.3 μ moles assuming reversible desorption from the two grams of soil with K_p equal to 12 l/kg. If the naphthalene was completely mineralized the nitrate demand would be 0.12 meqv according to:

A similar comparison is evident from data in Figure 10 with acclimated soil assuming that the nitrate demand results from the added naphthalene rather than from the residual, refractory soil organic carbon. These data show a nitrate demand of 0.081 meqv (20 mg/l NO_3^-) for 0.086 meqv total naphthalene (3.1 mg/l initial aqueous phase PAH, 2 grams of soil, and $K_p = 12$ l/kg). Hence, from these considerations it appears that about 50 and 90 percent of the naphthalene may have been mineralized to CO_2 for the experiments shown in Figures 6 and 10 respectively.

Data in Figure 3 can not be evaluated as readily to assess the extent of naphthalene mineralization, due to the uncertainty associated with precise determination of nitrate in a soil-water suspension by colorimetric procedures. This is evidenced by the nitrate demand for sample set 2 being less than that which may be attributed to mineralization of the labile fraction of the soil organic carbon. However, sample set 1 suggests that a significant portion of the naphthalene may have been mineralized to CO_2 , as nitrate decreased by approximately 40 mg/l NO_3^- for which approximately 22 mg/l NO_3^- may be ascribed to the mineralization of the labile fraction of the soil organic carbon. The nitrate data shown in Figure 8 correspond to acenaphthene degradation under nitrate-excess conditions. Unlike the results in Figure 6, these data do not show a demarcation between the nitrate reduction due to mineralization and PAH degradation. Nonetheless, the depletion of 40 mg/l NO_3^- can not all be attributed to the nitrate demand anticipated for labile soil organic carbon.

Nitrate Reduction: The reduction of nitrate in the soil-water systems was found to not entail dissimilatory reduction to ammonium as indicated by measurement of aqueous phase ammonium concentration. This observation is consistent with results from a study of soil carbon mineralization and denitrification kinetics in which there was very little reduction of nitrate to ammonium using an experimental apparatus similar to those employed here for determination of C_{max} and k_c (30). Dissimilatory reduction of nitrate has been shown to be significant in glucose-amended soils, but not in non-amended soils (12,39). Both assimilatory and dissimilatory nitrate reduction may be significant in the case of marine sediments (24,36).

Acclimated Soil: Experiments using acclimated soil showed that the degradation of naphthalene under denitrification conditions commenced without a several week acclimation period. The occurrence of an acclimation period may result from various factors including (43): enzyme induction, genetic mutation, insufficient supply of inorganic nutrients, preferential utilization of other organic compounds, and the time necessary for a small microbial population to increase in size for degradation of observable quantities of the organic compound. For the results reported here, it is believed that the acclimation period is not due to insufficient nutrients owing to the use of the mineral medium. The acclimation periods were much longer than the time usually required for enzyme induction which is typically minutes or hours (31). Although the period for enzymes to be induced may be longer at low substrate concentrations, other studies have concluded that long acclimation periods are not likely explained by induction (42,43).

Acclimation periods which have reproducible durations among replicate tests are thought not to be the result of mutation or the appearance of new genotypes (43). For this reason, genetic mutation is not probably the cause of the acclimation period due to the reproducibility of the acclimation period among individual samples of any one batch experiment, as well as the reproducibility among sample sets initiated at the same period in time (data in Figure 3, for example). The acclimation period may not be directly related to the preferential utilization of the mineralizable fraction of soil organic carbon over the PAH compound. Experiments which measured CO_2 evolution from the mineralization of soil organic carbon showed that most of the labile soil organic carbon was utilized by soil denitrifying microorganisms in less than three weeks, while the acclimation periods observed prior to PAH degradation varied from about two weeks to one month and more.

It is most probable that the acclimation period is a result of time necessary for an initially small population of PAH-degrading organisms to grow to sufficient levels in order to produce a detectable loss

of substrate PAH compound. This reasoning is consistent with the work of Wiggins et al. (43), who arrived at a similar conclusion for the case of microbial degradation of *p*-nitrophenol. This may also explain the increase in acclimation period with soil age. Over the time span of one year in which the PAH compound microbial degradation experiments were conducted, the acclimation period preceding naphthalene degradation increased from approximately two weeks to four weeks while that for acenaphthene increased from about two weeks to six weeks. This was probably a result of soil drying and storage on microbial activity. It has been shown by Sparling and Chesire (37) that initial microbial populations and their growth rates decreased with storage time over a period of six months.

The results of this study indicate that microbial degradation of acenaphthene and naphthalene under denitrification conditions may be an important process for assessing the fate of PAH compounds in anoxic soil-water systems. In this study the mass of both the PAH compound and the mineralizable fraction of the soil organic carbon were comparable, and nitrate depletion due to the mineralizable fraction of the soil organic carbon can arrest microbial degradation of the PAH compound under nitrate-limiting conditions. The sorption-desorption interactions with soil may limit the amount of total PAH compound available to the microorganisms, and this is a subject of further study.

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Figure 1.) Sorption isotherm for the solute naphthalene onto Barnes-Hamerly soil. $K_p = 12 \text{ l/kg}$ with $r^2 = 99.1$ percent.

Figure 2.) Soil organic carbon utilization under denitrification conditions with a soil-to-water ratio was 1:25.

Figure 3.) Microbial degradation of naphthalene under denitrification conditions with 1 gram of Barnes-Hamerly soil per 50 ml centrifuge tube.

Figure 4.) Microbial degradation of acenaphthene under denitrification conditions for a case in which nitrate was limiting. The initial nitrate was depleted to nondetectable levels in the first two weeks.

Figure 5. Aqueous naphthalene and nitrate concentrations under denitrification conditions. The nitrate was exhausted in 30 days and this resulted in the cessation of naphthalene degradation. After reintroduction of nitrate on day 38, the denitrification reactions resumed.

Figure 6.) Aqueous naphthalene and nitrate concentrations under denitrification conditions for a case in which nitrate was in excess. Naphthalene degraded from an initial concentration of 4.5 mg/l to nondetectable levels in 60 days with an acclimation period of approximately 30 days.

Figure 7.) Microbial degradation of naphthalene under denitrification conditions for a case in which nitrate was limiting. Aqueous naphthalene remained at 6 mg/l for a period of 11 weeks. The initial nitrate concentration of 9 mg/l was depleted to nondetectable levels in 20 days.

Figure 8.) Aqueous acenaphthene and nitrate concentrations under nitrate-excess conditions. Acenaphthene degraded from an initial concentration of 0.60 mg/l to nondetectable levels in 80 days with an acclimation period of approximately 42 days.

Figure 9.) Microbial degradation of acenaphthene under nitrate-limiting conditions. Aqueous acenaphthene remained at 0.6 mg/l for a period of 83 days.

Figure 10.) Microbial degradation of naphthalene under denitrification conditions utilizing acclimated soil. The initial aqueous naphthalene concentration was reduced from 3.1 mg/l to nondetectable levels in 16 days. No acclimation period was evident.

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