APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 1993, p. 2977–2983
0099-2240/93/092977-07$02.00/0

Vol. 59, No. 9

Nitrogen Limitation and Nitrogen Fixation during Alkane Biodegradation in a Sandy Soil

PATRICIA L. TOCCALINO,[1] RICHARD L. JOHNSON,[1] AND DAVID R. BOONE[1,2]*

Department of Environmental Science and Engineering[1] and Department of Chemical and Biological Sciences,[2] Oregon Graduate Institute of Science & Technology, 20000 N.W. Walker Road, P.O. Box 91000, Portland, Oregon 97291-1000*

Received 16 February 1993/Accepted 7 July 1993

We investigated nutrient limitations during hydrocarbon degradation in a sandy soil and found that fixed nitrogen was initially a limiting nutrient but that N limitation could sometimes be overcome by N_2 fixation. Hydrocarbon biodegradation was examined in an unsaturated sandy soil incubated aerobically at 20°C with propane or butane and various added nutrients. Propane and butane degradation proceeded similarly during the first 3 months of incubation. That is, bacteria in soil amended with N oxidized these hydrocarbons more rapidly than in controls without nutrient additions or in soil with added phosphate or trace minerals. Both propane- and butane-amended soil apparently became N limited after the initial available inorganic N was utilized, as indicated by a decrease in the rates of hydrocarbon degradation. After 3 months, propane and butane degradation proceeded differently. Bacteria in propane-degrading soil apparently remained N limited because propane degradation rates stayed low unless more N was added. In contrast, bacteria in butane-degrading soil appeared to overcome their N limitation because butane degradation rates later increased regardless of whether more N was added. Analyses of total N and acetylene reduction assays supported this apparent surplus of N in butane-amended soil. Total N was significantly ($P < 0.01$) higher in soil incubated with butane and no N amendments than in soil incubated with propane, even when the latter was amended with N. Acetylene reduction occurred only in butane-amended soil. These results indicate that N_2 fixation occurred in butane-amended soil but not in propane-amended soil.

Leaking underground storage tanks are a significant source of petroleum hydrocarbon contamination in soils and groundwater. There are 2 million underground tanks in the United States that store gasoline, and 90,000 of them are leaking (21). Efforts to clean up this extensive petroleum contamination have prompted research into in situ bioremediation. Depending on specific microbiological, chemical, and hydrogeological constraints, in situ hydrocarbon biodegradation is a cost-effective and environmentally sound remediation alternative or partner to pump-and-treat and vacuum extraction technologies (1). Although hydrocarbon biodegradation studies have been conducted in both groundwater (2, 5) and topsoil (8, 12) regions, fewer studies have been conducted in the unsaturated zone between these two regions.

In 1930, Schollenberger observed that soil exposed to natural gas contained more N than unexposed soil (25). Since that time, very few studies have compared hydrocarbon contamination with the occurrence of N_2 fixation in soils (7, 10, 13). Many field-scale bioremediation efforts have shown that fixed nitrogen additions to soils stimulate hydrocarbon biodegradation (8, 9, 19). Only a few studies have shown that N additions do not stimulate hydrocarbon biodegradation (17, 24), but these studies did not consider whether the lack of stimulation by N could be due to N_2 fixation.

We examined the effects of N on propane and butane biodegradation in an unsaturated sandy soil. Propane and butane were studied because volatile, low-molecular-weight hydrocarbons (especially butane) constitute a majority of the soil vapor phase after a gasoline spill and can be used to confirm and monitor subsurface contamination by soil-gas surveying (15). The soil (Columbia River soil) was taken from a Large Experimental Aquifer Program tank (9 m wide by 11 m long by 4.5 m deep), which is a unique field-scale experimental facility located at the Oregon Graduate Institute. The Large Experimental Aquifer Program tank is currently used to monitor the transport and fate of hydrocarbons leaked from underground storage tanks, and Columbia River soil was used to establish correlations between laboratory-scale and very-large-scale experiments.

These laboratory-scale experiments showed that N additions initially stimulated both propane- and butane-oxidizing organisms in Columbia River soil but that propane-amended soil became N limited whereas butane-amended soil eventually overcame its N limitation. Our results indicate that butane-oxidizing soil overcame N limitation by fixing N_2 and that N_2-fixing organisms grew in soil amended with butane but not in soil amended with propane.

(Portions of these results were presented previously [27].)

MATERIALS AND METHODS

Soil. A composite sample of Columbia River soil (originally from Scappoose, Oreg.) was collected from a 540-m^3 artificial aquifer at the Oregon Graduate Institute in 1989. The sample was stored at 10 to 15°C in a sealed plastic bucket until used. Available phosphorus (2.7 mg/kg of dry soil), organic carbon content (0.3%), and pH (6.56) of the original soil were determined by using previously described procedures (16, 18, 20). Total N (55 mg/kg of dry soil), NH_4^+-N (0.97 mg/kg of dry soil), and NO_3^--N (0.40 mg/kg of dry soil) were determined at the Oregon State University Soil Testing Laboratory. The volumetric water content of the soil is 8% at field capacity (FC), and the bulk density is

* Corresponding author.

1.52 g/cm^3 (14). The gravimetric water content (θ_g) at FC was therefore 5.3 ml of H_2O per 100 g of dry soil. The grain-size fractions of the sand based on the U.S. Department of Agriculture's classification of soil particle sizes (6) were as follows: 4% gravel (≥4 mm), 37% very coarse sand and gravel (1 to 4 mm), 17% coarse sand (0.5 to 1 mm), 26% medium sand (0.246 to 0.5 mm), 8% fine sand (0.175 to 0.246 mm), 7% fine and very fine sand (0.043 to 0.175 mm), and 1% silt (<0.043 mm) (14).

Experimental design. The soil was air dried for 3 days and sieved (<2 mm), and then 50-g (dry weight) portions of the soil were added to 161-ml serum bottles (Fisher Scientific, Tustin, Calif.). Ultrapure water (resistance, 18 MΩ-cm) was added to make θ_g equal to 4 or 4.5 ml of H_2O per 100 g of dry soil. Water activity (a_w) was measured with an SC-10A thermocouple psychrometer (Decagon Devices, Inc., Pullman, Wash.) and converted to water potential (ψ) by $\psi = [RT \cdot \ln(a_w)]/V_w$, where R is the universal gas constant, T is the absolute temperature, and V_w is the partial molal volume of water (22). The bottles were sealed with butyl rubber stoppers and aluminum crimp caps, shaken to distribute the water, and incubated for up to 600 days in the dark at 20°C (±0.5°C).

Propane or butane (Aldrich Chemical Co., Milwaukee, Wis.) was injected into the bottles; all hydrocarbon injections and sample removals were made with gas-tight syringes (Hamilton Co., Reno, Nev.). Sample replicates (4 to 12) were prepared for each experimental condition. To maintain aerobic conditions in the bottles, the bottles were flushed with room air when the O_2 level fell to approximately 10% and the hydrocarbon concentration was 0%. Additional hydrocarbons (0.24 to 2 ml of propane or 2.5 ml of butane) were added each time the bottles were flushed. Flushing the bottles with room air may also have increased soil pH that was lowered during incubation because of CO_2 accumulation; 8% CO_2 in the headspace decreased the soil pH from 6.56 to 6.17.

Sterile controls duplicating experimental conditions were prepared for all experiments. Empty sterile serum bottles injected with propane or butane served as additional controls to account for any sorption of the hydrocarbons to the butyl rubber stoppers. All sterile controls were autoclaved at 121°C for 30 min on 2 consecutive days before alkane addition.

Analytical methods. Hydrocarbon disappearance, CO_2 and CH_4 evolution, and O_2 consumption were measured by withdrawing serum bottle headspace gas (0.3 to 0.5 ml) with syringes equipped with sterile needles and injecting the gas into a fixed-volume sample loop on an HP-5890 gas chromatograph (Hewlett-Packard Co., Avondale, Pa.). The headspace volume was the volume of the serum bottle minus the volume of the soil and water; it included the soil pore-space volume. Propane and butane were separated in a fused-silica capillary column (25 m by 0.53 mm inside diameter; Chrompack International, Middelburg, The Netherlands) and detected by a flame ionization detector (FID). Flow rates were as follows: carrier gas (He), 4 ml/min; makeup gas (N_2), 34.5 ml/min; H_2, 41 ml/min; and air, 400 ml/min. Column and detector temperatures were 30 and 170°C, respectively.

Samples analyzed for O_2, CO_2, and CH_4 were split into two separate packed columns. In one column, O_2 was separated in a stainless-steel analytical column (6.4 mm by 48.3 cm; Molecular Sieve 5A 60/80; Alltech Associates, Inc., Deerfield, Ill.) and detected by a thermal conductivity detector. In the other column, CO_2 and CH_4 were separated in a stainless-steel analytical column (3.2 mm by 40.6 cm; Spherocarb 100/120 mesh; Analabs, Norwalk, Conn.) and detected by an FID. Prior to detection, the gas stream passed through a methanator, where the CO_2 in the gas stream was reduced to CH_4 by a Ni catalyst heated to 500°C. Helium was the carrier gas in both columns (40 ml/min to the thermal conductivity detector and 30 ml/min to the FID). Compressed air (400 ml/min) was the fuel gas to the FID, and H_2 (30 ml/min) fueled the methanator. Both column temperatures were held at 40°C for 1 min, increased to 80°C at 30°C/min, and then held at 80°C for 0.5 min. The detector temperatures were 105 and 225°C for the thermal conductivity detector and FID columns, respectively.

All peak areas were quantitated by comparison with standards made from the dilution of pure gases (Air Products and Chemicals, Inc., Allentown, Pa.) in N_2 and integrated with a model 900 series interface (Nelson Analytical, Inc., Cupertino, Calif.).

Effects of nutrients on propane biodegradation. Propane biodegradation was monitored under six nutrient conditions: (i) no added nutrients, (ii) N only, (iii) phosphorus (P) only, (iv) trace minerals (Tr) only, (v) N+P, and (vi) N+P+Tr. P (0.135 mg per bottle = 2.7 mg/kg of dry soil) was added in equimolar quantities of Na_2HPO_4 and NaH_2PO_4 to keep the pH neutral, and N (0.45 mg per bottle = 9 mg/kg of dry soil) was added as NH_4NO_3. The Tr solution was added to Tr-only bottles (2 ml) and to N+P+Tr bottles (0.5 ml). The Tr solution had a pH of 6 and contained the following (per liter of water): 5.0 mg of Na_2-EDTA · $2H_2O$, 1.5 mg of $CoCl_2 \cdot 6H_2O$, 1.0 mg of $MnCl_2 \cdot 4H_2O$, 1.0 mg of $FeSO_4 \cdot 7H_2O$, 1.0 mg of $ZnCl_2$, 0.4 mg of $AlCl_3 \cdot 6H_2O$, 0.3 mg of $Na_2WO_4 \cdot 2H_2O$, 0.2 mg of $CuCl_2 \cdot 2H_2O$, 0.1 mg of H_2SeO_3, 0.1 mg of H_3BO_3, and 0.1 mg of $NaMoO_4 \cdot 2H_2O$.

In all except the N+P+Tr bottles, θ_g was 4.0 ml of H_2O per 100 g of dry soil ($a_w = 0.997$, $\psi = -4 \times 10^5$ Pa, 75% of FC). In the N+P+Tr bottles, θ_g was 4.5 ml of H_2O per 100 g of dry soil ($a_w = 0.999$, $\psi = -1.4 \times 10^5$ Pa, 85% of FC). Propane (0.16 ml) was initially added to all bottles, resulting in a propane concentration of 0.1%. More propane (0.24 to 2 ml) was added after each propane addition was degraded. The soil had not been preexposed to propane.

Effects of N on butane biodegradation. All samples were preexposed to butane (0.13 ml) for 2 months, and then butane biodegradation was monitored under four different N conditions. N was added as $NaNO_3$ to levels of 0.05, 0.1, or 0.15 mg of N per bottle (1, 2, or 3 mg/kg of dry soil, respectively) or as NH_4Cl to 0.1 mg of N per bottle (2 mg/kg of dry soil). In all bottles, θ_g was 4.5 ml of H_2O per 100 g of dry soil. Butane (2 ml) was added to all bottles, resulting in a butane concentration of 1.2%. More butane (0.5 to 2.5 ml) was added after each butane addition was degraded. Controls included samples with no added N and samples with NaCl added at the same ionic strength as the nutrient solutions.

Acetylene reduction assay. Acetylene was introduced into the headspace of serum bottles or culture tubes to 0.07 atm (ca. 7 kPa) after first removing an equivalent volume of air. Cultures were incubated at 22°C in the dark. Controls without added acetylene accounted for any endogenous ethylene production. Headspace samples (0.5 ml) were analyzed for ethylene production with an HP-5890 gas chromatograph with a stainless-steel column (2 mm by 3 m) packed with Porapak N 80/100 mesh (Supelco, Bellefonte, Pa.) at 65°C. N_2 was the carrier gas (25 ml/min). Acetylene and ethylene were detected by using an FID at 250°C, and the integrated peaks were quantitated by comparison with

standards made from the dilution of pure gases (Aldrich) in N_2.

Numbers of organisms. A most-probable-number (MPN) assay was used to determine the numbers of propane- and butane-oxidizing organisms under various conditions. The assay was performed in a mineral medium with or without added N and with propane or butane as the sole source of carbon. The mineral medium contained the following (per liter of water): 0.4 g of $K_2HPO_4 \cdot 3H_2O$, 1.0 g of $MgCl_2 \cdot 6H_2O$, 0.4 g of $CaCl_2 \cdot 2H_2O$, and 10 ml of the Tr solution described above. An N-containing medium also included 0.1 g of $NaNO_3$ per liter as an N source. The medium was prepared by dissolving the constituents into CO_2-saturated water, adjusting the pH to 7.0 with NaOH, and dispensing 5-ml portions into 27-ml culture tubes. All culture tubes were capped with butyl rubber stoppers and aluminum crimp caps and autoclaved at 121°C for 20 min.

Air-dried soil (5 g) was added to 50 ml of sterile 1 mM sodium pyrophosphate (Sigma Chemical Co., St. Louis, Mo.) solution (26). The mixture was firmly shaken to dislodge bacteria from soil particles and settled for 3 min to allow the largest of the sand particles to fall to the bottom of the flask, and serial dilutions of the supernatant were prepared in the sterile medium. Dilutions (10^{-1} to 10^{-9}) were each inoculated into five tubes of mineral medium and into five tubes of mineral medium with added N. Propane (0.2 ml, about 0.5%) or butane (0.2 ml, about 0.5%) was added to each culture, and all cultures were incubated statically (horizontally) at room temperature for 10 weeks. Propane and butane were measured every 2 weeks for 10 weeks or until at least 50% of the hydrocarbon was degraded. Cultures were considered positive if 50% of the added hydrocarbon was degraded in comparison with that in uninoculated controls.

Isolation procedures. To isolate butane-oxidizing, N_2-fixing bacteria, transfers (10%) were made from the most-dilute, positive MPN cultures into a mineral medium with and without added N, and organisms from the MPN cultures were streaked and restreaked onto agar plates made from the mineral medium without N solidified with 15% purified-grade agar (Fisher Scientific). The mineral medium had a pH of 7 and no precipitate and contained the following (per liter of water): 0.014 g of Na_2SO_4, 0.4 g of $K_2HPO_4 \cdot 3H_2O$, 0.2 g of $MgCl_2 \cdot 6H_2O$, 0.02 g of $CaCl_2 \cdot 2H_2O$, and 10 ml of the Tr solution described above. The transferred cultures were incubated statically (horizontally) at 37°C after adding butane (0.2 ml). Cultures on agar plates were incubated at 37°C in an air-tight jar with 0.65% butane in the headspace. Controls included incubation of uninoculated transfer tubes as well as incubation of cultures on agar medium lacking substrate.

RESULTS

Effects of nutrients on propane biodegradation. During the first 40 days of incubation, propane degradation rates were similar under all nutrient conditions except when no nutrients were added (Fig. 1). More propane (0.24 ml) was added on day 47, and differences in the degradation rates of various treatments became apparent: soil with added N degraded propane much faster than those with other treatments (Fig. 1B). The degradation rate was independent of propane concentration when propane concentration was greater than about 0.05%, suggesting that the apparent K_m for propane oxidation was lower than 0.05% (data not shown).

Additions of multiple inorganic nutrients (N+P or N+P+Tr) did not enhance propane oxidation over that resulting from the addition of N alone. Each propane addition to N-amended bottles was degraded faster than the previous one to a maximum rate of 8.8 mg of C per day per kg of dry soil after the fifth addition on day 108. Propane additions after this one were degraded more slowly, at rates of about 1.7 mg of C per day per kg of dry soil (Fig. 2). Propane degradation was unaffected by water contents ranging from 75 to 85% of FC.

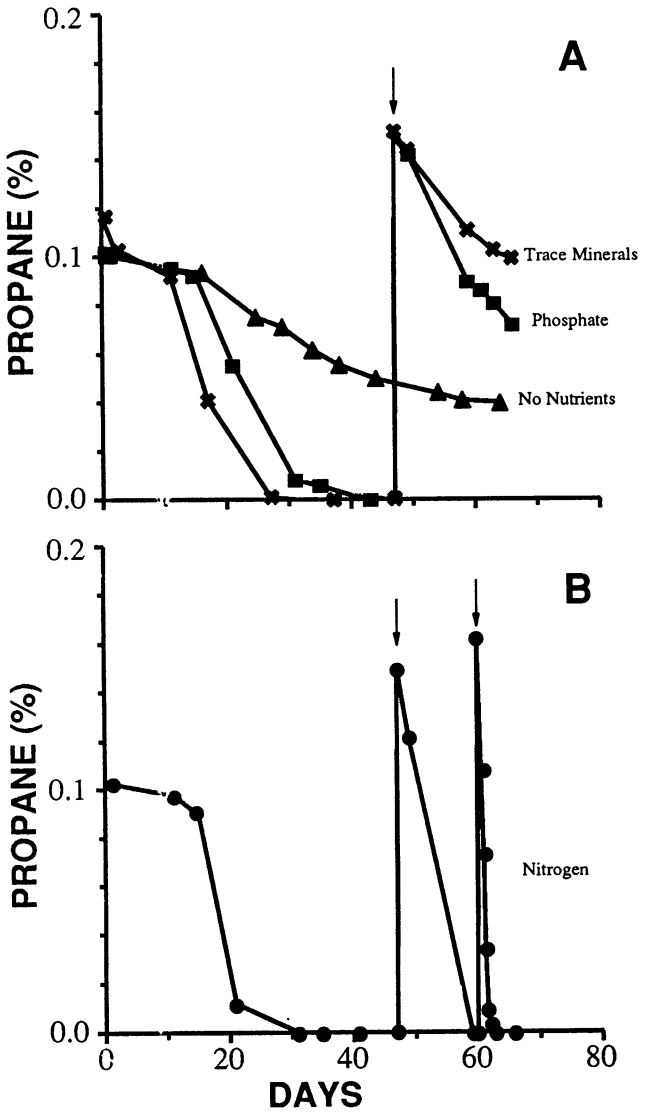

FIG. 1. (A) Effects of various nutrient amendments on propane biodegradation. The arrow indicates the addition of propane. (B) Effects of N only on propane biodegradation. Arrows indicate the addition of propane. Soils treated with N+P or N+P+Tr degraded hydrocarbon at the same rate as soils treated with N only.

In bottles with added N, a total of 19 mg of C was added as propane (0.53 mmol of propane), 12 mg of C was evolved as CO_2 (1 mmol of CO_2), and 1.81 mmol of O_2 was consumed over the course of the experiment (about 600 days). No CH_4 was produced, and sterile controls showed insignificant propane losses, CO_2 production, or O_2 consumption.

Effects of N on butane biodegradation. Nutrient studies

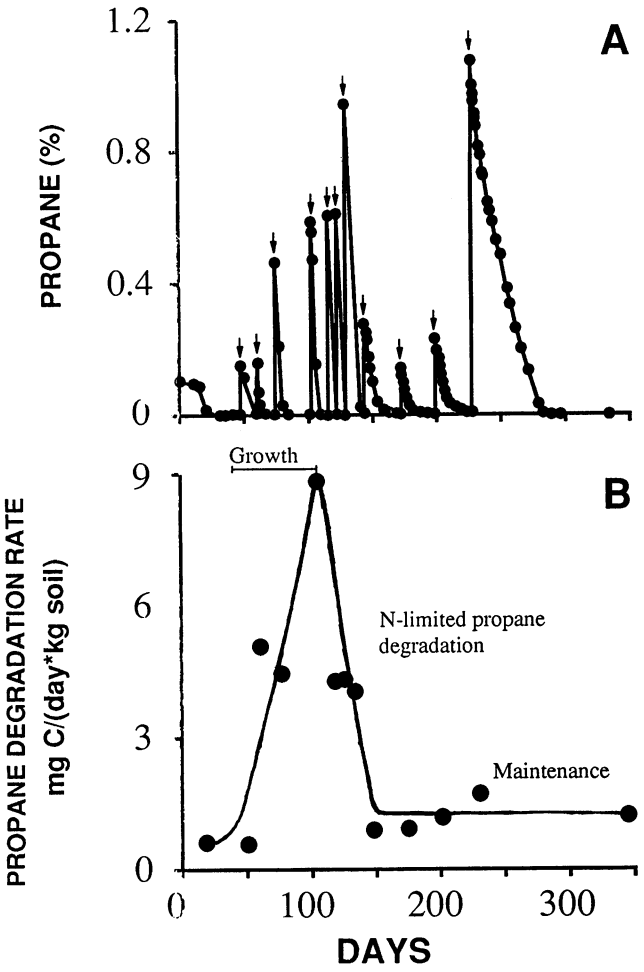

FIG. 2. (A) Propane degradation in N-amended soil (0.45 mg of N per bottle = 9 mg/kg of dry soil). Arrows indicate the addition of propane. Early time points are duplicates of data shown in Fig. 1. (B) Propane degradation rates, calculated from the slopes of the linear portions of curves shown in panel A (in some cases, only two points were used to determine a rate). The last propane degradation rate shown was calculated from additional propane degradation experiments carried out after day 300.

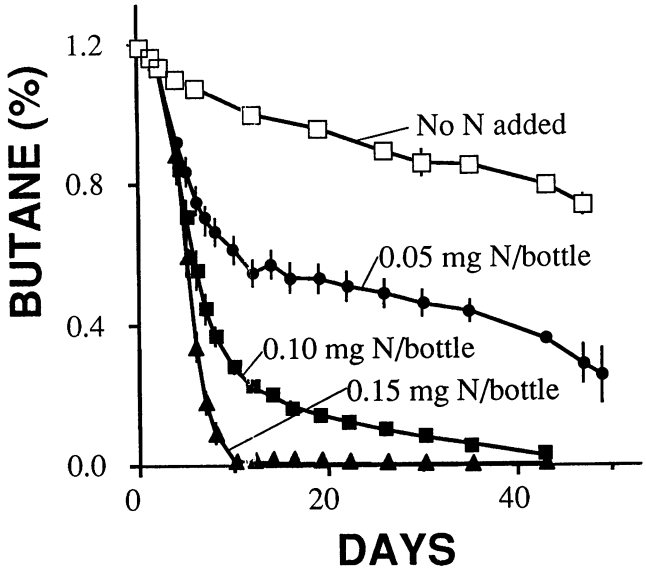

FIG. 3. Effects of various NO_3-N concentrations on butane biodegradation. Error bars represent the standard errors of the means.

with propane showed that N alone was the most-limiting nutrient in Columbia River soil. Therefore, in the butane biodegradation study, only N was supplied as an additional nutrient. During the first 7 weeks of incubation, butane degradation rates increased with increasing soil N concentrations (Fig. 3). Butane degradation rates ranged from 1.8 mg of C per day per kg of dry soil when no N was added to 11.4 mg of C per day per kg of dry soil in soil amended with 0.15 mg of N per bottle. Degradation rates later fell in all samples to about 2 mg of C per day per kg of dry soil, presumably after the available inorganic N was utilized (Fig. 4). This assumption was verified by plotting the amount of butane oxidized during the first 2 months of incubation versus the amount of N added to the bottles. The linear relationship between the N added and butane degraded (Fig. 5) suggested that butane degradation during the first 2 months was proportional to the available inorganic N (i.e., available inorganic N in the unamended soil plus added N). Assuming that butane degradation was proportional to available inorganic N, extrapolating this line back to an x intercept of -0.07 mg of added N per bottle indicates that the unamended soil contained 0.07 mg of available N per bottle (=1.4 mg of N per kg of dry soil). This was in agreement with the measured value of available inorganic N (1.37 mg of N per kg of dry soil). Measured available inorganic N was defined as the sum of NH_4^+-N (0.97 mg/kg of dry soil) and NO_3^--N (0.40 mg/kg of dry soil).

Butane biodegradation was unaffected by the form of N added; degradation rates were the same when N was added as $NaNO_3$ or as NH_4Cl. Soil samples with added NaCl degraded butane at a rate similar to samples with no N added (data not shown). After 3 months of incubation, butane was degraded in all samples at the same rate regardless of the initial N concentration, and butane degradation rates increased with time to 60 mg of C per day per kg of dry soil (Fig. 4).

A total of 131 mg of C was added as butane (2.7 mmol of butane), 66 mg of C was evolved as CO_2 (5.5 mmol of CO_2), and 9.8 mmol of O_2 was consumed over the course of the experiment (about 1 year). No CH_4 was produced, and sterile controls showed insignificant butane losses, CO_2 production, or O_2 consumption. Butane degradation rates, like propane degradation rates, appeared to be independent of substrate concentration when substrate concentration was high (greater than about 0.1% for butane).

Evidence for N_2 fixation in butane-amended soil. N became limiting when propane was used as a substrate and during the first 2 months of incubation with butane. However, during subsequent butane degradation, N limitation was apparently overcome. N_2 fixation would explain the rapid butane degradation in the absence of added N. Such N_2 fixation could be due to the activities of butane-degrading organisms per se or butane degraders could form associations with N_2-fixing bacteria which supply N to the butane degraders by cross-feeding. Four experiments were performed to verify that N_2 fixation occurred in butane-amended soil but not in propane-amended soil.

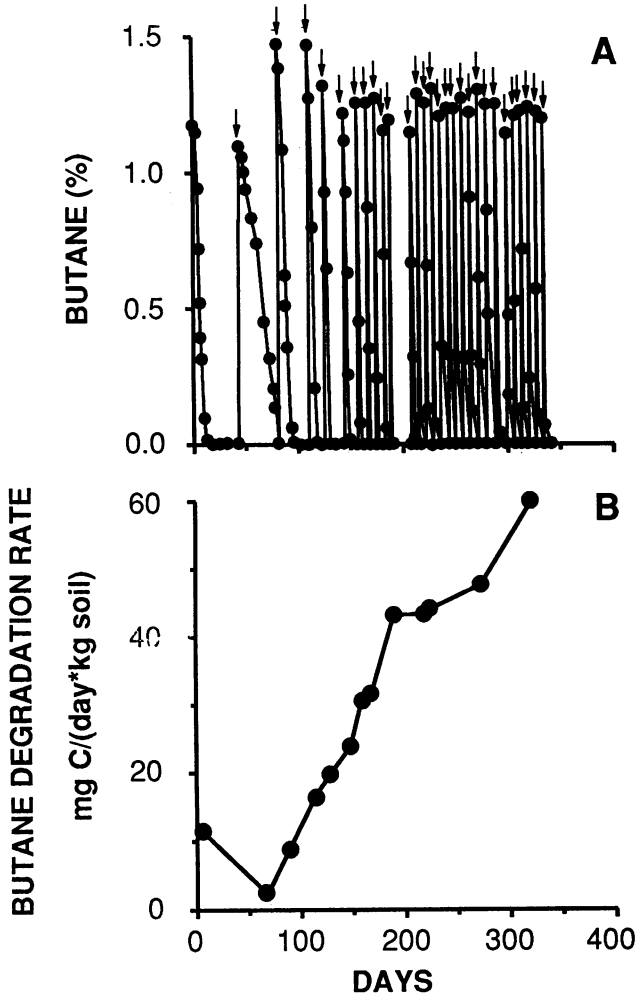

FIG. 4. (A) Butane biodegradation in N-amended soil (0.15 mg of N per bottle = 3 mg/kg of dry soil). Arrows indicate the addition of butane. Early time points are duplicates of data shown in Fig. 3 with 0.15 mg of N added per bottle (3 mg/kg of dry soil). (B) Butane degradation rates calculated from the slopes of the curves shown in panel A (in some cases, only two points were used to determine a rate).

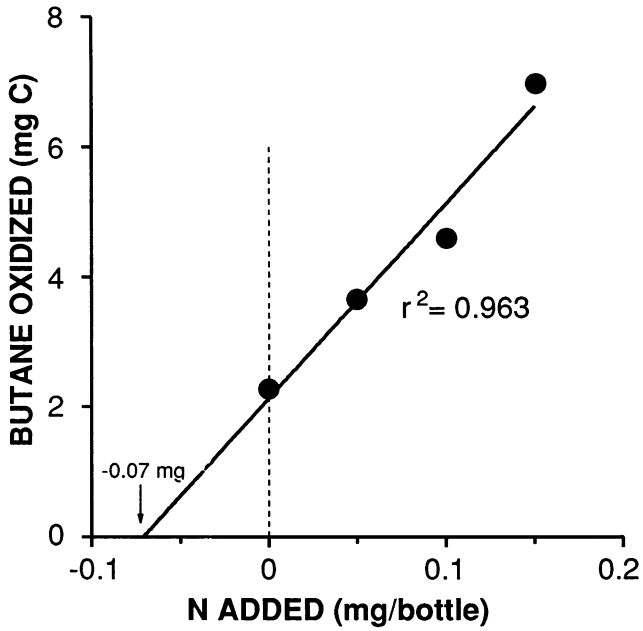

FIG. 5. Bioavailable N in Columbia River soil calculated from the amount of butane degraded during the first 2 months of incubation when various amounts of N were added to the soil.

The first experiment determined whether N was limiting in propane- and butane-amended soil. When more available inorganic N (0.15 mg of N as $NaNO_3$ per bottle) was added to propane-amended soil on day 287, the residual propane was quickly degraded after a short lag time of 3 days. Propane biodegradation rates increased from 0.5 to 6.7 mg of C per day per kg of dry soil after the supplemental N was added. In contrast, supplemental available inorganic N added to butane-amended soil had no effect on butane biodegradation rates, which remained constant at about 50 mg of C per day per kg of dry soil (data not shown).

In the second experiment, serum bottles were prepared with a 1:1 mixture of propane- and butane-degrading soil that was not amended with N, and both propane and butane were added as substrates. Organisms in the mixed soil degraded equal quantities of both compounds, and average maximum propane and butane degradation rates were approximately equal (27 mg of C per day per kg of dry soil). In propane-amended soil without N amendments, only 1.4 ml of propane was degraded in 180 days. In contrast, in the mixed soil without N amendments, 7 ml of propane was degraded in only 50 days. The maximum propane degradation rate in mixed soil was 37 mg of C per day per kg of dry soil, which was four times higher than the maximum propane degradation rate in unmixed soil with N amendments.

In the third experiment, the acetylene reduction assay demonstrated that N_2 was fixed (acetylene was reduced) in butane-amended soil but not in the original unamended soil, the propane-amended soil (Fig. 6), or the controls. Ethylene production rates in butane-amended soil averaged 2.1 μmol of ethylene per kg of dry soil per h.

In the last experiment, total Kjeldahl N was determined for the original soil and for propane- and butane-amended soil with and without N amendments. Propane-amended soil contained similar amounts of total N (55 to 65 mg of N per kg of dry soil) as the original soil (55 mg of N per kg of dry soil), whereas the butane-amended soil contained significantly ($P < 0.01$) more total N (85 to 95 mg of N per kg of dry soil) than the original soil, on the basis of a Student's t test analysis.

Numbers of organisms. The initial butane-oxidizing population (450/g of dry soil) was larger than the initial propane-oxidizing population (<10/g of dry soil) in the original soil. Hydrocarbon-amended soil contained significantly ($P < 0.05$) more hydrocarbon-degrading organisms than the original soil; propane-amended soil contained 680 propane-degrading organisms per g of dry soil, and butane-amended soil contained 6.8×10^5 butane-degrading organisms per g of dry soil. There were significantly ($P < 0.05$) more butane oxidizers than propane oxidizers in hydrocarbon-amended soil. The presence of N in the mineral medium only slightly increased the numbers of propane- and butane-oxidizing organisms counted. No organisms were detected in the uninoculated controls.

No ethylene was produced during the acetylene reduction assays of the MPN cultures over the 24-h incubation period

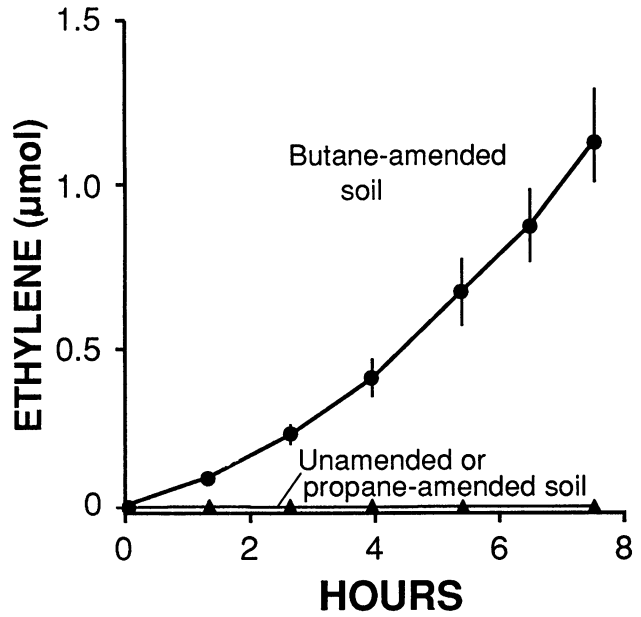

FIG. 6. Ethylene production in acetylene reduction assay soil samples. Error bars represent the standard errors of the means.

regardless of the dilution tested, the substrate utilized, or the presence or absence of N in the media.

Attempts to isolate organisms. No cultures that fixed N_2 and utilized butane as a sole source of C were isolated. Transfers made from positive MPN cultures did not grow unless (i) the transfers were made from MPN cultures amended with N or (ii) the transfers were made into media that contained N. In both cases, growth did not occur before at least 6 weeks of incubation. Colonies grew on all of the streak plates regardless of whether any butane was present as a substrate, suggesting that the organisms may have used one or more unknown catabolic substrates other than butane.

DISCUSSION

Differences between propane and butane biodegradation. Propane and butane degradation proceeded similarly during the first 2 to 3 months of incubation. That is, propane and butane degradation by microbes in this sandy soil was stimulated by N additions, and both propane- and butane-amended soil became N limited after the initial available inorganic N was utilized, as indicated by a decrease in degradation rates (Fig. 2 and 4). After 2 to 3 months, propane-degrading soil remained N limited, whereas butane-degrading soil overcame N limitation.

Propane biodegradation rates in N-amended soil followed a pattern consistent with the requirement of fixed N for growth of microbial populations. Our measurement of higher numbers of propane degraders after propane was degraded in soil samples indicated growth of these populations. The amount of propane degraded was much greater than the organic matter content of the soil, so it is unlikely that soil microbes could degrade so much propane without growth. Degradation rates were initially low and then increased. The increased degradation rate appears to be due to increases of propane-oxidizing populations. Later, degradation rates fell to a maintenance level (Fig. 2), presumably because available N was exhausted and microbial growth stopped. The rate of propane degradation in N-limited soil was relatively high (20% of the maximum degradation rate). This suggested that (i) propane degradation met the high maintenance-energy needs of a nongrowing population; (ii) the population of propane degraders was maintained at a constant number by an equal growth rate and microbial decay rate, perhaps the result of protozoal grazing of the bacterial population; or (iii) slowly growing propane-degrading bacteria were supplied N by N_2 fixation occurring at a rate much lower than that occurring in butane-degrading soils.

Whereas degradation rates in propane-degrading soil suggested that microbial growth depended on added N, butane degradation rates continued to increase without added N (Fig. 4), suggesting that N_2 was fixed in butane-amended soil.

Biodegradation equations. Mass balances for C and O_2 were calculated on the basis of the initial and final concentrations of CO_2, O_2, and hydrocarbons in the bottles. These calculations were made to better understand the nutrient requirements of propane- and butane-degrading bacteria. All of the added C that was not evolved as CO_2 was assumed to be incorporated into biomass. This assumption was made because actual measurements of biomass are highly variable (11). Biomass incorporation was 37.5 or 50% of the added C when propane or butane, respectively, was used as the substrate, which supports previous findings that about 40% of substrate C is assimilated into biomass (3). The following net equations for propane and butane degradation take into account the percentage of substrate mineralized and incorporated into biomass. The stoichiometric equation for propane mineralization is as follows: $C_3H_8 + 5O_2 \rightarrow 3CO_2 + 4H_2O$. The equation for stoichiometric conversion of propane to biomass is as follows: $C_3H_8 + 1.4O_2 + 0.3NH_4NO_3 \rightarrow 0.6C_5H_7NO_2 + 2.5H_2O$. The net equation, with 62.5% of propane mineralized and 37.5% converted to biomass, is as follows: $C_3H_8 + 3.65O_2 + 0.11NH_4NO_3 \rightarrow 0.22C_5H_7NO_2 + 1.87CO_2 + 3.4H_2O$.

For butane mineralization, the equation is as follows: $C_4H_{10} + 6.5O_2 \rightarrow 4CO_2 + 5H_2O$. The equation for butane incorporation into biomass is as follows: $C_4H_{10} + 0.7O_2 + 0.8NaNO_3 \rightarrow 0.8C_5H_7NO_2 + 2.2H_2O + 0.8Na^+$. On the basis of mineralization of 50% of butane, with 50% converted to biomass, the net equation for butane biodegradation is as follows: $C_4H_{10} + 3.6O_2 + 0.4NaNO_3 \rightarrow 0.4C_5H_7NO_2 + 2CO_2 + 3.6H_2O + 0.4Na^+$.

The biomass formula above ($C_5H_7NO_2$) is an empirical representation of microbial biomass derived from the ratios of elements in a typical microbial cell (23). There is very good agreement (0 to 7% difference) between the O_2/CO_2 and O_2/hydrocarbon ratios actually measured and ratios predicted by the above equations. In addition, the overall equations approximated how much N was needed to degrade the hydrocarbons before N became limiting to actively growing organisms. However, during nutrient- or C-limiting conditions, more of the substrate is expected to be utilized for maintenance functions (mineralization), and less of the substrate would be incorporated into biomass. Therefore, the overall equations described above can be manipulated on the basis of the fraction of C oxidized and the fraction assimilated into biomass. These propane and butane degradation equations suggest that the nutrient requirements for soil microbial communities can be quantitatively estimated.

Nutrient requirements. A separate method for estimating the N needed to satisfy the demands for cell synthesis requires data on the extent of C assimilation and the C/N

ratio of the cells formed. C assimilation was 37.5 or 50% of the substrate C in propane and butane oxidizers, respectively. Microbial cells have an average C/N ratio of 3.6 (4) to 10 (3, 8). Combining the figures for C assimilation and cell composition indicates that the decomposition of 100 mass units of substrate C requires 3.8 to 10.5 mass units of N for propane oxidizers and 5 to 14 mass units of N for butane oxidizers or a C/N ratio of 9.5 to 27 for propane oxidizers and 7 to 20 for butane oxidizers. These C/N ratios can be used to speculate on the microbial activities in the propane- and butane-amended soil.

On the basis of these ratios and the amount of N added to propane-degrading soil, 4.9 to 14 mg of C per bottle should have been degraded before N became limiting. In actuality, 3.7 mg of C per bottle was degraded before propane-amended soil appeared to become N limited, and a total of 19 mg of C per bottle was degraded over the course of the experiment.

Without N_2 fixation, butane-amended soil with no added N should have degraded only 0.5 to 1.4 mg of C per bottle before N became limiting, but 131 mg of C per bottle was actually degraded. These results suggested that butane-degrading soil obtained N by N_2 fixation. This suggestion was supported by total N accumulation and by acetylene reduction in butane-amended soil. The presence of surplus N in butane-degrading soil was also shown by a lack of response to additional N and by the stimulation of propane degradation when propane-degrading soil was mixed with butane-degrading soil.

ACKNOWLEDGMENTS

We thank Oregon State University's Soil Testing Laboratory for performing the nitrogen analyses and Wesley Jarrell (Oregon Graduate Institute) and Daniel Arp (Oregon State University) for valuable discussions.

This work was supported by the Oregon Oil Heat Commission.

REFERENCES

1. **Aelion, C. M., and P. M. Bradley.** 1991. Aerobic biodegradation potential of subsurface microorganisms from a jet fuel-contaminated aquifer. Appl. Environ. Microbiol. **57:**57–63.
2. **Aelion, C. M., C. M. Swindoll, and F. K. Pfaender.** 1987. Adaptation to and biodegradation of xenobiotic compounds by microbial communities from a pristine aquifer. Appl. Environ. Microbiol. **53:**2212–2227.
3. **Alexander, M.** 1977. Introduction to soil microbiology, 2nd ed. John Wiley & Sons, Inc., New York.
4. **Atlas, R. M.** 1988. Microbiology fundamentals and applications, 2nd ed. Macmillan Publishing Company, New York.
5. **Beloin, R. M., J. L. Sinclair, and W. C. Ghiorse.** 1988. Distribution and activity of microorganisms in subsurface sediments of a pristine study site in Oklahoma. Microb. Ecol. **16:**85–97.
6. **Brady, N. C.** 1984. The nature and properties of soils, 9th ed. Macmillan Publishing Company, New York.
7. **Coty, V. F.** 1967. Atmospheric nitrogen fixation by hydrocarbon-oxidizing bacteria. Biotechnol. Bioeng. **9:**25–32.
8. **Dibble, J. T., and R. Bartha.** 1979. Effect of environmental parameters on the biodegradation of oil sludge. Appl. Environ. Microbiol. **37:**729–739.
9. **Ellis, B., M. T. Balba, and P. Theile.** 1990. Bioremediation of oil contaminated land. Environ. Technol. **11:**443–455.
10. **Harper, H. J.** 1939. The effect of natural gas on the growth of microorganisms and the accumulation of nitrogen and organic matter in the soil. Soil Sci. **48:**461–466.
11. **Hulm, S. C., D. L. Castle, K. Cook, A. Self, and M. Wood.** 1991. Evaluation of soil microbial biomass methodology. Toxicol. Environ. Chem. **30:**183–192.
12. **Jones, J. G., and M. A. Edington.** 1968. An ecological survey of hydrocarbon-oxidizing micro-organisms. J. Gen. Microbiol. **52:** 381–390.
13. **Laguerre, G., B. Bossand, and R. Bardin.** 1987. Free-living dinitrogen-fixing bacteria isolated from petroleum refinery oily sludge. Appl. Environ. Microbiol. **53:**1674–1678.
14. **MacPherson, J. R.** 1991. Ph.D. dissertation. Oregon Graduate Institute of Science & Technology, Beaverton.
15. **Marrin, D. L., and H. B. Kerfoot.** 1988. Soil-gas surveying techniques. Environ. Sci. Technol. **22:**740–745.
16. **McLean, E. O.** 1982. Soil pH and lime requirements, p. 206–207. *In* A. L. Page, R. H. Miller, and D. R. Keeney (ed.), Methods of soil analysis, part 2. American Society of Agronomy, Madison, Wis.
17. **Morgan, P., and R. J. Watkinson.** 1992. Factors limiting the supply and efficiency of nutrient and oxygen supplements for the *in situ* biotreatment of contaminated soil and groundwater. Water Res. **26:**73–78.
18. **Nelson, D. W., and L. E. Sommers.** 1982. Total carbon, organic carbon, and organic matter, p. 565–570. *In* A. L. Page, R. H. Miller, and D. R. Keeney (ed.), Methods of soil analysis, part 2. American Society of Agronomy, Madison, Wis.
19. **Odu, C. T. I.** 1977. Oil degradation and microbiological changes in soils deliberately contaminated with petroleum hydrocarbons, p. 1–11. *In* publication number IP 77-005. Institute of Petroleum, London.
20. **Olsen, S. R., and L. E. Sommers.** 1982. Phosphorus, p. 421–422. *In* A. L. Page, R. H. Miller, and D. R. Keeney (ed.), Methods of soil analysis, part 2. American Society of Agronomy, Madison, Wis.
21. **OUST.** 1990. LUST trust fund monthly progress report, September 1990. Office of Underground Storage Tanks, U.S. Environmental Protection Agency, Washington, D.C.
22. **Papendick, R. I., and G. S. Campbell.** 1981. Theory and measurement of water potential, p. 1–22. *In* J. F. Parr, W. R. Gardner, and L. F. Elliott (ed.), Water potential relations in soil microbiology. Soil Science Society of America, Madison, Wis.
23. **Porges, N., L. Jasewicz, and S. R. Hoover.** 1956. Principles of biological oxidation, p. 35–48. *In* B. J. McCabe and W. W. Eckenfelder (ed.), Biological treatment of sewage and industrial wastes. Van Nostrand Reinhold, New York.
24. **Raymond, R. L., V. W. Jamison, J. O. Hudson, R. E. Mitchell, and V. E. Farmer.** 1978. Field application of subsurface biodegradation of gasoline in a sand formation, p. 1–112. *In* API publication no. 4430, project no. 307-77. American Petroleum Institute, Washington, D.C.
25. **Schollenberger, C. J.** 1930. Effect of leaking natural gas upon the soil. Soil Sci. **29:**261–266.
26. **Sharma, M. M., Y. I. Chang, and T. F. Yen.** 1985. Reversible and irreversible surface charge modification of bacteria for facilitating transport through porous media. Colloids Surf. **16:** 193–206.
27. **Toccalino, P. L., and D. R. Boone.** 1992. Effects of nitrogen additions on propane and butane biodegradation in sandy soils, p. 353, Q-107. Abstr. 92nd Gen. Meet. Am. Soc. Microbiol. 1992. American Society for Microbiology, Washington, D.C.