Optimization of Hydrocarbon Biodegradation in a Sandy Soil

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A dissertation submitted to the faculty of the
Oregon Graduate Institute of Science & Technology
in partial fulfillment of the
requirements for the degree
Doctor of Philosophy
in
Environmental Science and Engineering

October, 1992

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DEDICATION

To my Dad

Who always wanted me to be a Doctor

(He just didn't say what kind)

ACKNOWLEDGEMENTS

Dave, Rick, and Wes: thank you for your guidance, for not allowing me to falter on dead-ends for too long, for knowing when to push me and when to back off. Dr. Harmon, you opened the door to many possibilities, including this achievement. Thank you for your wisdom, advice, and caring.

My family provided me with much support. Tim believed in me, and made great sacrifices on my behalf. Without you, I may not have made this journey, and I would certainly still be washing bottles! From the beginning, my parents fostered in me a love of science and the drive to be the best I could be. Mom, thanks for your patience. Debbie (220 Pesta) dared me to be independent in the face of adversity. You taught me that anything is possible, and always listened to me. Greg kept me sane by being my best friend through the formidable years, and shared his wisdom with me. I am still learning to be like water. I owe my acceptance into OGI to Chris, who tutored me in calculus and diffy-Q until I could scream. You showed me kindness long after I didn't deserve it.

My friends were the backbone of my survival. The very best of my friends, Gwen, Carolyn, and Jerry always supported me, listened to me, and advised me. They always told me what I needed to hear, even when I didn't want to hear it. Choy, Leah, Kathy, Liannha, Tammy: sharing common goals, triumphs, and failures with you let me know that I was never alone. Our day-to-day conversations helped keep me going. Matt and Don not only provided invaluable technical assistance and saved me months of work, but also made me keep a sense of humor. Many people, including John St., Jim T., Stewart, Jay, Bernie, Mike, José, Tim M., and the people in my lab groups have helped me in many ways. Barry, Vivek, and John Si. showed me that having fun was an important part of the process. The staff at OGI has been indispensable, particularly Judi and Pam, Lorne, Alan, Doug, and the entire library staff.

I thank Dan Arp for his valuable comments, Oregon State University's Soil Testing Laboratory for performing the nitrogen analyses, and Elaine Ingham (Oregon State University, Microbial Biomass Service) for enumerating protozoa. I also thank the Oregon Oil Heat Commission for funding this project.

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ABSTRACT

Optimization of Hydrocarbon Biodegradation in a Sandy Soil

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Leaking underground storage tanks contaminate soils and groundwater with petroleum hydrocarbons. The objective of this research was to better understand hydrocarbon biodegradation in soil by examining the effects of soil moisture content, temperature, hydrocarbon concentration, hydrocarbon chain length, and nutrient additions on hydrocarbon biodegradation rates. Hydrocarbon biodegradation was examined in serum-bottles containing an unsaturated sandy soil incubated aerobically with hydrocarbons and various added nutrients. A majority of the experiments used propane and butane as catabolic substrates.

Temperature (15-20°C) and water contents (>75% of field capacity) had little effect on hydrocarbon biodegradation. Hydrocarbon concentrations greater than 0.5% of the serum-bottle headspace inhibited biodegradation unless the organisms were previously exposed to a lower concentration. Nutrient limitation studies showed that fixed nitrogen (N) was initially a limiting nutrient, but that N-limitation could be overcome by N_2 fixation under some conditions.

Propane and butane degradation proceeded similarly during the first 3 months of incubation. 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 bioavailable N was utilized, as indicated by a decrease in degradation rates. After 3 months, propane and butane degradation proceeded differently. Propane-degrading soil

apparently remained N-limited, because degradation rates stayed low unless more N was added. In contrast, butane-degrading soil appeared to overcome its N-limitation, because degradation rates continued to increase regardless of whether more N was added. Total-N analyses and acetylene-reduction assays supported this apparent surplus of N in butane-amended soil. Total N was significantly ($P<.01$) 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.

CHAPTER 1
INTRODUCTION

1.1 Purpose of Study

Leaking underground storage tanks are a significant source of petroleum hydrocarbon contamination in soils and groundwater. There are 2 million underground tanks in the U.S. that store gasoline, and 90,000 of them leaked between 1988 and 1990 alone (98). 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).

Hydrocarbon biodegradation in groundwater systems has been extensively studied (1, 2, 11, 114, 129) because the quality of drinking water is adversely affected by organic pollutants. However, the study of hydrocarbon biodegradation in the unsaturated zone has been primarily limited to the surface horizons in landfarming experiments (22, 31, 58, 63, 113). There have been few hydrocarbon biodegradation studies in the lower subsurface regions above the water table (131), but because contaminants entering groundwater frequently move through the unsaturated zone, an examination of the microbial communities in this region is needed.

The objective of this research was to examine how several physical and environmental factors affect the aerobic biodegradation of hydrocarbons in an unsaturated sandy soil. The soil used in this study (Columbia River soil) was taken from a Large Experimental Aquifer Program (LEAP) tank (Fig. 1.1), which is a unique field-scale experimental facility located at the Oregon Graduate Institute. The LEAP tank is current-

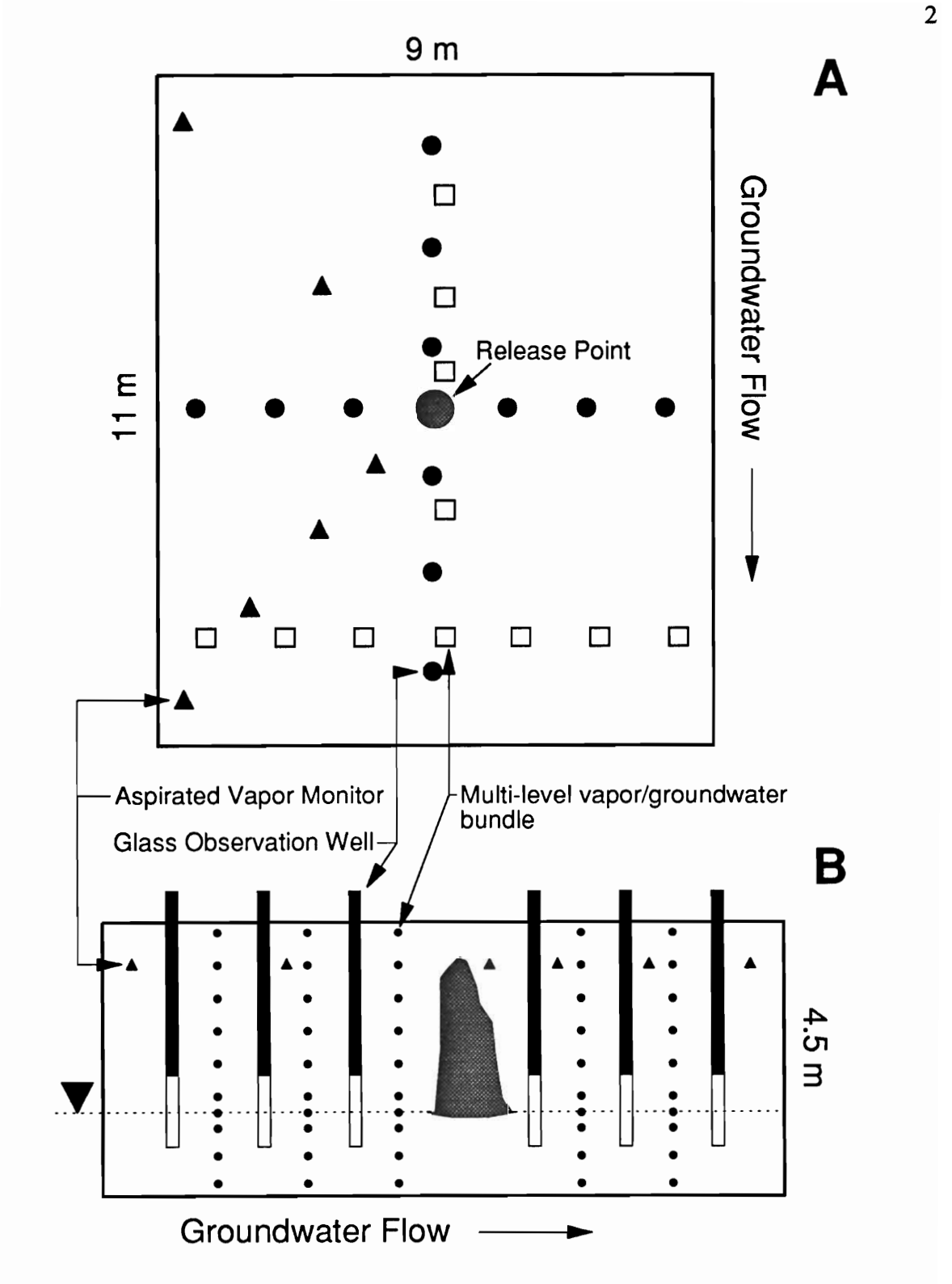


Figure 1.1 Plan view (A) and cross-sectional view (B) of LEAP tank.

ly used to model the transport and fate of hydrocarbons leaked from underground storage tanks, and soil from the LEAP tank was used in order to establish correlations between laboratory-scale and very large-scale experiments. Volatile, low-molecular-weight hydrocarbons were used in this study because that group of compounds comprise 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 (81). Propane and butane are components of natural gas [3% and 1%, respectively (145)], and gasoline [0.01% and 5-10%, respectively (61, 121)].

The effects of soil moisture content, temperature, hydrocarbon concentration, hydrocarbon chain length, and nutrient additions on hydrocarbon biodegradation were studied. The most significant result was that fixed nitrogen (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. Butane-oxidizing soil overcame N-limitation by fixing N_2, and N_2-fixing organisms grew in soil amended with butane but not in soil amended with propane.

1.2 Literature review

1.2.1 Background information

In situ biodegradation of organic contaminants in the unsaturated subsurface is a dynamic process which is initiated by the stimulation of the subsurface microflora to degrade contaminants in place. *In situ* biodegradation is site-specific, perhaps even microsite-specific, and its ultimate result is to convert organic wastes into biomass and harmless by-products of microbial metabolism. In order for *in situ* hydrocarbon biodegradation to take place, the soil matrix must be permeable to O_2 and nutrient solutions, and contaminant-degrading microorganisms must be present (132). Hydrocarbon degradation by microbes was first recognized in the late 1800's, and by 1946, the

role that microbes play in degrading petroleum pollutants was widely recognized (7).

1.2.2 Advantages and Disadvantages of Remediation Techniques

All treatment methods for contaminated soils have advantages and disadvantages. In addition to microbial degradation, other techniques used to remediate subsurface soils include enhanced volatilization, incineration, excavation, and soil washing/extraction. These techniques vary in cost and effectiveness (Table 1.1). There is considerable controversy over whether complete microbial degradation of hydrocarbons in soils is more rapid than other methods. An EPA study (37) found that the time needed for biorestoration to purify gasoline-contaminated aquifers is expected to be on the order of years rather than the shorter times needed for physicochemical techniques such as air stripping or carbon (C) adsorption.

However, for many spills, physical cleanup methods are too slow, too expensive, or cannot reduce the concentration of contaminant to a satisfactory level. In many such instances, the groundwater supply has to be abandoned or expensive activated C treatment systems must be employed (114). In some cases, the time required to treat subsurface pollution by using *in situ* biorestoration (years) is less than that of pump-and-treat procedures (decades) (133).

The above studies were conducted in saturated aquifer systems. Degradation rates in the unsaturated zone are expected to be faster than in saturated aquifer systems because O_2 diffusion to contaminants in the unsaturated zone is generally not limiting, except perhaps to the central portions of a hydrocarbon plume. When applicable, the biodegradation of organic compounds into inorganic constituents (CO_2 and H_2O) and cell mass offers a better solution to hazardous materials disposal than simply transferring the contamination from one medium of the environment to another (132).

Table 1.1 Advantages, disadvantages and costs of various hydrocarbon remediation techniques (37, 133)

REMEDIATION TECHNIQUE	ADVANTAGES	DISADVANTAGES	COST
Excavation and Disposal	-Can be 100% effective -Reduction in mobility -Reduction in volume	-Backhoes only reach depths of 45 feet -Excavation is difficult in congested areas -Increases risk of exposure -Transportation risks -Expensive landfill disposal -Not efficient for large quantities	$200 to $300/yd^3
Enhanced Volatilization	-Can remove up to 99.9% of organic compounds	-Some soil characteristics constrain movement of hydrocarbon vapors from the soil to the air -May create explosion hazard -Need to control dust -Need vapor phase treatment	$245 to $320/yd^3 (most effective with 15,000 to 18,000 tons of soil)
Incineration	-Eliminates 99.99% (or more) of hydrocarbon constituents by complete oxidation -Highly reliable -Reduction in volume	-Soil must be brought to the surface which increases risk of exposure -Usually appropriate only when contaminants other than volatiles are present -Permitting requirements may cause delay -Most expensive soil treatment method	$200 to $640/yd^3 (soil volumes less than 20,000 yd^3 greatly increase costs)
Venting	-Removes hydrocarbon vapors without excavation -Recovers up to 99% of gasoline components -Relatively easy to implement -Causes minimal disturbances to structures or pavement -Reduction in mobility	-Critical design parameters are undefined -Effectiveness is uncertain -Soil characteristics may impede vapor movement, create explosion hazards, or cause high levels of organic emission -May require vapor phase treatment	$15 to $20/yd^3 (more cost effective for greater than 500 yd^3 soil)
Soil Washing/ Extraction	-99% removal of volatile organics is possible (typical rates are less) -Reduction in mobility -Accelerated removal of contaminants	-Requires separation techniques such as distillation, evaporation and centrifugation -Large amounts of silts and clays in soil may impede separation of the solid and liquid after the washing phase -Pilot studies recommended before design and implementation -Less effective for textured soils	$150 to $200/yd^3
Microbial Degradation	-Greater than 99% removal efficiencies -Soil is treated *in situ* -Volatiles are completely degraded -Almost all soils contain indigenous microorganisms that can degrade hydrocarbons -Reduction in toxicity -Often cheapest alternative available -Works in saline and freshwater systems -Contaminants are completely destroyed to carbon dioxide, water, and biomass -Can be used in conjunction with other physiochemical corrective actions -Fast, safe and generally economical	-Partially degraded compounds may be more toxic than the original compound -Effectiveness depends on soil characteristics -Biologic systems subject to upset -Cannot be used where a quick start-up is needed -Addition of nutrients can cause problems in leachate -Can be inhibited by heavy metals and some organics -Labor and maintenance requirements may be high for long term treatment -Oxygen demand may drive system anaerobic	$66 to $123/yd^3

1.2.3 Hydrocarbon-degrading organisms in the subsurface

In order for *in situ* biodegradation to take place, contaminant-degrading microorganisms must be present. Early soil studies indicated that subsurface communities were not important to the ecology or fertility of soil because the number and activity of microbes decline dramatically with depth (38). However, subsurface microbes do exist, are metabolically active in deep unsaturated and saturated subsoils, and are often nutritionally diverse (133). Hydrocarbon-degrading microorganisms exist in most environments (7) because they have adapted to small-scale hydrocarbon exposure from human and natural activities (8, 20).

Subsurface bacteria in uncontaminated soils are relatively inactive in response to the stringent oligotrophic conditions of their environments, and are usually smaller (< 1.0 μm long) than those in eutrophic environments (11, 16). Gram-positive coccoid forms predominate in uncontaminated soils, whereas gram-negative cells are more prevalent in hydrocarbon-contaminated soils (89, 133). Small cell size, and hence the large surface-to-volume ratio for enhanced nutrient uptake, is a likely mechanism for survival in an oligotrophic environment such as the uncontaminated subsurface (15, 133).

Uncontaminated soils contain 10^5 to 10^6 hydrocarbon-degrading organisms per gram of soil (37, 63, 112), whereas soils with a history of hydrocarbon exposure contain 10^6 to 10^8 hydrocarbon degraders per gram of soil (37, 93, 113). Low numbers of hydrocarbon-utilizing microbes may initially limit the rate of hydrocarbon degradation in unpolluted soils, but the numbers of hydrocarbon utilizers may increase upon exposure to hydrocarbons to the point at which they no longer limit biodegradation (7, 8). Bacteria dominate the subsurface microflora, but low numbers of eukaryotic microorganisms (e.g. dormant forms of protozoa and fungi) are present, and the potential exists for competition at different trophic levels and regulation of bacterial numbers by grazing (11).

Indigenous soil microbes capable of degrading hydrocarbons include members of the genera *Achromobacter, Acinetobacter, Arthrobacter, Flavobacterium, Micrococcus,*

Nocardia, and *Pseudomonas* (37, 114, 133), and those that specifically degrade propane and butane are listed in Appendix B. More than 200 indigenous hydrocarbon-oxidizing organisms have been identified from soils, and these microbes will respond to hydrocarbon releases as long as the environmental conditions support their growth (37).

1.2.4 Factors affecting hydrocarbon biodegradation

The extent of hydrocarbon biodegradation in soils is influenced by the concentration of substrates, the availability of nutrients, soil moisture content, O_2 content, temperature, and several other physical and chemical conditions. Any of these factors may either restrict or promote the bacterial transformation of a compound.

1.2.4.1 Nutrients

The availability of essential nutrients to microorganisms often limits the rate of hydrocarbon degradation because natural environments rarely contain all the nutrients needed for cell synthesis at concentrations sufficient to allow growth to proceed at its potentially maximum rate (5). Both macronutrients (C, H, O, N, P, S, and K) and micronutrients are needed for optimal microbial growth, although micronutrients do not normally limit the growth of microbes in nature (37).

The release of hydrocarbons into soil environments containing low concentrations of inorganic nutrients often produces high C:N and C:P ratios, which limit microbial growth (8). Most studies (1, 31, 36, 50, 69, 118, 133) have shown that the addition of N-P-K fertilizers, urea-phosphate, and ammonium and phosphate salts accelerate the biodegradation of crude oil or gasoline in soil and groundwater. Other studies have shown that fertilizer amendments produce no increase in biodegradation rates (70, 87) or an increase only after a delay of several months to a year (94, 113). These contradictory findings have been attributed to the complex composition of soils and to other factors

such as N reserves and the presence of N_2-fixing organisms (17) (see section 1.5).

The specific nutrient-requirements for optimized microbial degradation of hydrocarbons are a site-specific factor that must be determined experimentally for each soil-contaminant problem (37, 133). The chemistry of a site affects the nutrient formulation needed for microbes. Nutrients may sorb onto particles, undergo complexation reactions, or precipitate out of solution, and therefore be unavailable to the microflora (132). In order to estimate the amount of nutrients needed to satisfy the demands for cell synthesis, data are required on the extent of C assimilation and the C-to-nutrient ratio of the cells formed (31, 37).

1.2.4.2 Soil moisture content

Because microbes require water to carry out metabolic processes, an understanding of the relationship between moisture content and microbial degradation is important. The amount of water a soil holds varies with time due to changes in precipitation, drainage, and evapotranspiration (37). Heavy rains can considerably alter the physical, chemical and microbial characteristics of a soil (63), and can limit O_2 diffusion to microbes (see section 1.2.4.5).

Soil moisture can be expressed as gravimetric water content (ratio of the weight loss in drying to the dry weight of the sample), volumetric water content (ratio of the volume of water present to the total volume of the sample) (49), or as field capacity (FC) (fraction of water remaining after free drainage of a saturated soil has ceased) (18). However, measurements of water content on a volume or weight basis do not indicate how much water is available to the microbes. Therefore, instead of relating microbial growth to water content, it is more desirable to describe the water relations of microbes in thermodynamic terms such as water activity (a_w), which provides a measure of the degree to which microbes are stressed for water (41, 115). The a_w term is a measure of the amount of free water in a system. Numerically, $a_w = P/P_0$ where P and P_0 are the

vapor pressures of solution and pure solvent, respectively. For an ideal pure solvent, $a_w = 1$; in all other cases $a_w < 1$ (41).

The water potential (ψ) directly measures the amount of work that must be done by an organism to obtain water, and is related to a_w 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 (99). ψ is the sum of matric, osmotic or solute, and pressure potentials, but the latter component is negligible in most terrestrial environments (41, 42, 48). When $\psi > -1$ bar, the effects of the osmotic potential are negligible because of the extreme dilution (42). The matric potential is a reflection of surface tension forces acting at menisci of water columns in capillary pores (41), and is the largest component of ψ in most unsaturated soils (99).

The amount of water that is bioavailable is the moisture retained in the soil between FC ($\psi = -0.1$ bar) and the permanent wilting point ($\psi = -15$ bar) (18). Microbial activity is maximum at a water content where the limiting effects of substrate diffusion and O_2 supply are equal (122). Generally, microbial activity in soil is optimal at $\psi = -0.1$ bar, and decreases as the soil becomes either waterlogged near zero water potential or drier at large, negative water potentials (4, 31, 102).

Some studies have shown that bacterial activity in soil is highly sensitive to water content, where bacterial activity decreases rapidly as ψ falls to between -0.5 and -3 bars (42). Other work has shown that water content has little effect on microbial activity, where the optimal aerobic biodegradation of organic material in soil occurred when the moisture content was 30% to 90% of FC (31, 111), perhaps because hydrocarbons can render some soil surfaces hydrophobic, reducing the water holding capacity of the soil, thus increasing the availability of the water that is present (31).

Microbes need to be in direct contact with the liquid phase in order to extract necessary nutrients from the environment (115). Soil water can affect bacterial activity by restricting movement of the bacteria to new sources of nutrients, and by restricting the metabolism of established colonies through nutrient deficiencies (42).

1.2.4.3 Temperature

Temperature affects the physiological reaction rates of cells and most of the physicochemical characteristics of the environment including soil volume, redox potentials, diffusion, surface tension, and water structure. Because the activities of microbial cells are governed by thermodynamics, changes in soil temperature affect microbial activity (102).

The temperature in the upper subsurface may vary seasonably or diurnally, but the soil temperature between 9 and 18 m in depth is constant, approximating the mean air temperature of a particular region. Because of the high specific heat of water, wet soils are less subject to large diurnal changes than dry soils (102). Typically, hydrocarbons are degraded more slowly at lower temperatures (8, 131), because low-molecular-weight hydrocarbons are less volatile and more water soluble, and the viscosity of heavier hydrocarbons increases at low temperatures (8, 68). The rate of a chemical reaction is a direct function of temperature and generally follows the relationship described by Arrhenius: $k = Ae^{-E/RT}$, where k is the reaction velocity, A is the frequency of molecular collisions, E is the activation energy of the reaction, R is the gas constant, e is the base of the natural logarithm, and T is the absolute temperature (86, 102). However, E is not always constant with temperature, and tends to decrease with increased temperature. Therefore, Arrhenius plots of bacterial specific growth rate may vary from linearity (86). Also, temperature is not usually the major limiting factor for hydrocarbon degradation in the environment except as it relates to other factors such as the physical state of the hydrocarbon or whether liquid water is available for microbial growth (8).

The majority of microorganisms that degrade petroleum hydrocarbons are mesophiles and thermophiles, although degradation by psychrophiles has been reported (37). Optimal temperatures for growth of hydrocarbon-degrading microbes is between 20°C and 35°C (8, 37, 131). Biodegradation can occur at most soil temperatures once the microbes become acclimated to that temperature (37).

1.2.4.4 Hydrocarbon Concentration

The rate of hydrocarbon biodegradation in soils can be inhibited if the hydrocarbon concentration is too high or too low, and the concept of a maximum or threshold concentration for microbial degradation of hydrocarbons may apply to soil ecosystems (68). Two studies have shown dose-response relationships between hydrocarbon concentration and degradation. Dibble and Bartha (31) reported increases in CO_2 evolution over the range of 1.25 to 5% hydrocarbon mass per dry weight of soil, no CO_2 increases at 10%, and decreases in rates at 15%. Similarly, Zvyagintsev et al. (147) reported that hydrocarbon degradation was minimal at low levels (0 to 0.07%) of petroleum, increased with increasing petroleum levels (0.07 to 30%), and rapidly decreased at high levels (>30%).

At high hydrocarbon concentrations, degradation rates are limited by microbial toxicity (37, 131) or nutrient or O_2 limitation, rather than substrate availability (68). Microbial toxicity may occur even if the indigenous microbial population is acclimated to the contaminant (133). At lower concentrations of contaminants, microbes can become adapted to the compounds, and may be able to overcome the effects of toxicity and degrade the compounds. However, substrates may become unavailable to organisms when petroleum concentrations are low, thus limiting the rate of hydrocarbon biodegradation (37).

Laboratory tests which use chemical concentrations greater than those found in nature may not correctly assess the rate of biodegradation in natural ecosystems (14). The minimum concentration of hydrocarbon that can be achieved by *in situ* biorestoration is unknown and may be site-specific, but levels below detection limits (1 to 2 µg of hydrocarbon per kg of soil) can be achieved (133).

1.2.4.5 O_2 content

The initial steps in the bacterial catabolism of aliphatic, cyclic, and aromatic hydrocarbons involve the oxidation of the substrate by oxygenases, for which molecular O_2 is needed (8, 68, 132). O_2 can serve as the terminal electron acceptor in microbial metabolism (23, 67, 103), and the availability of O_2 in soils is dependent on rates of microbial O_2 consumption, the type of soil, whether the soil is waterlogged, and the presence of utilizable substrates which can lead to O_2 depletion (17). The concentration of O_2 can be the rate-limiting variable in hydrocarbon biodegradation in both soil and groundwater (68).

Reports vary in the amount of O_2 needed to oxidize hydrocarbons in soil. Values as low as 0.3 mg of O_2 per mg of hydrocarbon have been reported (8), but other studies (23, 131) show that the complete oxidation of 1 mg of hydrocarbon to CO_2 and H_2O requires 3 to 8 mg of O_2. Less O_2 is required when microbial biomass is generated or when oxidation is not complete (131). The concentration of O_2 in air-saturated water is about 10 mg/liter, but most aerobic bacteria can grow at dissolved O_2 concentrations as low as 0.05 mg/liter, and some at even lower levels (39). Since most methods commonly used for measuring dissolved O_2 have a lower detection limit of about 0.1 mg/liter, it is possible that aerobic bacteria can mediate redox reactions in situations that might appear to be anaerobic based on the lack of detectable O_2 (39).

Aerobic conditions generally exist throughout the unsaturated zone, but hydrocarbons floating above the groundwater can decrease the O_2 concentration above the water table to less than 1% because available O_2 near the hydrocarbon source is rapidly consumed (56, 57). In the body of the hydrocarbon plume, O_2 transport is often rate-limiting and the consumption of O_2 and hydrocarbon can be approximated as an instantaneous reaction (16). Anoxic conditions may exist in microenvironments in soils that have significant amounts of O_2 in the soil gas, or in soils that have their pore spaces filled with water or hydrocarbons, thus excluding O_2. Microbial degradation of

hydrocarbons under negative redox potentials (anaerobic conditions) is not significant, and can produce noxious by-products (7, 8, 37). Organisms that utilize nitrate or sulfate as terminal electron acceptors under anaerobic conditions generally cannot catabolize the saturated hydrocarbons found in petroleum (131).

1.2.4.6 pH

Soil pH can be highly variable, ranging from 2.5 in mine spoils to 11.0 in alkaline deserts (17), and soil pH influences the rate of hydrocarbon biodegradation by affecting the sorption of ionizable compounds and limiting the types of microorganisms in the subsurface (133). Most heterotrophic bacteria and fungi favor a pH near neutrality, but fungi are more tolerant of acidic conditions than are bacteria (9). Fungi can degrade petroleum products, but the rate of degradation is less than that attained by a mixed fungi-bacterial community found in neutral to slightly alkaline soils (37).

A pH range from 5 to 9 is appropriate for many organisms, but a pH near 7 is most conducive to microbial activity in soils (31, 131), perhaps because the availability of macronutrients in soils is maximized at a neutral pH (37). Although near-neutral pH values are optimal for many soil microbes, soil is comprised of microhabitats, each with its own pH (5).

1.2.4.7 Alternate substrates

Alternate substrates are organic compounds that are present in soils in addition to the contaminant substrates. The presence of secondary organic substrates in soils may either enhance or inhibit microbial degradation of the contaminant substrates.

Alternate substrates enhance the biodegradation of xenobiotic substrates when they are less preferentially utilized than the xenobiotics, or when the xenobiotics are present in low concentrations. In the uncontaminated subsurface, alternate substrates are usually

present as refractory humic substances, which resist degradation (133, 135). Under these conditions, xenobiotic substrates added to the soil may be easier to degrade than humic material. Microbial populations can metabolize low levels of organic compounds at accelerated rates when an ancillary C source is supplied. Cell-specific degradation rates may remain unchanged, but absolute removal rates are increased by virtue of increased biomass (74).

Alternate substrates inhibit the biodegradation of xenobiotic substrates when they are more preferentially utilized than the xenobiotics. Swindoll et al. (128) and Van Eyk and Bartels (136) found that additions of alternate C sources, such as glucose, malate, or amino acids, inhibited the mineralization of xenobiotic substrates because they were more easily degraded than the xenobiotics.

1.2.4.8 Previous exposure and adaptation

Prior exposure of a microbial community to natural or anthropogenic sources of hydrocarbons is important in determining how rapidly subsequent hydrocarbon inputs can be biodegraded (68). Adaptation is defined as an increase in the rate of degradation with exposure to a chemical and can play a major role in determining biodegradation rates (2, 3, 123).

Adaptation is not directly proportional to the preexposure concentration of the substrate; there is a threshold concentration below which there is no detectable adaptation of the community (123). Active microbial communities are produced by preexposure to contaminant concentrations above this threshold, and higher preexposure concentrations cause only slight increases in biodegradation rates. Adapted communities are influenced more by test concentrations than by preexposure concentrations (123).

The time needed for adaptation depends on the rate of growth of the microorganisms that degrade the material (64) as well as the time required for the original number of microbes to increase to a size that is adequate to cause detectable degradation.

Adaptation is accompanied by a significant increase in hydrocarbon-utilizing populations in areas where inhibitory contaminant levels are initially present (2, 37, 113, 133). Once the communities in highly contaminated sites adapt, biodegradation rates may be controlled by hydrogeological characteristics, such as the rate of O_2 replenishment, rather than by the inherent abilities of the microbial community to degrade hydrocarbons (128).

1.2.4.9 Hydrocarbon structure

The type of hydrocarbon present in soil determines in part the ability of microbes to degrade it. In general, aliphatic hydrocarbons are more readily oxidized than aromatic or naphthenic compounds (7, 63), and polar hydrocarbons degrade more slowly than non-polar hydrocarbons (113). The *n*-alkanes are considered the most readily degraded components in a hydrocarbon mixture, and biodegradation of alkanes up to *n*-C_{44} has been shown (133).

Some studies have shown that *n*-alkanes of intermediate chain length (C_{10}-C_{20}) are most rapidly utilized by microbes, whereas long-chain hydrocarbons ($>C_{22}$) are not readily degraded because they are so insoluble (8, 37, 107, 133). In contrast, other studies (63, 131) show that long-chain hydrocarbons (C_{22}-C_{34}) are the most readily oxidized. Hydrocarbons $<C_{10}$ have relatively high solvent-type toxicity because they partition into cell lipids and are therefore not readily degraded (37, 131, 133).

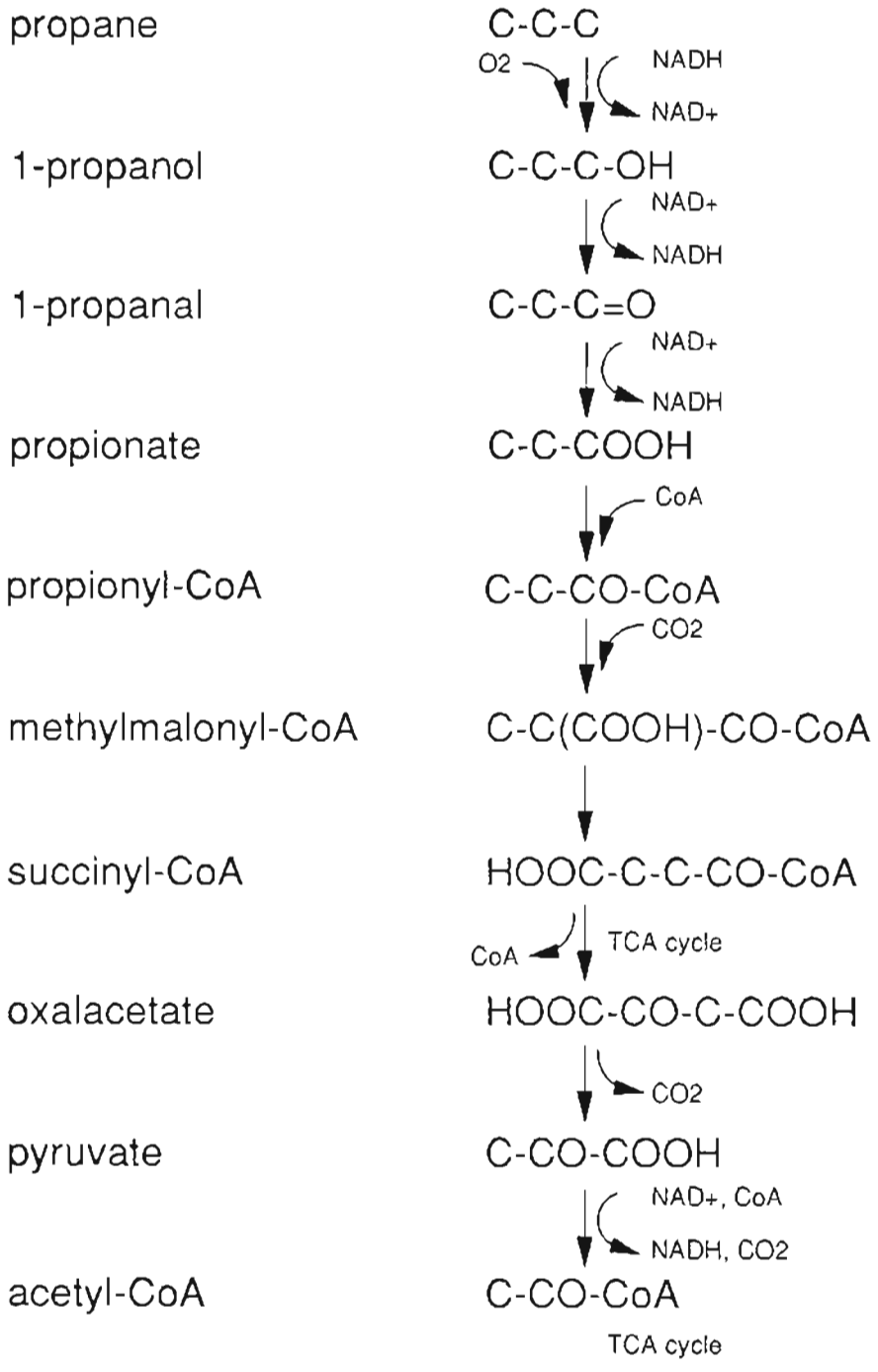
Alkenes are also substrates for microbial growth (137), but they are more resistant to microbial attack than alkanes (19, 133). Alkane-grown bacteria can often degrade alkenes due to the broad substrate specificity of alkane monooxygenases which are responsible for the initial oxidation of alkanes. Alkene utilizers also contain monooxygenases with a broad substrate specificity, but enzymes from these organisms generally do not hydroxylate alkanes (137, 138). Alkene-oxidation studies have been confined primarily to the degradation of terminal alkenes, and there are few data on the oxidation of internal alkenes (19).

Generally, the larger and more complex the structure of the hydrocarbon, the more slowly it is oxidized (22, 83, 131). Methyl branching increases the resistance of hydrocarbons to microbial attack (83, 106, 133) because methyl branches interfere with the beta-oxidation sequence which catabolizes alkanes (107, 117), and can inhibit the initial oxidation step altogether (8).

1.3 Pathways of alkane biodegradation

Alkanes can be oxidized terminally or subterminally. Terminal oxidation involves the oxidation of the terminal methyl group to a primary alcohol, then to an aldehyde and a monocarboxylic acid. The fatty acid is then degraded via beta oxidation into shorter fatty acids and acetyl coenzyme A, with the eventual release of CO_2 (7, 67, 107). In subterminal oxidation, the beta C of the alkane is oxidized to form a secondary alcohol followed by a methyl ketone (8, 52, 67). Both propane (10, 26, 125) and butane (52, 79, 85) can be degraded terminally or subterminally (Figs. 1.2 to 1.5).

Although each bacterial species generally metabolizes only a narrow range of similar hydrocarbons, some species produce enzymes which are less specific. Cooxidation is the process by which a microbe oxidizes a substance without being able to use the energy obtained from the oxidation to support its growth (7, 103). Methanotrophs are organisms capable of utilizing methane as their sole source of C and energy, and the initial oxidation of methane is catalyzed by an enzyme called methane monooxygenase (MMO) (73, 139). MMO has an extremely broad substrate specificity, and is able to oxidize or hydroxylate a wide variety of aliphatic, straight chain, branched, aromatic, and halogenated compounds (6, 51, 126). Methanotrophic organisms were used to cooxidize propane and butane in order to determine the degradation pathways presented in Figs. 1.2 to 1.5, as MMO catalyzes the oxidation of various *n*-alkanes to the corresponding primary and secondary alcohols (101).



ATPs produced: 1 NADH = 3 ATP
TCA (tricarboxylic acid) cycle gives 12 ATPs per acetyl-CoA and 5 ATPs from succinate to oxalacetate. Total ATP = 23

Figure 1.2 Terminal propane-oxidation pathway.

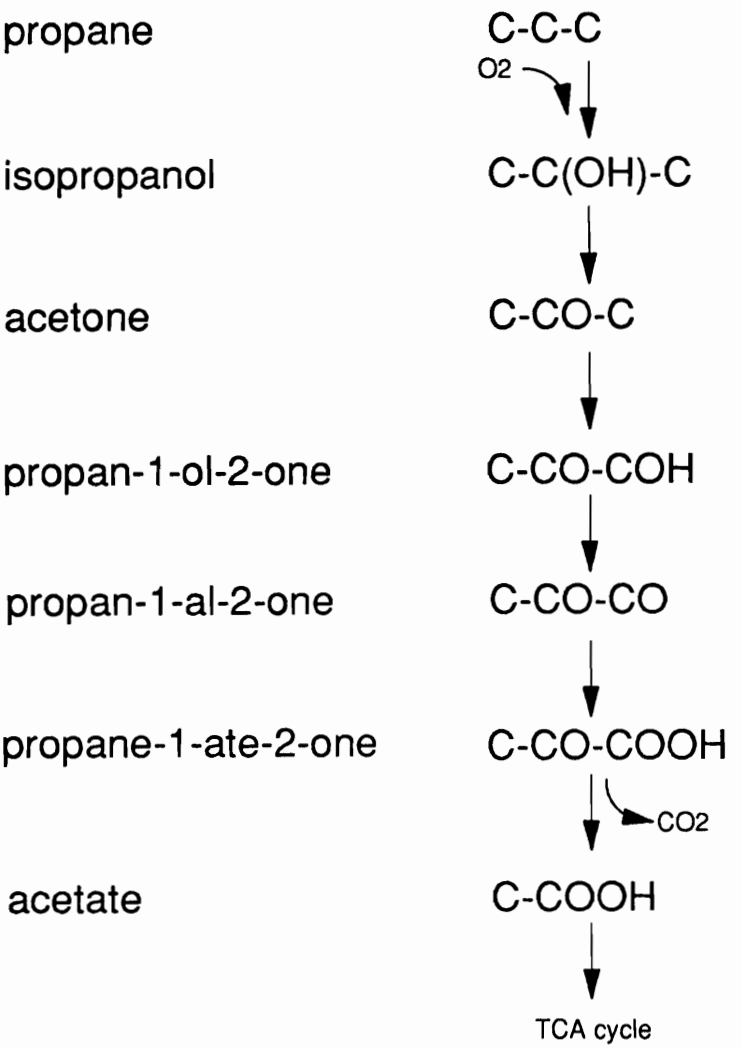
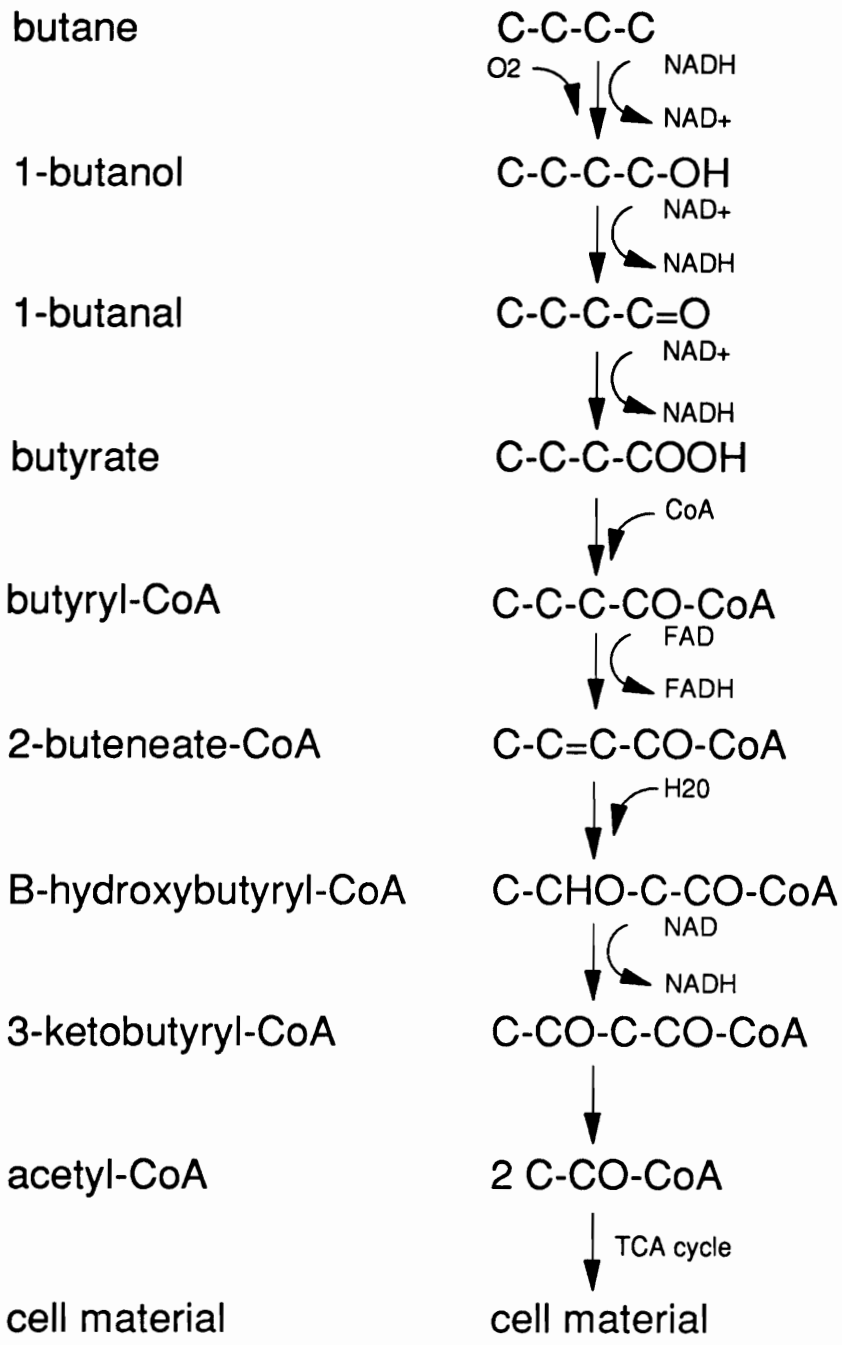


Figure 1.3 Subterminal propane-oxidation pathway.



ATPs produced: 1 NADH = 3 ATP, 1 FADH = 2 ATP
TCA (tricarboxylic acid) cycle gives 12 ATPs per acetyl-CoA
Total ATP = 32 ATP - 2 ATP for CoA activation = 30 ATP

Figure 1.4 Terminal butane-oxidation pathway.

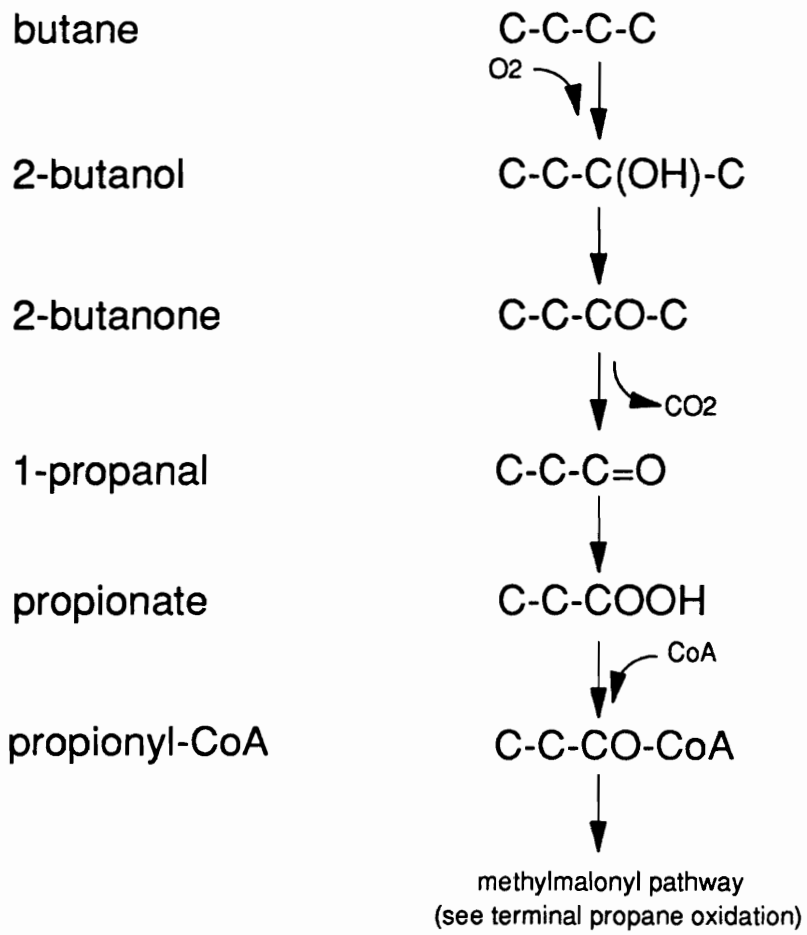


Figure 1.5 Subterminal butane-oxidation pathway.

Cooxidation may play a major role in the degradation of hydrocarbon mixtures such as gasoline, and may be the primary way that hydrocarbons are degraded to the parts-per-billion level (37). Assessing the role of cooxidation in natural environments is difficult, since multiple microbial populations are present, and the degradation rates of hydrocarbons are different when the mediator is a pure culture than when it is a mixed culture (8, 143).

1.4 Transport of contaminants in the unsaturated zone

After a gasoline spill, hydrocarbons can exist in the unsaturated zone in four phases: free product retained in pore spaces by capillary forces, solutes sorbed onto particles, vapor in the soil air, and dissolved species in the soil water (Fig. 1.6) (37).

1.4.1 Liquid hydrocarbons

The liquid phases of hydrocarbons migrate both vertically and horizontally through unsaturated soils. Vertical migration is a result of gravity, and is most common in highly permeable formations, whereas horizontal migration is mainly due to capillary forces, and is most common in less-permeable formations (37). Vertical penetration of liquid hydrocarbons is impeded when the threshold of residual saturation is reached, when an impermeable layer is in the path of the hydrocarbons, or when hydrocarbons reach the water table (37, 131). Hydrocarbons trapped between individual soil particles remain behind the main body of hydrocarbon and slowly leak contaminants into the soil over a long period of time. Residual hydrocarbons can occupy from 15 to 40% or more of the pore space (133), and infiltrating rainfall or a fluctuating water table can flush the residual hydrocarbons from the soil matrix into the groundwater (37).

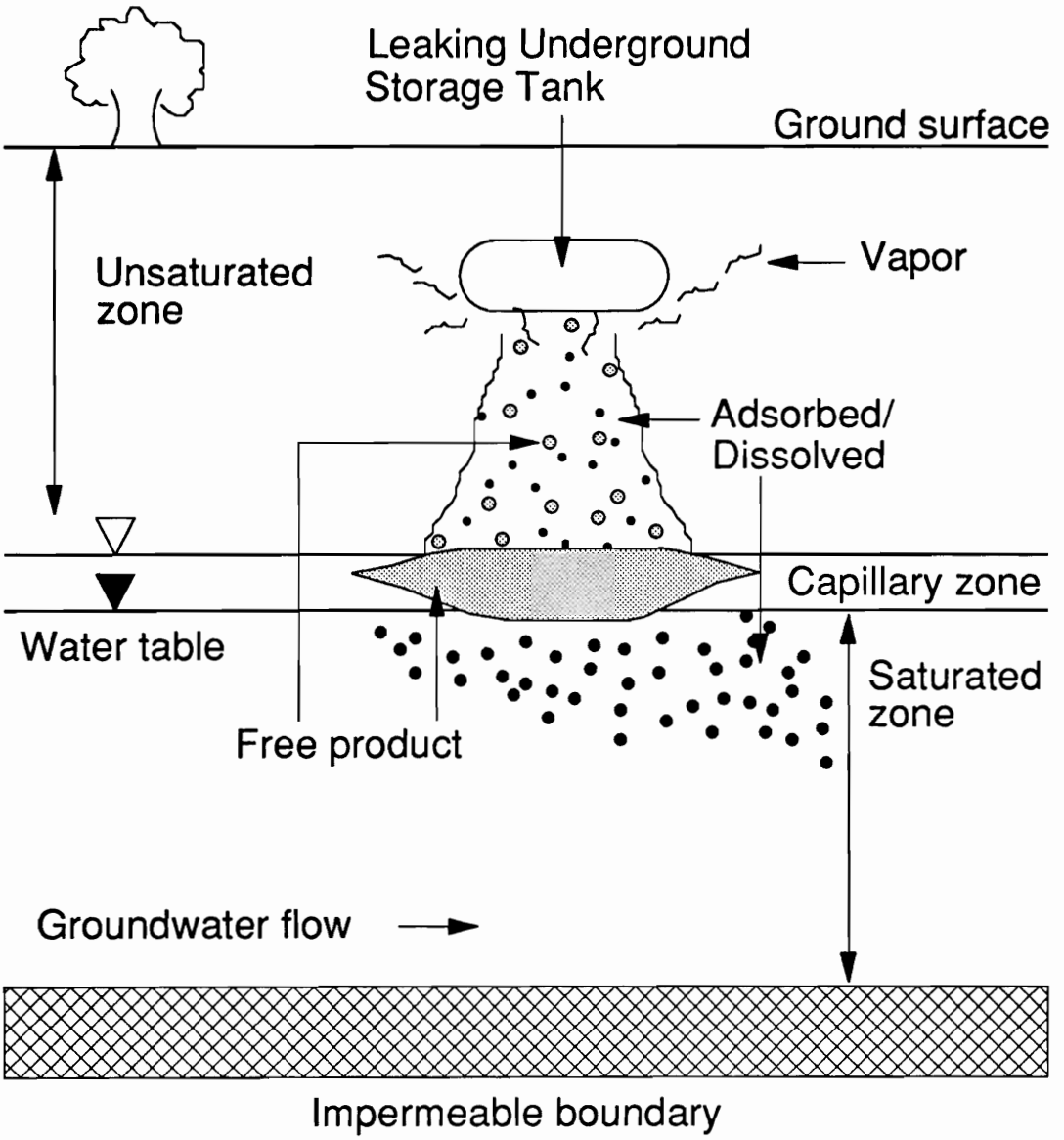


Figure 1.6 Schematic of the subsurface environment and four phases of contamination.

1.4.2 Gaseous hydrocarbons

The chief modes of vapor transport in soil are diffusion and advection. Diffusion is the scattering of particles by random molecular motions and net movement is generally away from areas of high concentration towards areas of low concentration. Advection is the process by which vapors are transported by the bulk motion of the gases flowing down-gradient as a result of a pressure gradient (39). In order for vapors to move, soil and rock formations must be dry enough to allow interconnection of air passages among the soil pores (37). Other processes that influence contaminant mobility in the subsurface include sorption, ion exchange, chemical precipitation, and complexation reactions (133).

1.4.3 Transport of propane and butane

In order to determine how propane and butane will be transported through unsaturated soils, the chemical properties of the compounds must be considered (Table 1.2).

Table 1.2 Chemical properties of propane and butane

Property	Propane	Butane	Source
Molecular weight	44.11	58.13	(72)
Boiling point, ^{o}C	-42.07	-0.5	(72)
Density, g/cm^3	.5005	.5788	(72)
Water solubility, g/m^3	62.4	61.4	(76)
Vapor pressure, atm	9.29	2.4	(76)
Henry's constant, H, atm/M	706.64	946.46	(76)
Dimensionless H, water/air	.034	.025	(76)
log K_{ow}	2.32	2.86	(75)
log $K_{oc} = 0.544\log(K_{ow}) + 1.377$	2.64	2.93	(65)

A mass balance equation (Equation 1) can determine what the distribution of propane or butane will be in the gas phase, aqueous phase, or sorbed onto solids for the experimental conditions described in the Materials and Methods section (e.g., there is no liquid hydrocarbon phase).

Eqn. 1 $$M_T = M_g + M_w + M_s$$

where M_T is the total mass (which is known), M_g is the mass in the gas phase, M_w is the mass in the aqueous phase, and M_s is the mass sorbed onto solids. Breaking the phases down into the component parts gives Equation 2.

Eqn. 2 $$M_T = C_g V_g + C_w V_w + C_s W_s$$

where C_g is the concentration of the gas, V_g is the volume of the gas phase (123.25 ml), C_w is the aqueous concentration, V_w is the volume of water (2 ml), C_s is the sorbed concentration, and W_s is the mass of soil (50 g). Equation 3 describes all of the phases in terms of the aqueous phase.

Eqn. 3 $$M_T = (C_w/H)V_g + C_w V_w + K_p C_w W_s$$

where H is the dimensionless Henry's constant, and K_p is the soil/water distribution coefficient which is the product of K_{oc} (organic C/water partition coefficient) and f_{oc} (the fraction of organic C, 0.003). Collecting terms leads to Equation 4:

Eqn. 4 $$M_T = C_w(V_g/H + V_w + K_p W_s)$$

Equation 4 can be solved for C_w, then C_w is substituted into Equation 3 to solve for C_g and C_s. These equations show that the majority of propane added to the

experimental bottles will partition to the gas phase (98.15%), with only small amounts to the aqueous phase (0.05%) and sorbed onto solids (1.8%). Likewise, butane also equilibrates mainly into the gas phase, (97.4%), with small amounts in the aqueous phase (0.04%) and sorbed onto solids (2.56%).

1.5 N_2 fixation

Input of inorganic N to the biosphere by N_2 fixation is rate-limiting for biological productivity on most areas of the sea or land surface of this planet. The ability to fix N_2 is restricted to bacteria called diazotrophs (21, 109). The first product of N_2 fixation is ammonia, which is nearly always assimilated as rapidly as it is formed (109). Although only 6 electrons are needed to reduce N_2 to $2NH_3$, recent measurements show that 8 electrons are actually consumed, with 2 electrons being lost as hydrogen gas for each mole of N_2 reduced (20, 34).

N_2 fixation is dependent on nitrogenase, an enzyme found only in prokaryotic organisms, including aerobic and anaerobic photosynthetic and non-photosynthetic eubacteria and archaeobacteria (35). Nitrogenase is a non-specific enzyme that can catalyze the reduction of N_2, acetylene, hydrogen cyanide, cyanogen, hydrogen azide, carbon monoxide, and some other low molecular weight compounds with triple bonds (35). Because nitrogenase reduces acetylene to ethylene, the extent of nitrogenase activity in a soil can be estimated by measuring the amount of ethylene formed in soil exposed to acetylene. An ethylene-produced to N_2-fixed ratio of 4 can then be used to estimate the amount of N_2 fixed:

$$N_2 + 8H^+ + 8e^- \rightarrow 2NH_3 + H_2$$
$$4C_2H_2 + 8H^+ + 8e^- \rightarrow 4C_2H_4$$

However, caution should be used in the use of the acetylene-reduction assay for

precise measurements of N_2 being fixed in nature (12), because the ratios of ethylene produced to N_2 fixed in natural systems can be higher than the theoretical ratio of 4, particularly in soils (146). In some cases (127), this overestimation of N_2 fixed is due to long-term (>6 h) incubations with acetylene which may have produced anomalous results (46).

N_2 fixation is a very energy-intensive reaction, consuming as many as 16 mol of ATP per mole of N_2, and may proceed symbiotically in association with plants, or nonsymbiotically (35, 124). This review focuses on nonsymbiotic N_2 fixation, where the active organisms are free-living in soil or water and fix N_2 if fixed N is limiting. Because nonsymbiotic N_2 fixers can survive in both aerobic and anaerobic environments, they appear to be able to maintain an intracellular environment in which nitrogenase is not inactivated by oxidizing conditions (21, 35).

Free-living aerobic or microaerophilic diazotrophs are a diverse group, representing over 22 genera (34). All aerobes require O_2 for metabolic processes, but since nitrogenase is an O_2-sensitive enzyme, aerobic diazotrophs have to protect their nitrogenase from irreversible inactivation by O_2, and to prevent inhibition of activity due to oxidation of their electron-donating systems to nitrogenase (34, 88). Several strategies for providing an environment compatible with the presence of O_2 and nitrogenase function have evolved. These include transient protection during O_2 stress (conformational protection), maintenance of a low ambient dissolved O_2 tension by strongly increased respiration, and provision of a diffusion barrier (membrane or slime) which allows colony formation, where O_2 concentrations in the center of the colony will be low (34, 88, 124). At high O_2 concentrations, large amounts of substrate are needed to protect the N_2-fixing systems, so only a minimum of ATP is generated under these conditions (88).

Because diazotrophs are metabolically and physiologically diverse, there are few habitats from which bacterial N_2 fixation is excluded. In addition to aeration, factors such as temperature, pH, moisture, and availability of C sources influence the extent of growth.

In most soil samples, anaerobic N_2-fixing activity appears to exceed aerobic activity, and soils lacking organic matter normally support only low populations of N_2-fixing bacteria (21). Free-living diazotrophs substantially contribute to specialized environments with high C:N ratios (e.g., compost heaps), and can assist in the degradation of recalcitrant substances such as cellulose. In principle, diazotrophs will augment biological activity in any ecosystem with a high C:N ratio (110, 124). N_2 fixation occurs only in environments which are deficient in fixed N (34), and adding fixed N to soils results in a temporary reduction in nitrogenase activity (40, 44, 88).

1.5.1 N-limitation and N_2 fixation

There is substantial evidence that N limits net primary production much of the time in most terrestrial and marine ecosystems (109), but it would seem that N_2 fixers should have a competitive advantage wherever N is limiting, and that their activity in turn should reverse N-limitation (141). Vitousek and Howarth (141) examined both how the biogeochemistry of the N cycle could cause limitation to develop, and how N-limitation could persist as a result of processes that prevent or reduce N_2 fixation. The three main biogeochemical mechanisms that favor N-limitation are: 1) in terrestrial ecosystems, N is derived primarily from the atmosphere, so N is nearly absent from new soils; 2) N is mobile and crosses ecosystem boundaries through leaching to aquatic systems or volatilization and denitrification to the atmosphere; and 3) organic N is directly C-bonded (C-N), and multiple enzymes are needed to break down these organic compounds before N can be released as available forms (141).

Because the above mechanisms lead to N-limitation, N_2 fixers should have an enormous competitive advantage, and their activity should alleviate N-limitation. However, there are two main mechanisms that prevent N_2 fixation from responding to and reversing N-limitation: 1) energetic constraints on the colonization or activity of N_2 fixers (43); and 2) limitation of N_2 fixers or fixation by another nutrient (P, Mo, Fe) (32).

1.5.2 N_2 fixation and hydrocarbon oxidation

In 1930, Schollenberger showed that soil exposed to natural gas contained more exchangeable ammonium than in ungassed soil, and hypothesized that the increase in N was due to biological actions under nearly anaerobic conditions (119). In 1939, Harper examined the effects of natural gas on microbial growth and the accumulation of N in the soil. Total N averaged 0.24% in gassed soils and 0.09% in ungassed soils. The increase in total N was assumed to be due to N_2-fixation because *Clostridia* were present in the gassed soil, and because some *Clostridia* species are known to fix N_2 (47). Since that time, a few studies have compared hydrocarbon contamination with the occurrence of N_2 fixation in soils, but the results of these studies are variable; sometimes organic C additions stimulate N_2 fixation (66, 92, 95), sometimes they do not (45, 134), and at other times N_2 fixation may or may not be stimulated by hydrocarbons, depending on the hydrocarbon concentration (71). Many field-scale bioremediation efforts have shown that fixed N additions to soils stimulate hydrocarbon biodegradation (31, 93). A limited number of studies have shown that N additions do not stimulate hydrocarbon biodegradation (70, 87), but these studies did not consider whether the lack of stimulation by N could be due to N_2 fixation.

The first time that hydrocarbon-oxidizing organisms were associated with N_2-fixing capabilities was in 1964 when Davis, Coty, and Stanley reported that methane-oxidizing bacteria isolated from garden soil, pond mud, oil-field soil, and soil exposed to natural gas were capable of fixing atmospheric N_2 (29). Coty later observed that N_2 fixation occurred in the presence of gaseous or liquid alkanes, aromatics, and a naphthenic acid (27), but was unable to isolate a pure culture that fixed N_2 and oxidized hydrocarbons. Roy et al. reported the isolation of an *Azospirillum* sp. that fixes N_2 while utilizing *n*-dodecane as the sole C source (116) (see Appendix C). It is presently unclear whether hydrocarbon oxidizers fix N_2 or whether hydrocarbon oxidizers and N_2-fixers are separate organisms in soil.

CHAPTER 2
MATERIALS AND METHODS

2.1 Characterization of soil

Columbia River soil from Scappoose, Ore. was collected in 1989 and stored at 10-15°C in a sealed plastic bucket until used. The original properties of the sand (Table 2.1) were determined in order to quantify any changes in the soil.

2.2 Experimental design

The soil was air-dried for 3 days, sieved (<2 mm), then 50 g (dry weight) portions of sand were added to 161-ml serum-bottles (Fisher Scientific, Tustin, Calif.). Ultrapure water (18 MΩ, resistivity) was added to a known water content, and then the bottles were sealed with butyl-rubber stoppers and aluminum crimp caps, shaken to distribute the water, and incubated in the dark. Propane or butane (Aldrich Chemical, Milwaukee, Wis.) was injected into the bottles; all hydrocarbon injections and sample removals were made with gas-tight syringes (Hamilton, Reno, Nev.). Sample replicates (4 to 12) were prepared for each experimental condition. Bottles were periodically flushed with room air to maintain aerobic conditions ($p_{O_2} > 0.1$) and to prevent pH lowering due to CO_2 accumulation.

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.

Table 2.1 Characteristics of unamended Columbia River soil

Characteristic			Value	Method or Reference
Total N			55 mg/kg soil	a
NO_3^--N			0.4 mg/kg soil	a
NH_4^+-N			0.97 mg/kg soil	a
Available N (NO_3^--N + NH_4^+-N)			1.37 mg/kg soil	(60)
Available phosphorus			2.7 mg/kg soil	(96)
Iron			7.5 mg/kg soil	(97)
Organic C content			0.3% (w/w)	(90)
pH			6.56	(84)
Bulk density			1.52 g/cm^3	(78)
Porosity			0.35	(62)
Gravimetric water content (θ_g) at FC			5.3 ml H_2O/100 g dry soil	(78)
Grain size fractions, mm	≥4.0	Gravel[b]	4%	(78)
	4.0 - 1.0	Very coarse sand, gravel	37%	
	1.0 - 0.71	Coarse sand	8%	
	0.71 - 0.5	Coarse sand	9%	
	0.5 - 0.246	Medium sand	26%	
	0.246 - 0.175	Fine sand	8%	
	0.175 - 0.043	Very fine and fine sand	7%	
	<0.043	Silt	1%	

[a]Determined at Oregon State University's Soil Testing Laboratory
[b]U.S. Department of Agriculture classification of soil particle sizes (18)

2.3 Analytical methods

2.3.1 Gas chromatography

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, Avondale, Pa.). All peak areas were quantified by comparison with standards made from the dilution of pure gases (Air Products and Chemicals, Allentown, Pa.) in N_2, and integrated with a Nelson Analytical 900 Series Interface (Nelson Analytical, Cupertino, Calif.).

Propane and butane were analyzed with a flame-ionization detector (FID) and a fused-silica capillary column (25 m by 0.53 mm, i.d.) (Chrompack International, The Netherlands). Flow rates were: carrier (He), 4 ml/min; make-up, (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 to two separate packed columns. O_2 was analyzed with a thermal conductivity detector (TCD) on a 6.4 mm by 48.3 cm stainless-steel analytical column (Molecular Sieve 5A 60/80, Alltech Associates, Inc., Deerfield, Ill.). CO_2 and CH_4 were analyzed with an FID on a 3.2 mm by 40.6 cm stainless-steel analytical column (Spherocarb 100/120 mesh, Analabs, Norwalk, Conn.). After the CO_2 was separated from CH_4, the CO_2 was reduced to CH_4 with a Ni catalyst heated to 500°C (methanator). Helium was the carrier gas to both the TCD (40 ml/min) and FID (30 ml/min). 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, were 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 TCD and FID columns, respectively.

2.3.2 Acetylene-reduction assay

Acetylene was introduced into the headspace of serum-bottles or culture-tubes to 0.07 atm after first removing an equivalent volume of air. Headspace samples (0.5 ml) were analyzed for ethylene production with an HP-5890 gas chromatograph equipped with an FID on a 2 mm by 3 m stainless-steel analytical column (Porapak N, 80-100 mesh, Supelco, Bellefonte, Pa.). N_2 was the carrier gas (25 ml/min), and the temperatures of the column and detector were 65 and 250°C, respectively. Acetylene and ethylene peaks were quantified by comparison with standards made from the dilution of pure gases (Aldrich) in N_2. Samples were stored at 22°C in the dark during the assay. Controls without added acetylene accounted for any endogenous ethylene production.

2.4 Preliminary studies: finding optimal conditions for biodegradation

2.4.1 Temperature and soil water content studies

Propane degradation was monitored under 3 water contents (4, 4.5, and 5 ml of H_2O per 100 g of dry soil) and 2 temperatures (14 and 22°C ±2°C), and butane degradation was monitored under 5 water contents (3.5, 4, 4.5, 5, and 5.5 ml of H_2O per 100 g of dry soil) and 2 temperatures (15 and 20°C ±0.5°C). Water activity (a_w) was measured with an SC-10A thermocouple psychrometer (Decagon Devices, Inc., Pullman, Wash.). Propane (0.16 ml) was initially added to all propane-degrading bottles, and more propane (0.16 to 2.5 ml) was added if the initial propane aliquot was degraded. Butane (0.17 ml) was initially added to all butane-degrading bottles, and more butane (1.0 ml) was added after the initial butane aliquot was degraded.

2.4.2 Hydrocarbon concentration and chain-length studies

The effects of hydrocarbon concentration on degradation rates were tested with

ethane (1.3, 6.6, and 14 ml) and propane (0.16, 0.74, 1.5, and 4.6 ml) additions while incubated at the same θ_g (4.0 ml of H_2O per 100 g of dry soil) and temperature (10°C for 2.5 months, then 14°C ±2°C). The effect of chain length [C_1 to C_4 alkanes (~6.7 ml)] on degradation rates was also tested under the same temperature and water content conditions.

2.5 Effects of nutrients on propane biodegradation

Propane biodegradation was monitored under 6 nutrient conditions: 1) no added nutrients, 2) N only, 3) phosphorus (P) only, 4) trace minerals (Tr) only, 5) N & P, and 6) N & P & Tr. P (0.135 mg/bottle) was added in equimolar quantities of Na_2HPO_4 and NaH_2PO_4 to keep the pH neutral, and N (O.45 mg/bottle) 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 was pH 6 and contained (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 bottles, θ_g was 4.0 ml of H_2O per 100 g of dry soil, except for the N & P & Tr bottles where θ_g was 4.5 ml of H_2O per 100 g of dry soil. Propane (0.16 ml) was initially added to all bottles, and more propane (0.24 to 2 ml) was added after each propane aliquot was degraded. All bottles were incubated at 20°C ±0.5°C.

2.6 Effects of N on butane biodegradation

2.6.1 N concentration

All samples were pre-exposed to butane (0.13 ml) for 2 months, and then butane biodegradation was monitored under 4 different N conditions. N was added as $NaNO_3$ to levels of 0.05, 0.1, or 0.15 mg of N per bottle, or as NH_4Cl to 0.1 mg of N per bottle.

In all bottles, θ_g was 4.5 ml H_2O per 100 g of dry soil. Butane (2 ml) was added to all bottles, and more butane (0.5 to 2.5 ml) was added after each butane aliquot was degraded. Controls included samples with no added N and samples with NaCl added at the same ionic strength as the nutrient solutions. All bottles were incubated at 20°C ±0.5°C.

2.6.2 Form of N

This experiment was designed to determine whether gaseous forms of N stimulate butane degradation in soil, because gaseous forms of N would be easier to pump into contaminated subsurface soils than aqueous forms of N. N was added as NO (0.2 or 15 ml), NO_2 (0.2 or 10 ml), NH_3 (0.2 or 1 ml), and N_2O (10 ml). In all bottles, θ_g was 4.0 ml of H_2O per 100 g of dry soil. Butane (2.5 ml) was added to all bottles including controls with no added N, and all samples were incubated at 20°C ±0.5°C .

2.7 Microbiological methods

2.7.1 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 mineral media with or without added N, and with propane or butane as the sole source of C. The mineral media contained (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 previously described. N-containing media also included 0.1 g of $NaNO_3$ per liter as a N source. Precipitates formed in the mineral media, so the media was equilibrated with CO_2 to remove the precipitate, adjusted to pH 7.0 with NaOH, and dispensed in 5-ml aliquots into test tubes. All of the test tubes were capped with butyl-rubber stoppers and aluminum crimp caps, and were autoclaved at 121°C for 20 min.

Air-dried sand (5 g) was added to 50 ml of sterile 0.001 M sodium pyrophosphate (Sigma Chemical Co., St. Louis, Mo.) solution (120). The mixture was firmly shaken to dislodge bacteria from soil particles, settled for 3 min to allow large sand particles to fall to the bottom of the flask, and serial dilutions of the supernatant were prepared in the sterile media. Dilutions were each inoculated into 5 tubes containing mineral medium, and into 5 tubes containing mineral medium with added N. Propane (0.2 ml) or butane (0.2 ml) was added to each tube, and all tubes 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. Tubes were considered positive if 50% of the added hydrocarbon was degraded as compared to uninoculated controls.

Numbers of protozoa in unamended and propane-amended soil were enumerated by the Oregon State University Microbial Biomass Service.

2.7.2 Isolation of organisms

To isolate butane-oxidizing, N_2-fixing bacteria, transfers (10%) were made from the most dilute positive MPN tubes amended with butane into mineral media with and without added N. Organisms from the MPN tubes were also streaked and re-streaked onto agar plates made from mineral media without N plus 15% purified-grade agar (Fisher Scientific). The mineral media had a pH of 7, had no precipitate, and contained (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 as described above. The transfer tubes were incubated at 37°C statically (horizontally) after adding butane (0.2 ml). Agar plates were incubated at 37°C in an air-tight jar with 0.65% of butane in the headspace. Controls included uninoculated transfer tubes as well as agar plates incubated without any substrate.

CHAPTER 3
RESULTS

3.1 Preliminary studies: finding optimal conditions for biodegradation

3.1.1 Temperature and soil water content studies

The effects of temperature and water content on both propane and butane biodegradation were highly variable and were not reproducible. Therefore, only general trends will be reported.

The a_w was high in all samples (Table 3.1), yet propane biodegradation was greatest when θ_g was at least 85% of FC, regardless of the incubation temperature. In general, water content had little effect on propane degradation at 22°C, but wetter soil degraded propane much faster than drier soil at 14°C. Higher temperatures favored biodegradation in drier soil, but the most rapid propane degradation occurred at lower temperatures in soil near FC (Fig. 3.1).

Table 3.1 Relationship between θ_g, a_w, and ψ in water content experiments

θ_g (ml H_2O/100 g dry soil)	% of FC	a_w	ψ (bar)[a]
3.5	66	ND[b]	ND
4.0	75	0.9979	-2.8
4.5	85	0.9993	-0.95
5.0	94	0.9995	-0.68
5.5	104	0.9994	-0.81

[a] $\psi = \{RT \cdot \ln(a_w)\}/V_w$ (see Section 1.2.4.2)
[b] ND = not determined

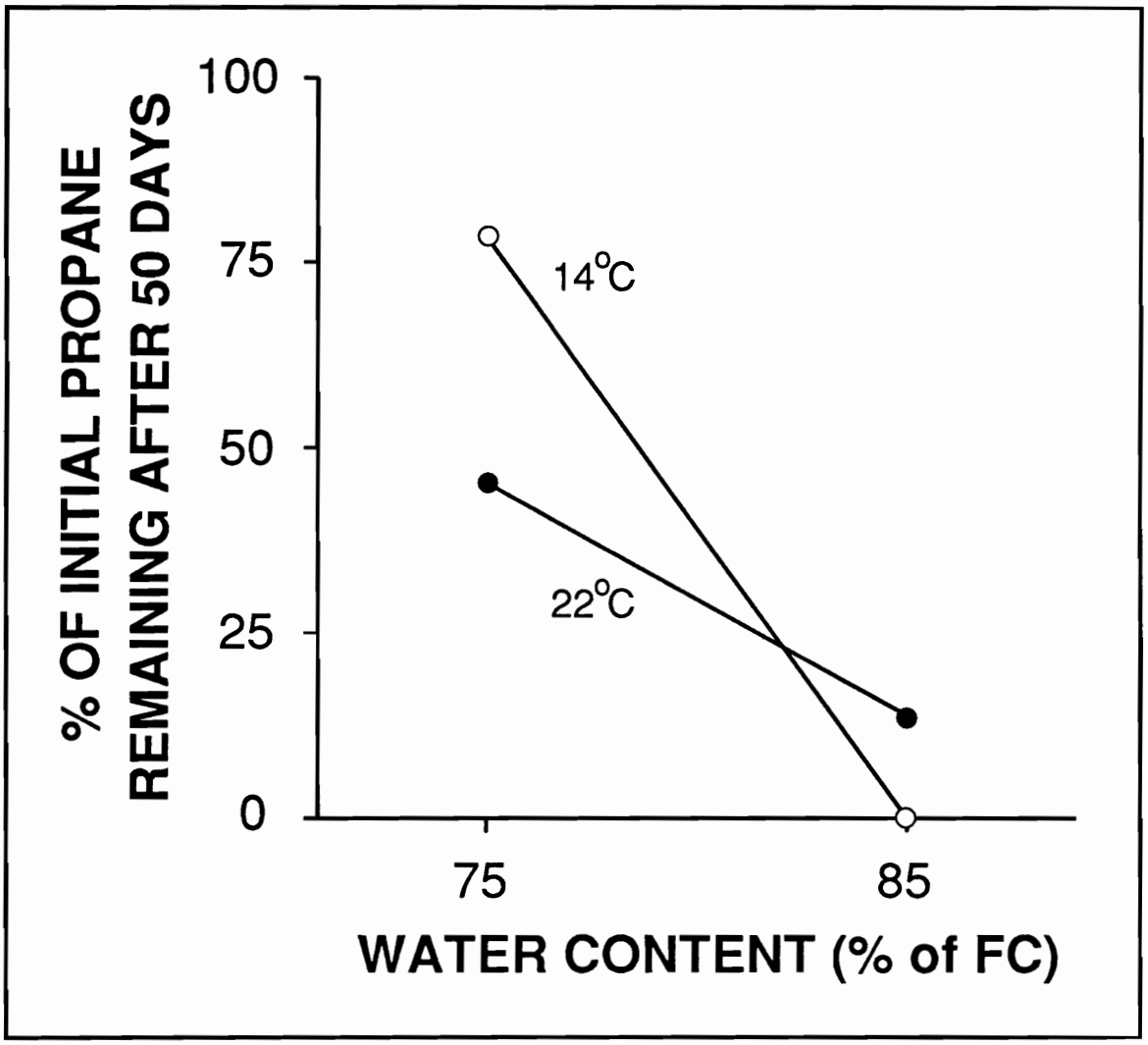


Figure 3.1 Relationship between temperature and water content on propane degradation after 50 days of incubation.

The first butane aliquot was degraded faster at 15°C than 20°C at all water contents except at 66% of FC, whereas the second butane aliquot was degraded much faster at 20°C than 15°C at all water contents. In general, butane degradation was not affected by water content at either temperature, except that degradation of the second butane aliquot was inhibited when the water content was only 66% of FC at both temperatures (Fig. 3.2). There were some similarities between propane and butane degradation; wetter soil degraded both compounds faster than drier soil at low temperatures, and high temperatures favored biodegradation in drier soil (Fig. 3.1). The effect of the interaction between water content and temperature on hydrocarbon degradation was significant ($P<.01$), based on an analysis of variance (ANOVA). Butane degradation was generally faster than propane degradation at all temperatures.

3.1.2 Hydrocarbon concentration and chain-length studies

Hydrocarbon degradation decreased with increasing hydrocarbon concentrations for both propane (Fig. 3.3 A) and ethane (Fig. 3.3 B). Hydrocarbon degradation was inhibited when the initial hydrocarbon concentration was >0.5% of the headspace volume (Fig. 3.3 A). There was no significant ethane degradation over the course of the experiment (400 days), even at the lowest concentration (0.8% of the headspace volume) (Fig. 3.3 B).

The chain-length experiments were unsuccessful because in these experiments, the initial hydrocarbon concentration was 4% of the headspace volume, which was shown to be inhibitory in the hydrocarbon concentration study above. There was no significant ethane, propane, or butane degradation over 400 days, but methane was eventually completely degraded (Fig 3.4).

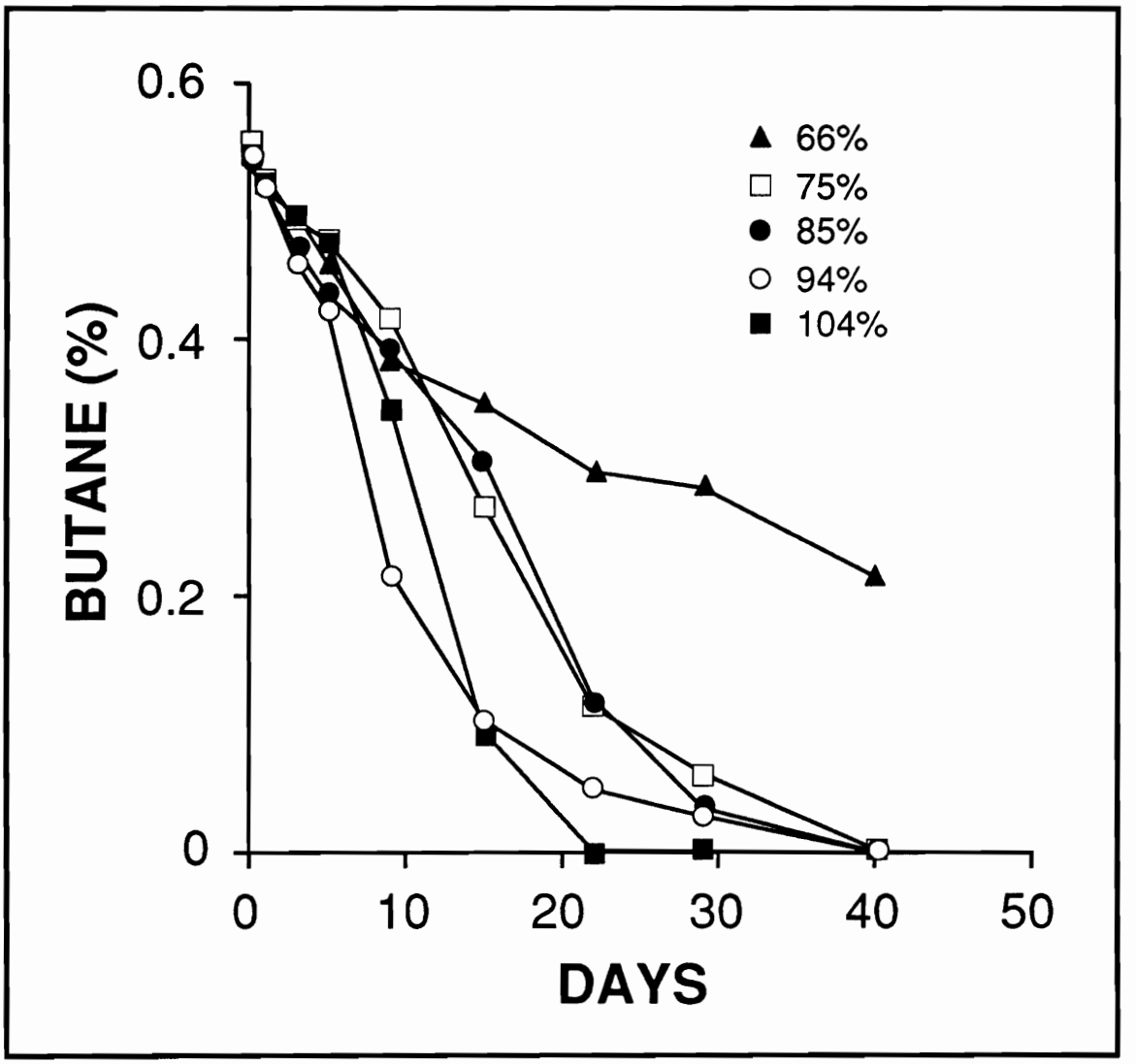


Figure 3.2 Effect of water content (% of FC) on butane degradation at 20°C.

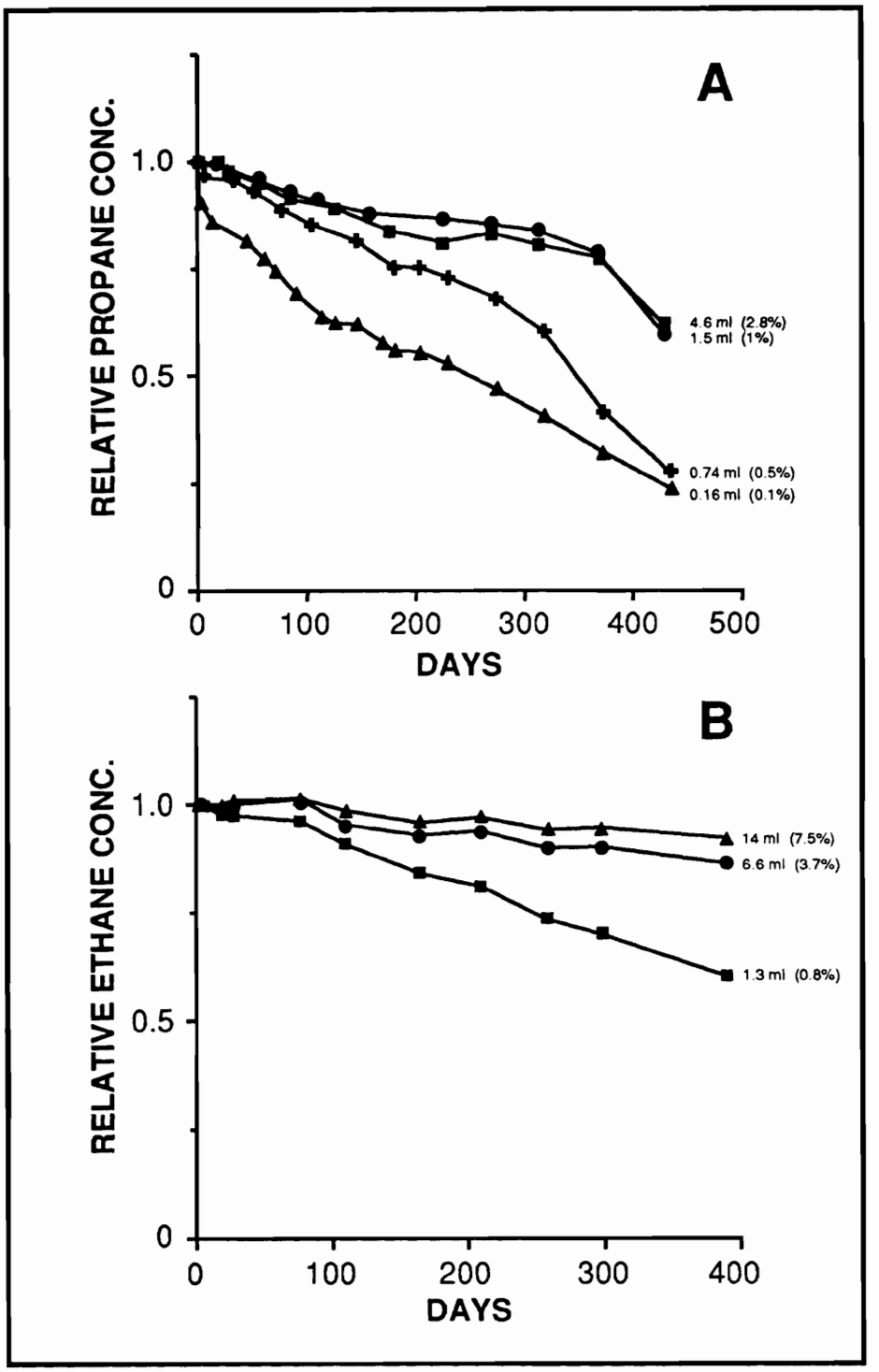


Figure 3.3 Effects of propane (A) and ethane (B) concentration on hydrocarbon degradation. Values in parenthesis represent the measured % of headspace concentration at day 0.

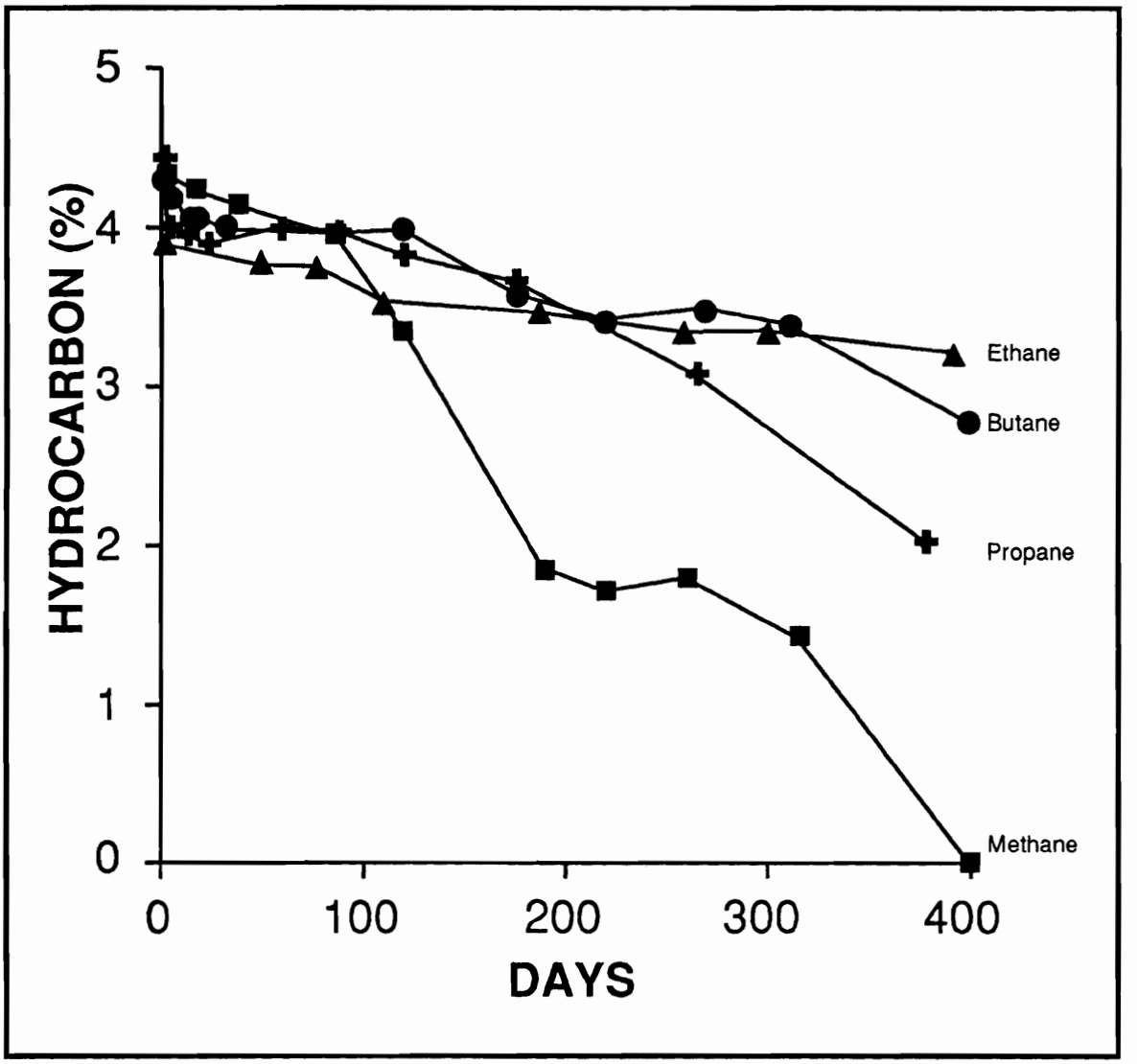


Figure 3.4 Effect of chain length on hydrocarbon degradation.

3.2 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. 3.5). 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. 3.5 B). 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 aliquot added 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 soil, until the fifth aliquot on day 108, after which degradation rates fell to a constant level of 1.7 mg of C per day per kg of soil (Fig. 3.6). Propane degradation was unaffected by water contents ranging from 75 to 85% of FC.

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.

3.3 Effects of N on butane biodegradation

3.3.1 N concentration

Nutrient studies with propane showed that N alone was the most limiting nutrient in Columbia River soil. Therefore, this butane biodegradation study used only N as an additional nutrient. During the first 2 months of incubation, butane degradation rates increased with increasing N concentrations from 1.8 mg of C per day per kg of soil when no N was added, to 11.4 mg of C per day per kg of soil when 0.15 mg of N was added per bottle (Fig. 3.7). Degradation rates later fell in all samples to about 2 mg of C per

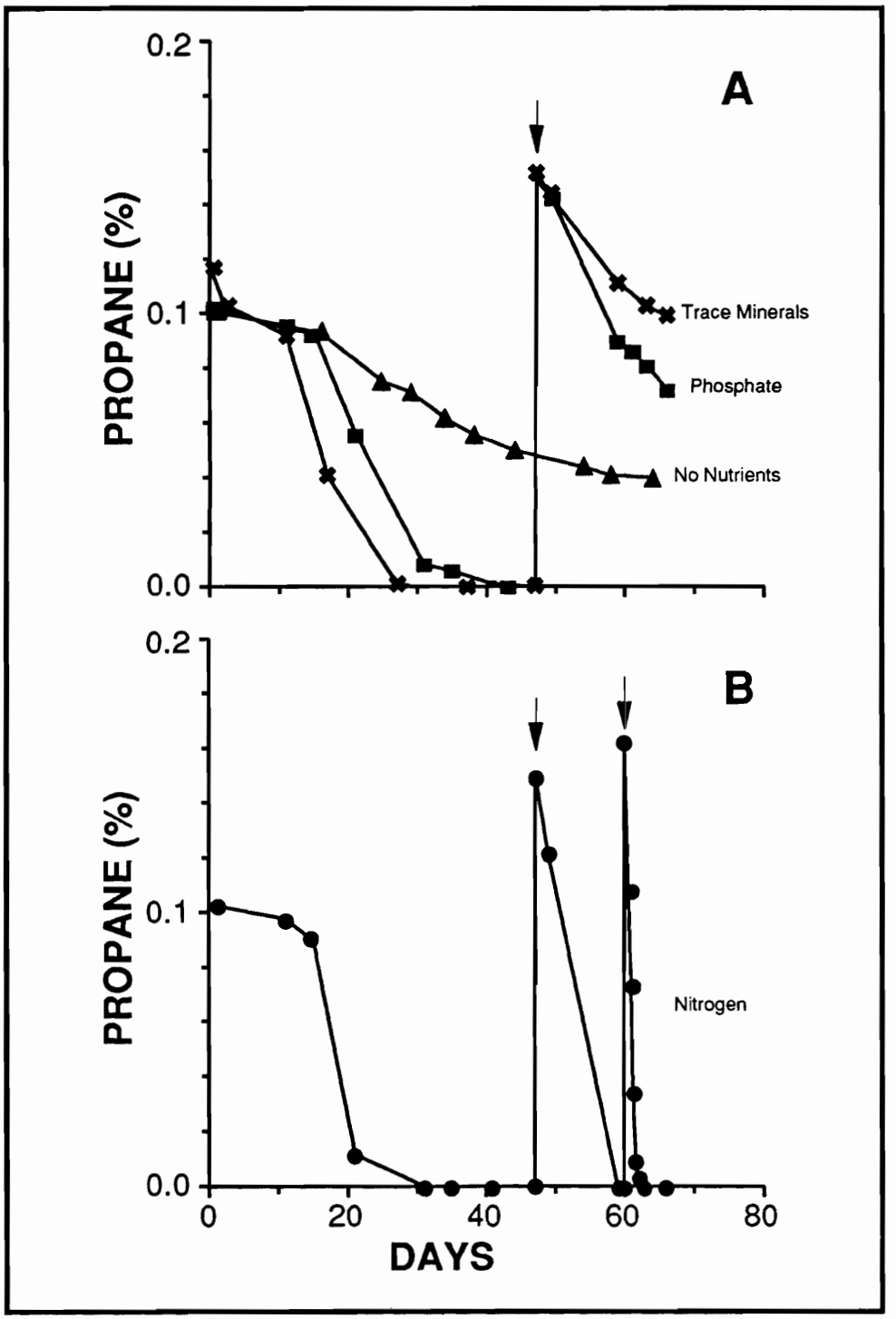


Figure 3.5 A) Effects of various nutrient amendments on propane biodegradation. Arrow indicates the addition of propane. B) Effect of N only on propane biodegradation. Arrows indicate the addition of propane. Data points for the N & P and N & P & Tr treatments fell on the N only line.

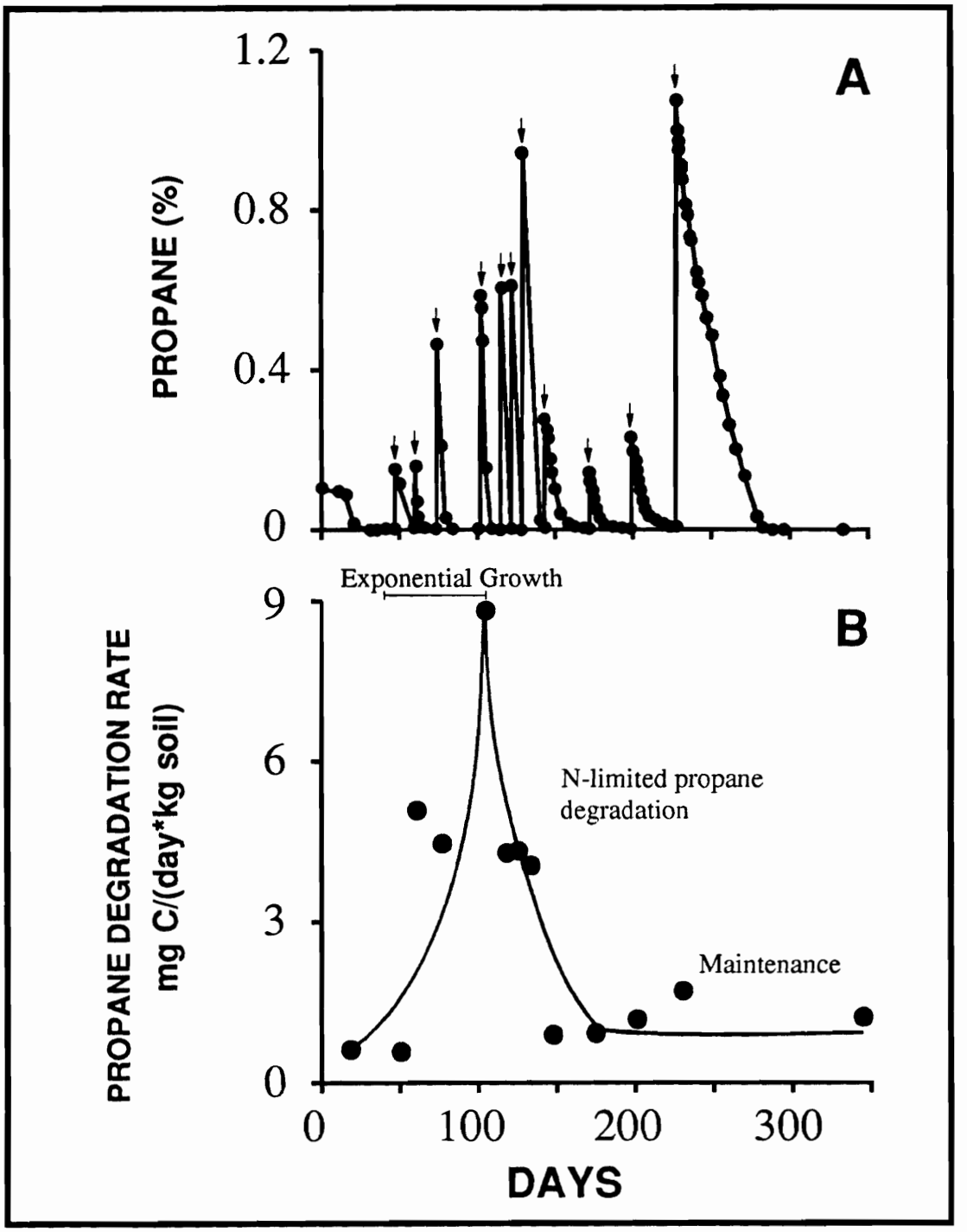


Figure 3.6 A) Propane degradation in N-amended soil (0.45 mg N/bottle). Arrows indicate the addition of propane. Early time points are the same as those shown in Figure 3.3 B) Maximum propane degradation rates over time derived from A).

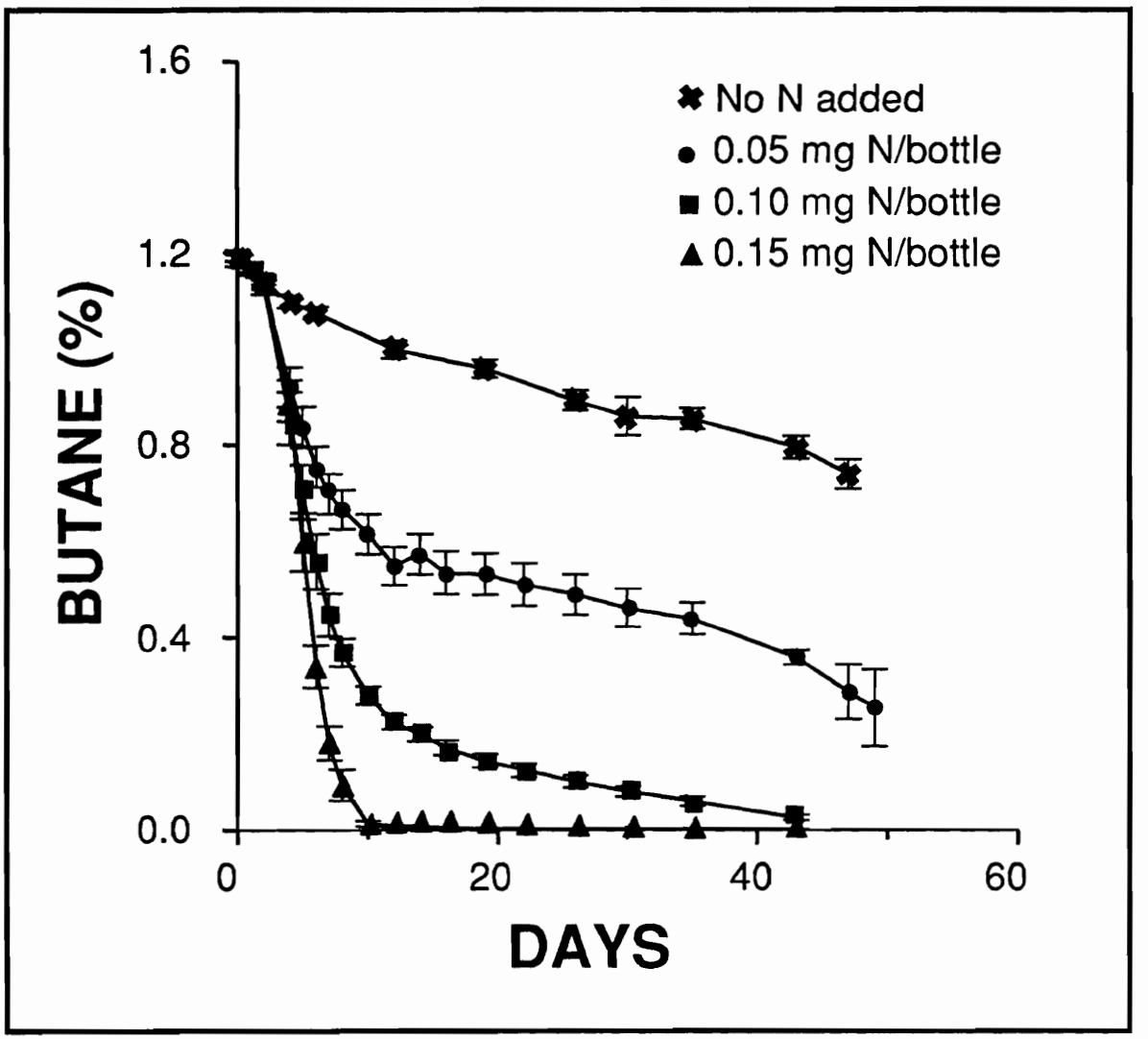


Figure 3.7 Effects of various NO_3-N concentrations on butane biodegradation. Error bars represent the standard error of the mean.

day per kg of soil, presumably after the bioavailable N was utilized (Fig. 3.8). 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. 3.9) suggested that butane degradation during the first 2 months was a function of bioavailable N. Extrapolating this line indicated that the calculated initial bioavailable N (0.07 mg N/bottle = 1.4 mg N/kg soil) was in agreement with the measured value of bioavailable N (1.37 mg N/kg soil) listed in Table 2.1.

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. Controls with added NaCl behaved the same as samples with no N added. 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 soil (Fig. 3.8).

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. No CH_4 was produced, and sterile controls showed insignificant butane losses, CO_2 production, or O_2 consumption.

3.3.2 Form of N

Butane biodegradation was initially stimulated by ionic forms of N, but adding N in gaseous forms to the soil was not generally successful in increasing butane biodegradation rates. Although NO and NO_2 gases are not a direct part of the N cycle in soil (109), both gases undergo chemical reactions that convert them to potentially bioavailable forms of N. NO reacts instantly with O_2 to form NO_2 ($2NO + O_2 \rightarrow 2NO_2$), and NO_2 reacts with water to form nitric and nitrous acids ($2NO_2 + H_2O \rightarrow HNO_3 + HNO_2$) (28). Nitric acid is a strong acid, and completely dissociates to H^+ and NO_3^- ions

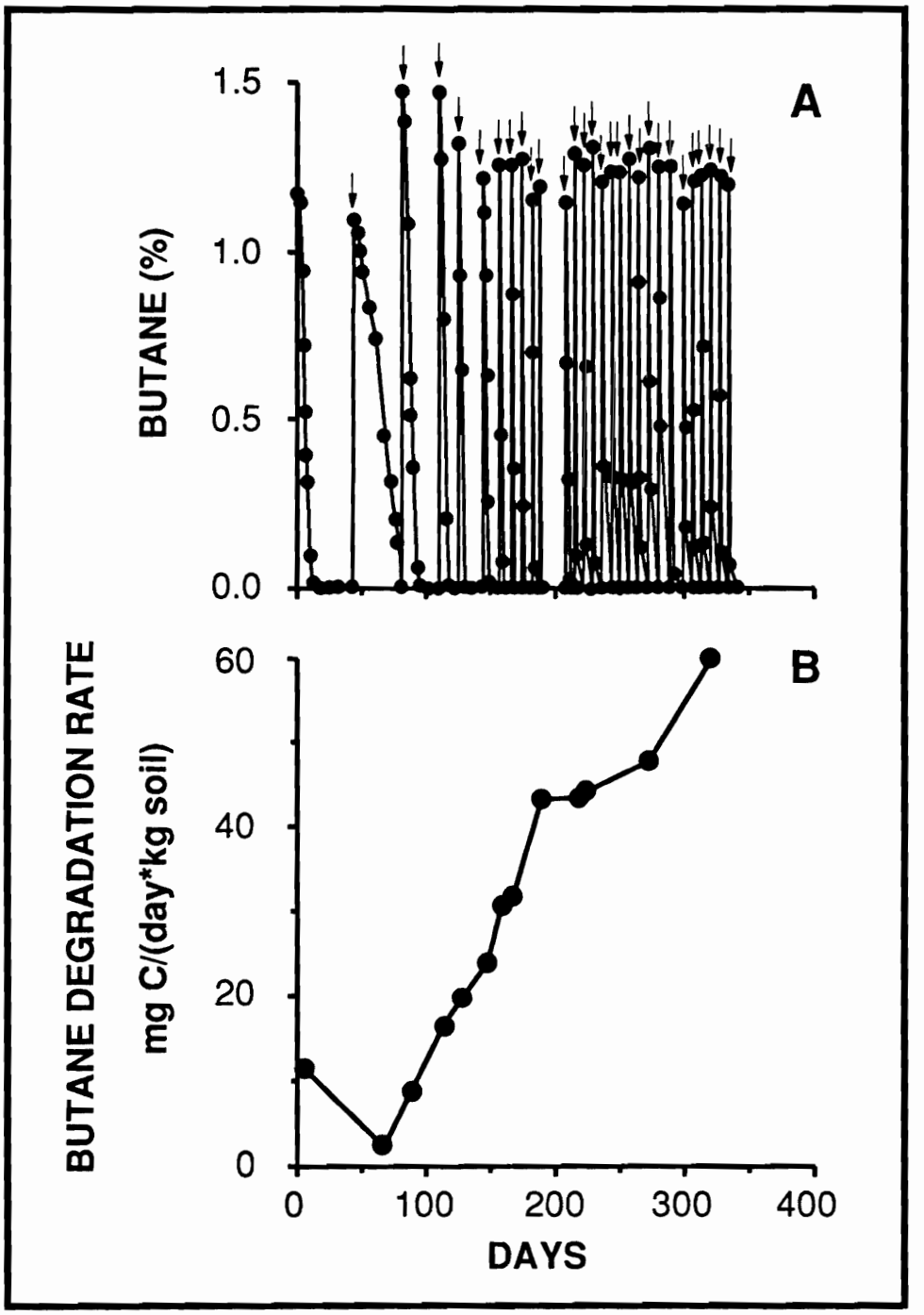


Figure 3.8 A) Butane biodegradation in N-amended soil (0.15 mg N/bottle). Arrows indicate the addition of butane. Early time points are the same as those shown in Figure 3.5 with 0.15 mg N/bottle added. B) Maximum butane degradation rates over time derived from A).

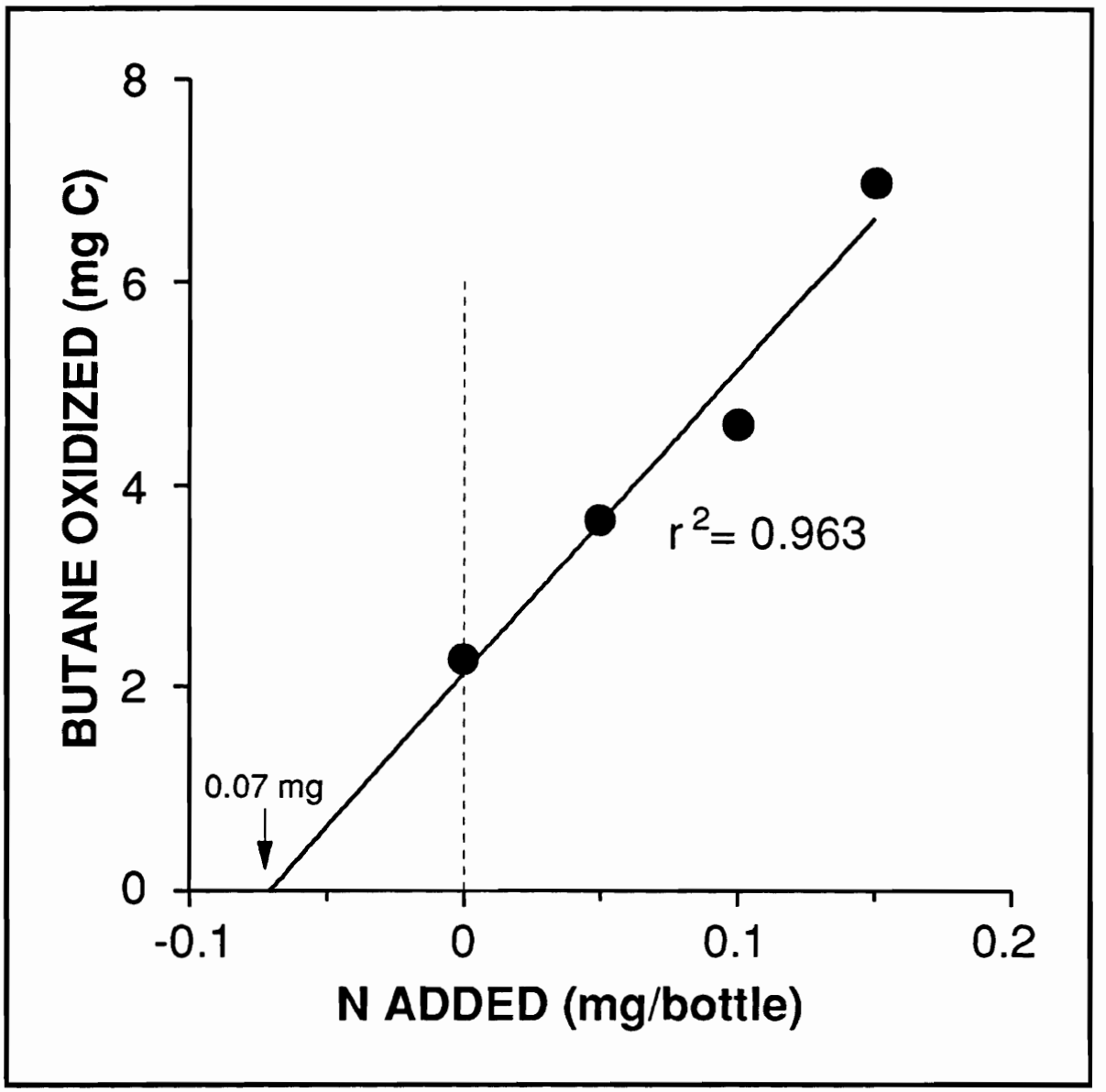


Figure 3.9 Bioavailable N in Columbia River soil calculated from the amount of butane degraded when various amounts of N were added to the soil.

in water (82). Nitrous acid is a weak acid, and also dissociates to form some NO_3^- ions in water (82). Therefore, both NO and NO_2 gases can react to form NO_3^- in soil water, which can be biologically converted to organic N by an assimilation process (109).

There was no difference in butane biodegradation when N was added as N_2O or as low concentrations of NO, NO_2, or NH_3, as compared to controls with no added N. High concentrations of NO and NO_2 inhibited butane degradation, presumably because the soil pH decreased from near neutral to 5.5 after exposure to these gases. N_2O had no effect on butane degradation, probably because it is relatively unreactive at room temperature (28). NH_3 at moderate concentrations was the only gas tested that clearly stimulated butane degradation (Fig. 3.10). However, NH_3 may not be easily dispersed in soil because it is highly soluble in water, and dissociates to the NH_4^+ ion (28). Cations do not travel as far or as fast as anions in soils because soils are generally negatively charged (39).

3.4 Evidence for N_2 fixation in butane-amended soil

N apparently became limiting when propane was used as a substrate, but not when butane was used as a substrate. N_2 fixation would explain this apparent surplus of N in butane-amended soil. The following 4 experiments were designed to determine whether N_2 fixation occurred in butane-amended soil, but not in propane-amended soil.

The first experiment determined whether N was limiting in propane- and butane-amended soil. When more bioavailable 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 (Fig. 3.11 A). Propane biodegradation rates increased from 0.5 to 6.7 mg of C per day per kg of soil after the supplemental N was added. In contrast, supplemental bioavailable 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 soil (Fig. 3.11 B).

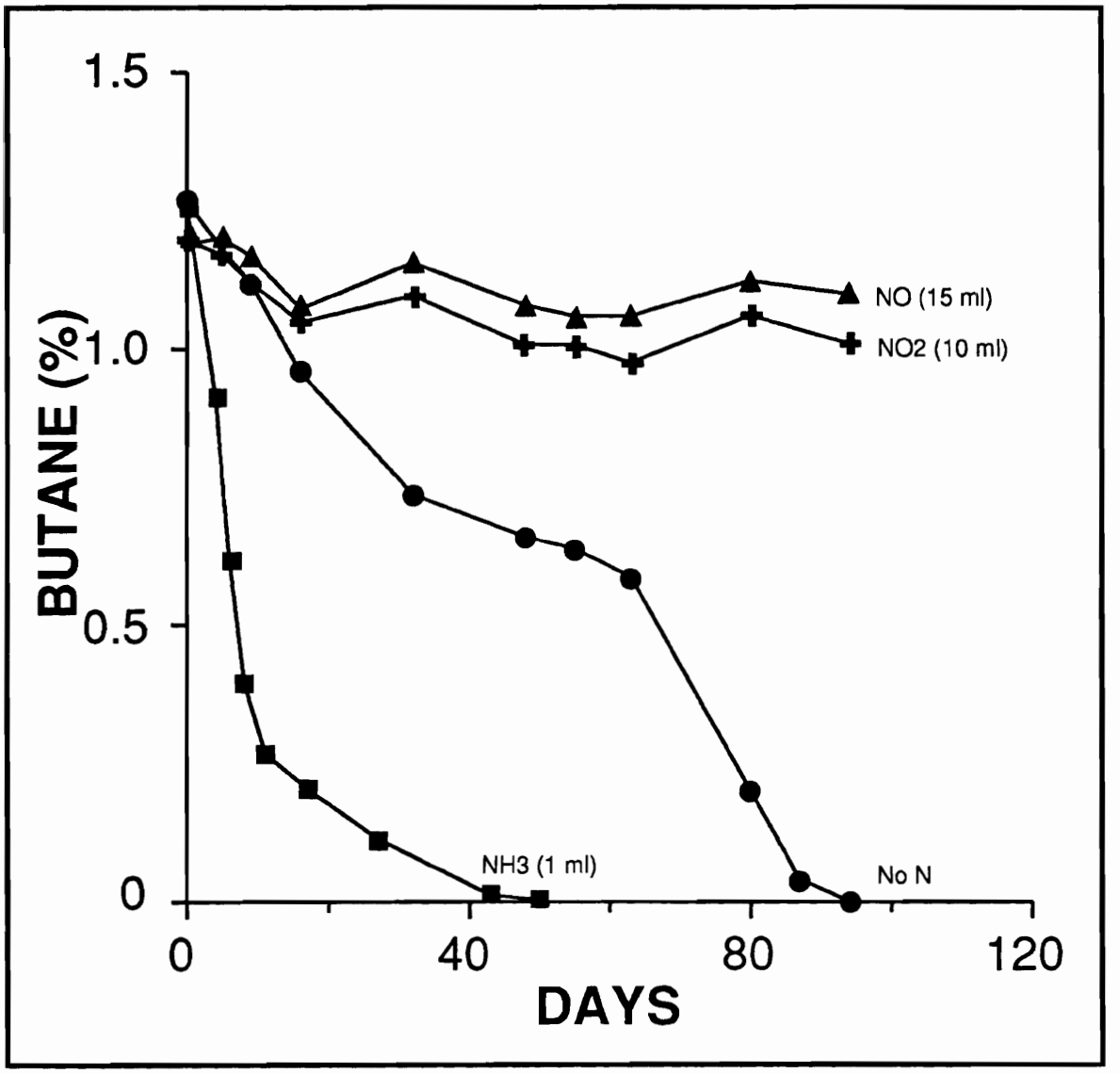


Figure 3.10 Effect of various gaseous forms of N on butane degradation.

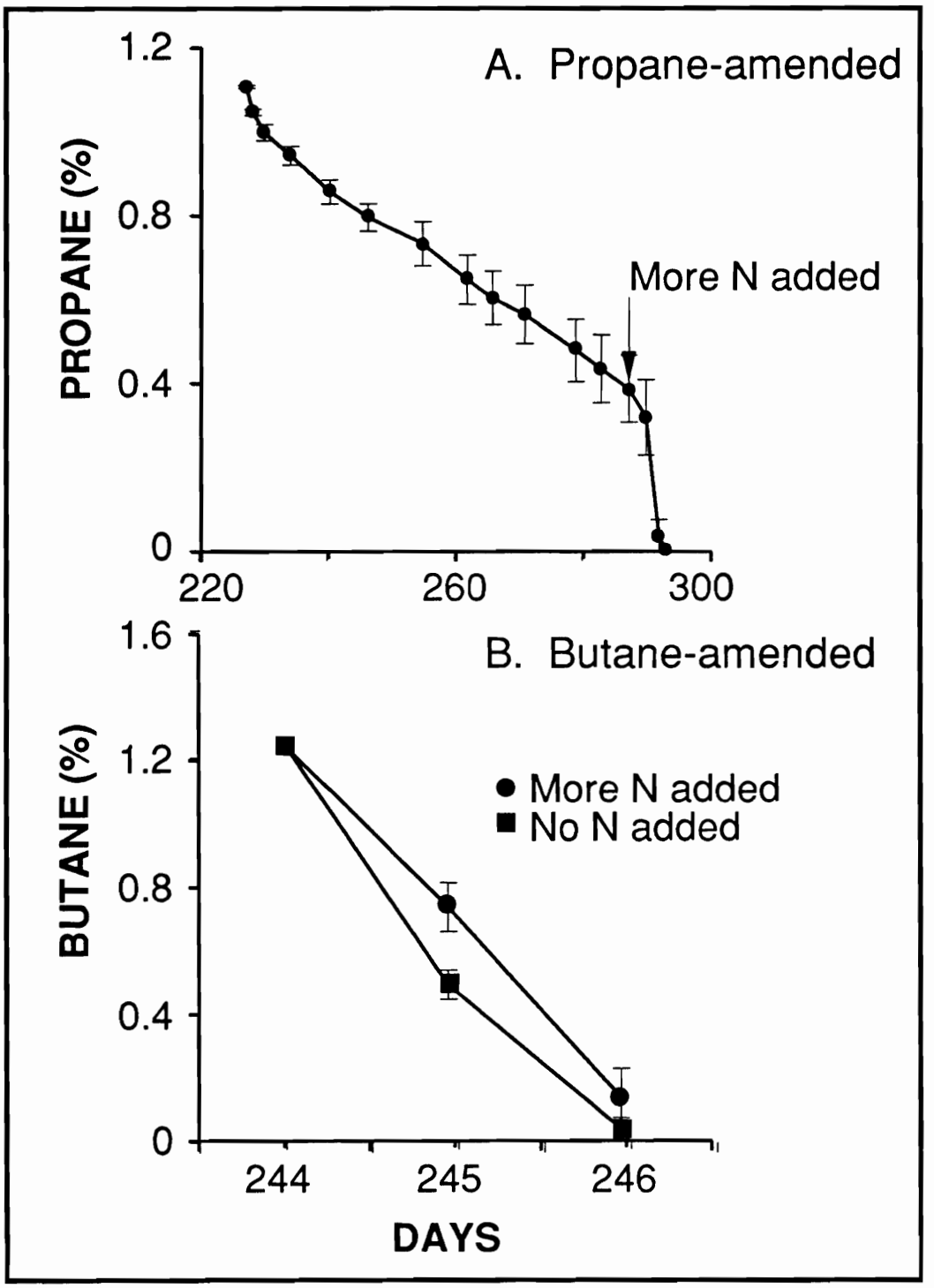


Figure 3.11 Effect of additional bioavailable N on propane (A) and butane (B) biodegradation.

Secondly, serum-bottles were prepared with a 1:1 mixture of propane- and butane-degrading soils that were 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 soil) (Fig. 3.12). 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 was 37 mg of C per day per kg of soil, which was 4 times higher than the maximum propane degradation rates in unmixed soil with N amendments.

Thirdly, the acetylene-reduction assay demonstrated that N_2 was fixed (acetylene was reduced) in butane-amended soil, but not in the original soil, the propane-amended soil (Fig. 3.13), or in the controls. Rates of N_2 fixation in butane-amended soil increased with decreasing amounts of added N. Ethylene production rates ranged from 1.5 µmol per kg of dry soil per hour in soil with added N to 2.8 µmol per kg of dry soil per hour in soil with no added N. Ethylene production rates and an ethylene to NH_3 ratio of 2 were used to estimate that the amount of N_2 fixed in butane-amended soil was 37 mg of N per kg of soil, resulting in a total N value of 92 mg of N per kg of soil. Appendix D lists N_2-fixation rates in other studies.

Lastly, total Kjeldahl N was determined for the original soil, and propane- and butane- amended soil with and without N amendments. Propane-amended soil contained similar amounts of total N (55-65 mg N/kg soil) as the original soil (55 mg N/kg soil), whereas the butane-amended soil comprised significantly ($P<.01$) more total N (85-95 mg N/kg soil) than the original soil, based on a Student's *t* test analysis.

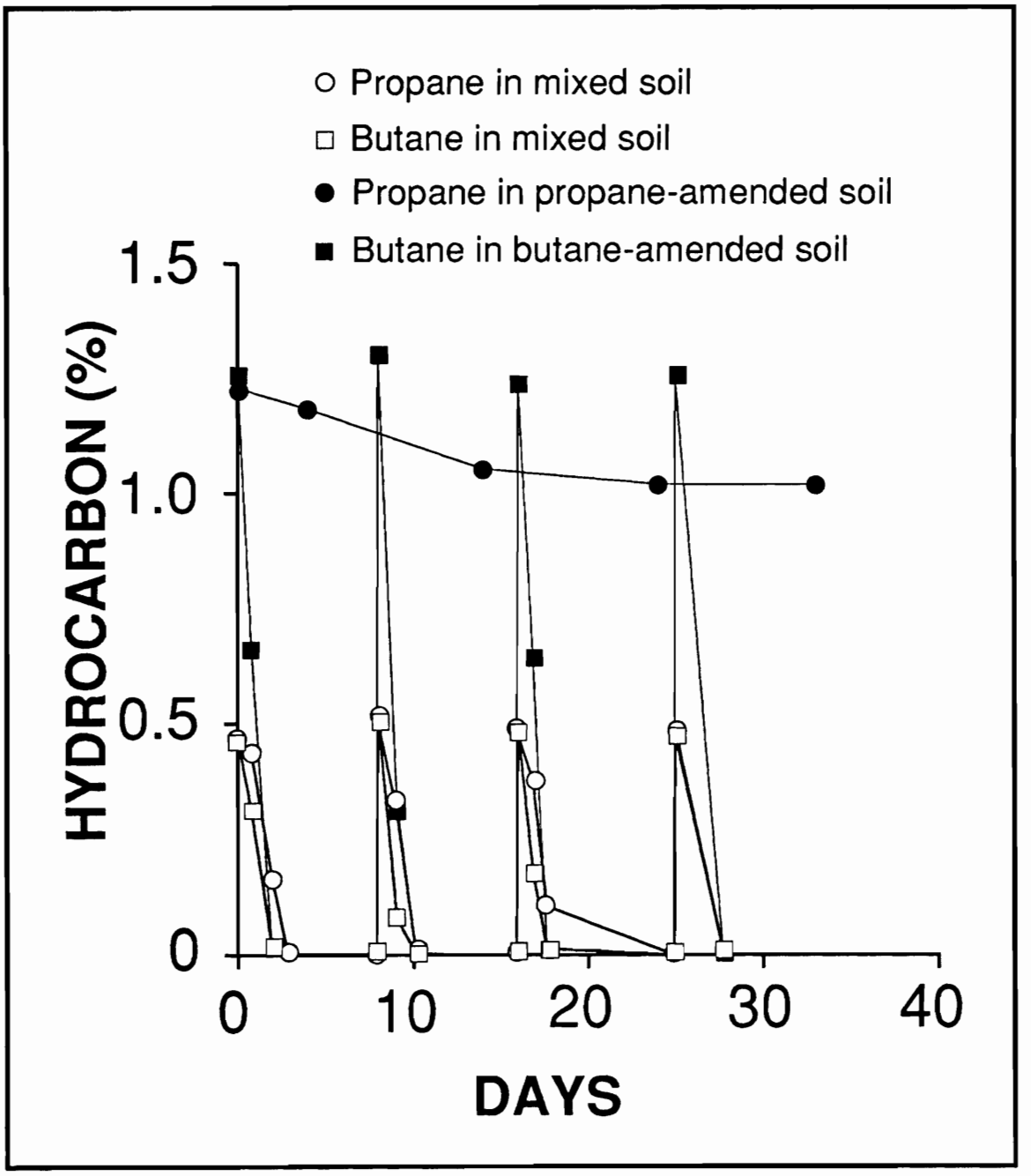


Figure 3.12 Effect of mixing propane- and butane-amended soils together on propane and butane biodegradation.

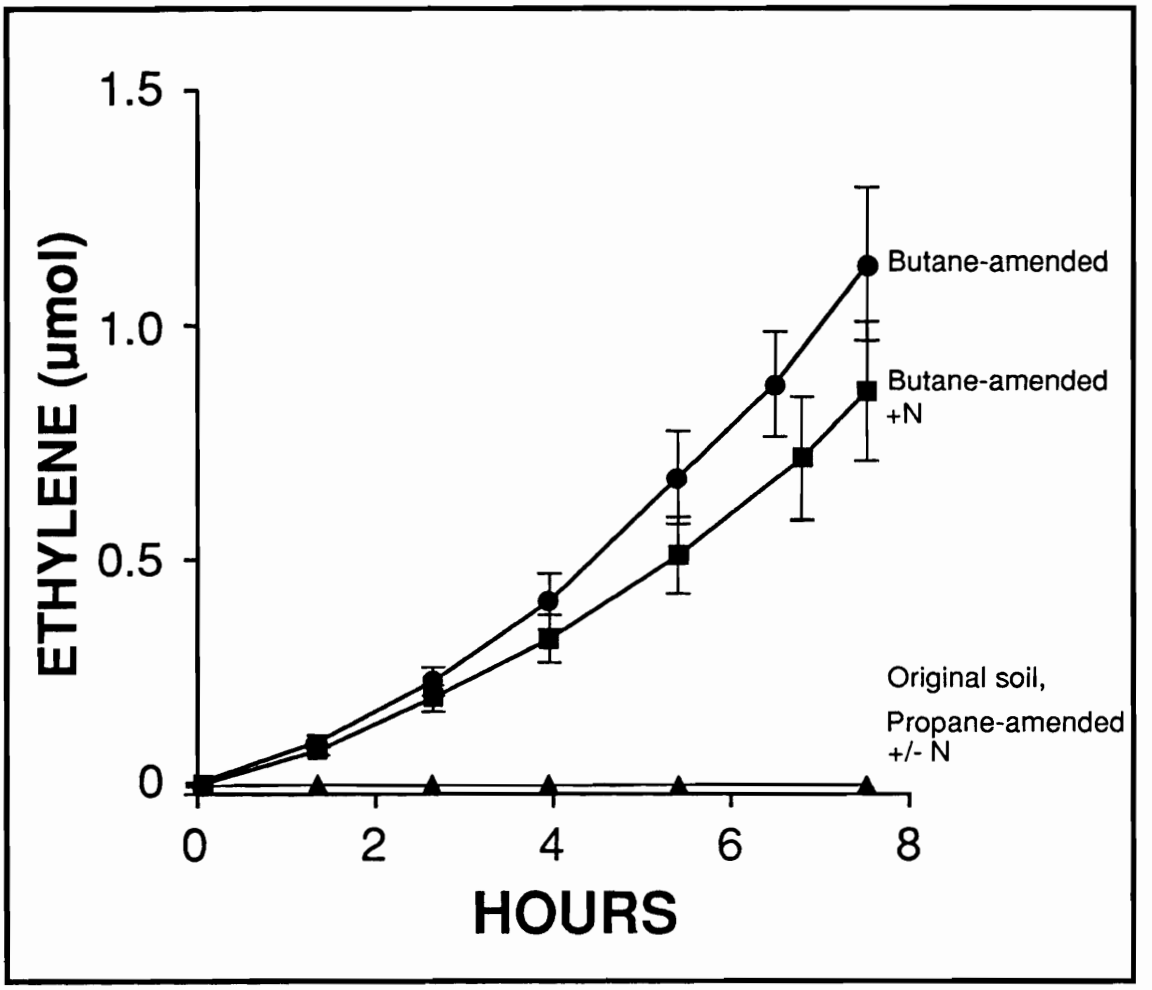


Figure 3.13 Ethylene production in acetylene-reduction assay soil samples. Error bars represent the standard error of the mean.

3.5 Microbiological procedures

3.5.1 Numbers of organisms

The initial butane-oxidizing population was larger than the initial propane-oxidizing population in the original soil, and propane- and butane-amended soil contained more propane- and butane-oxidizing organisms, respectively, than the original soil (Table 3.2). The presence of N in the mineral media increased the numbers of propane- and butane- oxidizing organisms counted, and there were significantly more butane oxidizers than propane oxidizers in hydrocarbon-amended soil.

Table 3.2 Numbers of propane- and butane-oxidizing organisms

	Numbers of Propane-degraders per µg of soil		Numbers of Butane-degraders per µg of soil	
Soil	In medium without N	In medium with N	In medium without N	In medium with N
Unamended	<0.01	<0.01	4.5 (1.4 - 15)[a]	7.8 (2.4 - 26)
Propane-amended	6.8 (2.1 - 22)	22 (6.7 - 73)	ND[b]	ND
Butane-amended	ND	ND	6,800 (2,100 - 22,000)	33,000 (10,000 - 110,000)

[a]Numbers in parentheses are the 95% confidence range from MPN tables.
[b]ND = not determined.

In the acetylene-reduction assays of the MPN tubes, no ethylene was produced in any MPN tubes over 24 h, regardless of the dilution tested, the substrate utilized, or the presence or absence of N in the media.

Unamended Columbia River soil contained 600 protozoa per g of dry soil.

Propane-amended soil with and without added nutrients contained 500 and 5,700 protozoa per g of dry soil, respectively.

3.5.2 Attempts to isolate organisms

Transfers made from positive MPN tubes did not grow unless 1) the transfers were made from MPN tubes amended with N, or 2) the transfers were made into media that contained N. In both cases, growth did not occur until after at least 6 weeks of incubation. Colonies grew equally well on all of the streak plates regardless of whether any substrate was present. Therefore, no cultures that fixed N_2 and utilized butane as a sole source of C were isolated.

CHAPTER 4
DISCUSSION

4.1 Microbial activity of Columbia River soil

The microbial community in the Columbia River soil was active, but required time to adapt to propane and butane before rapid degradation commenced. MPN analysis and slow degradation rates indicated that few hydrocarbon-degrading organisms were initially present in the soil, but exposure to propane or butane increased the numbers of propane- and butane-degrading organisms, respectively. The organisms in Columbia River soil were able to degrade both propane and butane, contrasting other reports that show hydrocarbons $<C_{10}$ are not readily degraded because they dissolve cell lipids (37, 131, 133).

4.2 Optimal conditions for hydrocarbon biodegradation

Surprisingly, temperature and water content were not independent factors affecting propane and butane biodegradation over the range examined, but neither factor had a significant effect on degradation rates as long as the soil was quite moist (~80% of FC). This result contrasts with studies that show that aerobic hydrocarbon biodegradation in soil containing more organic C (3.2%) than Columbia River soil is optimal when the moisture is 30% to 90% of FC (31, 111), presumably because this soil could retain more water than Columbia River soil. Because the a_w was approximately the same at all water contents tested in this study, differences in the quantity of free water (thickness of water film surrounding soil grains) rather than in a_w may have been important.

The initial hydrocarbon concentration to which the soil microbial population was

exposed was an important factor in hydrocarbon biodegradation. If the initial concentration was low (≤0.5% of the headspace), then the microbial community was able to adapt to the hydrocarbons, and could later degrade higher concentrations (1.5% of the headspace). Initially high hydrocarbon concentrations (>0.5% of the headspace) inhibited hydrocarbon degradation. After a gasoline spill, the vapor phase equilibrium concentration of total petroleum hydrocarbons can be estimated by multiplying the mole fraction of gasoline components by their vapor pressures (80). In general, the concentration of total hydrocarbons can range from 5,000 to 30,000 ppm (0.5 to 3%) in the soil gas after a gasoline spill, which are levels initially inhibitory to the organisms in Columbia River soil.

4.3 Differences between propane and butane biodegradation

4.3.1 N-limitation and degradation rates

Propane and butane degradation proceeded similarly during the first 3 months of incubation. Propane and butane degradation was stimulated by N additions, and both propane- and butane-amended soil became N-limited after the initial bioavailable N was utilized, as indicated by a decrease in degradation rates. After 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. Degradation rates were initially low, then increased when numbers of propane oxidizers increased, or after a lag phase during which little growth occurred. Later, degradation rates fell to a maintenance level, presumably because available N was exhausted.

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

4.3.2 Biodegradation equations

Mass balances for C and O_2 were calculated based on the initial and final concentrations of CO_2, O_2, and hydrocarbons in the bottles. 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 largely variable (55). Estimated biomass incorporation was 37.5% or 50% of the added C when propane or butane, respectively, were used as the substrates, which supports previous findings that about 40% of substrate C is assimilated into biomass (4). The following net equations for propane and butane degradation take into account the percentage of substrate mineralized and incorporated into biomass.

Propane:

Mineralization: $C_3H_8 + 5O_2 \rightarrow 3CO_2 + 4H_2O$

Biomass Incorporation: $C_3H_8 + 1.4O_2 + 0.3NH_4NO_3 \rightarrow 0.6C_5H_7NO_2 + 2.5H_2O$

Overall Equation: $C_3H_8 + 3.65O_2 + 0.11NH_4NO_3 \rightarrow 0.22C_5H_7NO_2 + 1.87CO_2 + 3.4H_2O$

Butane:

Mineralization: $C_4H_{10} + 6.5O_2 \rightarrow 4CO_2 + 5H_2O$

Biomass Incorporation: $C_4H_{10} + 0.7O_2 + 0.8NaNO_3 \rightarrow 0.8C_5H_7NO_2 + 2.2H_2O + 0.8Na^+$

Overall Equation: $C_4H_{10} + 3.6O_2 + 0.4NaNO_3 \rightarrow 0.4C_5H_7NO_2 + 2CO_2 + 3.6H_2O + 0.4Na^+$

The biomass formula above is an empirical representation of microbial biomass derived from the ratios of elements in a typical microbial cell (108). There is very good agreement between the O_2:CO_2 and O_2:hydrocarbon ratios actually measured and ratios predicted by the above equations (Table 4.1). 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 above can be manipulated based on 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.

Table 4.1 Measured and predicted ratios between the components of the biodegradation equations

Soil	O_2:CO_2 Meas[b]	Pred[c]	O_2:Hydrocarbon Meas	Pred	N[a]:Hydrocarbon Meas	Pred
Propane-amended	1.81	1.95	3.4	3.65	0.36	0.22
Butane-amended	1.78	1.8	3.6	3.6	0.1	0.4

[a]N = bioavailable N (bioavailable N in unamended soil + added N)
[b]meas = measured value
[c]pred = predicted value

4.3.3 Nutrient requirements and N_2 fixation

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 estimated as 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 (9) to 10 (4, 31). 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.

Based on these ratios and the amount of N added to propane-degrading soil, 4.9 to 14 mg of C should have been degraded before N became limiting. In actuality, 3.7 mg

of C was degraded before propane-amended soil appeared to become N-limited, and a total of 19 mg of C was degraded over the course of the experiment. Propane degradation continued in N-limited soil, suggesting that 1) propane degradation met the high maintenance-energy needs of a non-growing population (the maintenance degradation rate was 20% of the maximum degradation rate), or 2) N was recycled, possibly by protozoal grazing of the bacterial population. However, protozoa in propane-amended soil with added N were not more numerous than in unamended soil.

Without N_2 fixation, butane-amended soil with no added N should have only degraded 0.5 to 1.4 mg of C before N became limiting, but 131 mg of C 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 adequate bioavailable 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.

4.4 Isolation of a butane-oxidizing, N_2-fixing organism

Only one study (27) has reported the isolation of a butane-oxidizing, N_2-fixing organism from soil, but this culture was apparently not pure (88). We were unsuccessful in determining whether butane oxidizers fix N_2 or whether butane oxidizers and N_2-fixers are separate organisms in Columbia River soil.

If the butane oxidizers can fix N_2, then our culture medium did not allow the organisms to express their ability to fix N_2, perhaps because the N_2-fixers could not survive in completely aerobic media, as compared to a soil matrix which possibly contains anaerobic microenvironments and solid surfaces. The acetylene-reduction assay supports this hypothesis because ethylene was produced only in butane-amended soil, but not in butane-amended liquid MPN cultures. We do not know if the inability of propane-amended soil to fix N_2 was due to a lack of a hydrocarbon-oxidizing N_2-fixing organism

that could utilize propane as a substrate, or due to the inability of the organism to fix N_2 while using propane.

If the butane oxidizers are separate organisms from N_2-fixers, then the degradation of butane may produce some extracellular products that are available to N_2-fixers as a substrate, whereas propane degradation may not produce these extracellular products. For example, cyst formation in some N_2-fixing organisms is closely related with the synthesis and accumulation of poly-β-hydroxybutyric acid (PHB) (88). The biosynthesis of PHB is a mechanism for the accumulation of a readily available reserve of C, reducing power, and energy (88). Butanol and β-hydroxybutyric acid [intermediates of butane oxidation (see Figure 1.4)], are precursors for the synthesis of PHB, and promote cyst formation (20, 88). Cyst-forming N_2 fixers in soil have a survival advantage because cysts resist desiccation and show negligible endogenous respiration (20, 88). Therefore, butane-amended soil may contain butanol and β-hydroxybutyric acid which can be used by N_2 fixers, whereas propane-amended soil may contain extracellular products that are unavailable to N_2 fixers.

4.5 Conclusions

1. The microbial population in Columbia River soil is active and can degrade both propane and butane.
2. Temperatures ranging from 15 to 20°C had little effect on hydrocarbon biodegradation.
3. Water contents needed to be ~80% of FC for maximum hydrocarbon biodegradation.
4. Initial hydrocarbon concentrations >0.5% of the headspace were inhibitory.
5. N was the most limiting nutrient in Columbia River soil, and was initially limiting to both propane- and butane-degrading organisms.
6. NO, NO_2, and N_2O gases had no effect on or inhibited butane degradation, whereas NH_3 stimulated butane degradation.

7. Propane degraders remained N-limited unless more N was added to the soil, whereas butane degraders overcame N-limitation by fixing N_2.
8. The nutrient requirements of propane and butane degraders can be estimated.
9. Columbia River soil contained more butane degraders than propane degraders, and exposure to either hydrocarbon increased the numbers of organisms in the soil.

4.6 Future work

This work can continue in both fundamental and applied research directions. The fundamental research projects include:

1. Isolating propane- and butane-degrading organisms.
2. Determining why butane stimulates N_2 fixation and propane does not.
3. Determining whether butane oxidizers fix N_2 or whether butane oxidizers and N_2 fixers are separate microbial populations in Columbia River soil.
4. Probing butane-oxidizer genes for N_2-fixing capability.

Projects that lean towards applied research include:

1. Determining if butane stimulates N_2 fixation in a variety of soil types and contaminant conditions in the laboratory and on a larger scale (e.g., in columns).
2. Determining which hydrocarbons stimulate N_2 fixation in soils, and whether gasoline-contaminated soils can fix N_2 since gasoline contains butane.
3. Determining whether butane oxidizers can cometabolize other contaminants such as chlorinated solvents.
4. Examining the water content/temperature relationship more closely.

4.7 Significance and applications of study

Some hydrocarbons have been shown to stimulate N_2 fixation in soils, yet hydrocarbon degradation at a majority of field-scale remediation sites is enhanced by N additions (1, 31, 36, 50, 69, 118, 133) (see section 1.2.4.1). This study raises the question of why any hydrocarbon-contaminated field site is N-limited.

One potential application of this study is to use butane as a N source in N-limited subsurface soils by stimulating *in situ* N_2 fixation (if it is determined that butane alone stimulates N_2 fixation in soils). Butane added to the vadose zone would diffuse fairly homogeneously throughout the soil, thus providing a uniform substrate for N_2 fixers. Ionic forms of N do not travel very far through the subsurface, clog up well screens, and can leach into groundwater.

This study has shown that the nutrient requirements of propane and butane degraders can be quantitatively estimated based on an overall reaction comprised of the fractions of added C mineralized and incorporated into biomass. It is therefore feasible that the nutrient requirements of other hydrocarbons can be estimated in batch tests that determine how much of the added substrate is mineralized. Upon extrapolating from batch tests, it is possible to use the results of this study to optimize hydrocarbon biodegradation in excavated soils, and in soils contaminated with residual hydrocarbons after excavation or pump-and-treat procedures. The amount of N needed can be calculated, and this study suggests that temperature and soil moisture content do not need to be rigorously controlled.

Bioremediation can also be used in conjunction with soil vacuum extraction. Soil vacuum extraction is the process of applying a vacuum to an unsaturated zone to collect vapor-phase contaminants and stimulate their removal from the soil by encouraging their volatilization. These systems are attractive for soil remediation because of their low cost, ease of implementation, and apparently favorable results in many instances of gasoline contamination in permeable soils. However, the cost increases significantly when

treatment is required for the off-gas (33). The results of this study can aid in the design of a bioreactor or soil column that can be used in the clean-up of the off-gas from soil vacuum extraction procedures.

Finally, another potential application is use butane oxidizers to cometabolize other contaminants (e.g., trichloroethylene (TCE) and other chlorinated solvents). Because alkane monooxygenases are similar to the monooxygenases of methanotrophs that oxidize and dechlorinate halogenated compounds, it is possible that other alkanes can also cometabolize TCE. Both natural gas (144) and propane (142) have been shown to stimulate the cometabolism of TCE, so it is feasible that butane additions will not only stimulate N_2 fixation, but also cometabolize TCE. There is great interest in implementing processes to remove TCE from drinking-water supplies, because TCE is an Environmental Protection Agency priority pollutant, a common groundwater contaminant, a potential carcinogen, and anaerobic degradation of TCE in groundwater leads to the formation of the potent carcinogen vinyl chloride (6).

CHAPTER 5

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APPENDIX A

Chemicals used and their Chemicals Abstracts Service (CAS) registry numbers

Chemical Name	CAS #
Gases:	
Methane	74-82-8
Ethane	74-84-0
Propane	74-98-6
Butane	106-97-8
Acetylene	74-86-2
Ethylene	74-85-1
Oxygen	7782-44-7
Carbon dioxide	124-38-9
Inorganic Compounds:	
Sodium phosphate, dibasic	7558-79-4
Sodium phosphate, monobasic	7558-80-7
Ammonium chloride	12125-02-9
Ammonium nitrate	6484-52-2
Sodium nitrate	7631-99-4
Sodium pyrophosphate decahydrate	13472-36-1

APPENDIX B

Propane- and butane-oxidizing organisms

Propane-oxidizing Organisms

Name	Source(s)
Acinetobacter calcoaceticus (ATCC 19140)	(53, 54)
Acinetobacter sp. strain CRL67	(53, 54)
Actinomyces sp. strain CRL66	(53, 54)
Alcaligenes eutrophus (ATCC 17697)	(53, 54)
Alcaligenes sp. (ATCC 15525)	(53, 54)
Arthrobacter petroleophagus (ATCC 21494)	(53, 54)
Arthrobacter simplex (ATCC 21032)	(53, 54)
Arthrobacter sp. strain CRL60	(53, 54)
Arthrobacter strain B2	(125)
Arthrobacter strain B3aP	(125)
Arthrobacter strain PrIO3	(125)
Brevibacterium insectiphilum (ATCC 15528)	(53, 54)
Brevibacterium sp. (ATCC 14649)	(53, 54)
Brevibacterium sp. strain JOB5	(140)
Brevibacterium sp. strain CRL52, CRL56	(53, 54)
Corynebacterium sp. strain CRL63	(53, 54)
Mycobacterium album strain 7E1B1W (ATCC 29676)	(13, 53, 54, 104)
Mycobacterium album strain 7E4	(13)
Mycobacterium convolutum	(142)
Mycobacterium convolutum strain NPA-1 (ATCC 29674)	(104)
Mycobacterium convolutum Strain R-22 (ATCC 29671)	(13)
Mycobacterium lacticolum	(104)
Mycobacterium paraffinicum (ATCC 12670)	(53)

Mycobacterium rhodochrous strain 7E1C (ATCC 19067)	(13, 104)
Mycobacterium rhodochrous strain A-78 (ATCC 29670)	(13, 53)
Mycobacterium rhodochrous strain OC2A (ATCC 29675)	(104)
Mycobacterium rhodochrous Strain OFS (ATCC 29672)	(13, 53, 54, 104)
Mycobacterium rhodochrous W-21	(142)
Mycobacterium rhodochrous W-24	(142)
Mycobacterium rhodochrous W-25	(142)
Mycobacterium rubrum var. *propionicum*	(104)
Mycobacterium smegmatis 422	(125)
Mycobacterium sp. strain CRL51, CRL62	(53, 54)
Nocardia asteroides	(104)
Nocardia brasiliensis	(104)
Nocardia caviae	(104)
Nocardia coelicaca	(104)
Nocardia convoluta	(104)
Nocardia madurae	(104)
Nocardia neoopaca (ATCC 21499)	(53, 54)
Nocardia rubropertincta	(104)
Nocardia sp. strain CRL55, CRL57, CRL64	(53, 54)
Pseudobacterium subluteum	(104)
Pseudomonas caudatus	(104)
Pseudomonas crucurae NRRL B-1021	(53, 54)
Pseudomonas fluorescens NRRL B-1244	(53, 54)
Pseudomonas ligustri (ATCC 15522)	(53)
Pseudomonas liquefaciens	(104)
Pseudomonas multivorans (ATCC 17515)	(53)
Pseudomonas multivorans (ATCC 17616)	(54)
Pseudomonas putida (ATCC 17453)	(53)
Pseudomonas sp. strain CRL53, CRL54, CRL58, CRL65	(53, 54)

Butane-oxidizing Organisms

Name	Source
Arthrobacter sp. CRL70	(54)
Arthrobacter strain AK 19	(85)
Arthrobacter strain AK 117	(85)
Arthrobacter strain J	(85)
Arthrobacter strain P4	(85)
BG 28, unknown genus	(85)
Brevibacterium Strain AK 22	(85)
Mycobacterium vaccae strain Et32	(30)
Mycobacterium vaccae strain 3b	(30)
Mycobacterium vaccae strain H12	(30)
Pseudomonas sp. CRL71	(54)

Organisms that Oxidize both Propane and Butane

Name	Source(s)
Brevibacterium fuscum (ATCC 15993)	(53, 54)
Brevibacterium ketoglutamicum	(104)
Brevibacterium sp. strain CRL61	(53, 54)
Methylobacter bovis CRL M1Y	(52, 100)
Methylobacter capsulatus Y	(100, 101)
Methylobacter sp. CRL 5	(52)
Methylobacter sp. CRL M6	(52, 100)
Methylobacterium organophilum CRL26	(51, 52, 100, 101)
Methylobacterium organophilum XX	(52, 100)
Methylococcus capsulatus Bath	(24)
Methylococcus capsulatus CRL M1	(51, 52, 100)
Methylococcus capsulatus CRL 24	(101)
Methylococcus capsulatus Texas	(100)
Methylococcus capsulatus Y	(52)
Methylocystis parvus OBBP	(52, 100)
Methylomonas albus BG8	(52, 100)
Methylomonas methanica CRL-21	(100)
Methylomonas methanica S_1	(52, 100)
Methylomonas sp. CRL 8	(52)
Methylomonas sp. CRL 10	(52)
Methylomonas sp. CRL 17	(101)
Methylomonas sp. CRL M4	(52)
Methylomonas sp. CRL M6P	(52)
Methylosinus trichosporium CRL 15	(52, 100, 101)
Methylosinus trichosporium OB3b	(51, 52, 91, 100, 101)
Methylosinus sporium 5	(52, 100)
Mycobacterium vaccae JOB5 (ATCC 29678)	(13, 26, 25, 104, 105, 139, 142)
Mycobacterium vaccae Strain E20	(30)
Nitrosomonas europa (ATCC 19178)	(59)

Nocardia paraffinicum (ATCC 21198)	(10, 53, 54, 77)
Nocardia vaccinii TB1	(137)
Pseudomonas butanovora sp. nov	(130, 139)
Pseudomonas methanica	(67)

APPENDIX C

Hydrocarbon-oxidizing, N_2-fixing bacteria

Name	Substrate	Source
Azospirillum sp. ANK BI-11	dodecane	(116)
Mycobacterium butanitrificans	Butane	(27)
Mycobacterium vaccae Strain 3b	Ethane, butane	(30)
Mycobacterium vaccae Strain H12	Butane, Hexane	(30)
Strain BPD1 no species assigned	Oily Sludge	(66)
Strain BPD2 no species assigned	Oily Sludge	(66)
5 out of 6 isolates	Oil Sand	(95)

APPENDIX D

N_2-fixation rates in contaminated and uncontaminated sites

N_2-fixation rates at hydrocarbon-contaminated sites

Rate	Rate [µmol C_2H_4/(kg soil*h)][a]	Source
838.1 nmol fixed N_2/g wet sludge	189[b]	(66)
144-240 mg N/g soil (323-538 kg/ha/yr)	2.3	(95)
31 µmol $C_2H_2/m^2/h$	0.14	(71)
20 µmol $C_2H_2/m^2/h$	0.09	(71)
100 nmol C_2H_4/ml C_2H_2/h	[c]	(116)
3.17 mg $N/m^2/d$	0.2[d]	(134)
0.6 nmol C_2H_4/g dry wt/h	0.6	(134)
5.64 mmol $C_2H_4/m^2/h$	25	(45)

N_2-fixation rates at uncontaminated sites

Rate	Rate [µmol C_2H_4/(kg soil*h)]	Source
6-12 mg N/g soil (13-26 kg/ha/yr)	0.1	(95)
19 µmol $C_2H_2/m^2/h$	0.08	(71)
20 µg N/g soil/d	89	(92)
2.23 mg $N/m^2/d$	0.1[d]	(134)
0.3 nmol C_2H_4/g dry wt/h	0.3	(134)
4.09 mmol $C_2H_4/m^2/h$	18	(45)

[a]Assumptions made in conversions: 1) C_2H_4:NH_3 ratio = 3:2, 2) soil depth = 15 cm = plough layer, 3) bulk density = 1.5 g/cm^3, so 1 m^2 of soil = 225 kg of soil
[b]Based on a 48 h sample
[c]Measured in a culture (not soil)
[d]Based on soil depth = 5 cm

APPENDIX E

Larger-scale (column) experiment

Background

The purpose of performing column experiments was 2-fold: 1) to examine any correlations between laboratory-scale experiments and larger-scale (column) experiments, and 2) to determine if any gaseous forms of N pumped through a column could be used to stimulate butane biodegradation. Although I made the columns to do this study, the experiment was never carried out because N_2 fixation became the primary focus of my work. This appendix is meant to serve as a record not only of my intent, but also to record how the columns were made in the event that future investigators use the columns in their work.

Materials needed per column

-PVC Pipe, Schedule 40, 6" diameter, 8' long
-PVC Pipe, Schedule 40, 6" diameter, 1'8.25" long
-3 PVC endcaps, 6" diameter, schedule 40
-1 PVC "collar", made from flared end of PVC pipe (~6.25" diameter)
-2 Screens, stainless steel mesh, 6" diameter
-2 Screens, aluminum or stainless, 1/8" holes, 6" diameter
-Humidifier with precision o.d. 1/4" glass, 75 ml capacity, 50 ml water
-1/8" and 1/4" copper tubing
-2 Snap valves
-3 Needle valves
-3 Flow meters, calibrated
-1 3-way connector (Swagelok)
-1 Compressed Air tank
-1 Tank with hydrocarbon/nitrogen mix
-Sand, ~2 ft^3 (~56 L)

-Pea Gravel, ~3 L
-3 1/4" pipe thread to 1/8" Swagelok fittings
-5 Needles, 16 gauge, 4" long for sampling ports
-5 1/8" pipe thread to 1/16" Swagelok fittings machined out (drill bit #51) (for sampling ports)
-5 Teflon stoppers (plugs for needles)
-Plywood, 4' x 10' x 3/4", 1 sheet
-Wood, ~12-15 ft^2, 1" thick
-14 1' pieces of 3/8" diameter all-thread with washers and nuts to fit
-Taps, 1/8" NPT
-Large hook to hang column assembly onto.
-3 pieces 2-3" diameter PVC pipe, 12', 6', and 3'.
-Fitting to connect a hose to 1/8" Swagelok.
-2 1/4" Teflon ferrules
-Several 1/8" and 1/4" Swagelok ferrules, nuts, and endcaps.
-2 1/4" to 1/8" Swagelok adaptors

Procedure

A. Build a housing for column out of plywood (use Fig. E1 as a guide).

B. Build wooden supports for column to hang onto wall, and screw the braces onto studs in the wall.

C. Build the column by:

Drill a 1/4" hole in the center of 3 endcaps, then tap the endcap holes with 1/4" NPT, and screw in one 1/4" pipe thread-to-1/8" Swagelok fitting using teflon tape to make a good seal. Drill 1/8" holes for sampling ports spaced equidistant along column, then tap the sampling port holes with 1/8" NPT, and screw in 1/8" pipe thread-to-1/16" Swagelok fittings using teflon tape to make a good seal.

Vertically align the 8' and 1'8.25" PVC pipes, caulk them together, then duct tape them together, and put the collar on. Caulk, duct tape, and hose clamp the collar onto the PVC pipes, and let the caulk dry while the column hangs vertically overnight. The next day, place an endcap at the end of the 1'8.25" PVC pipe, then caulk and screw the endcap onto the PVC pipe. Secure the entire column in the plywood housing (step A)

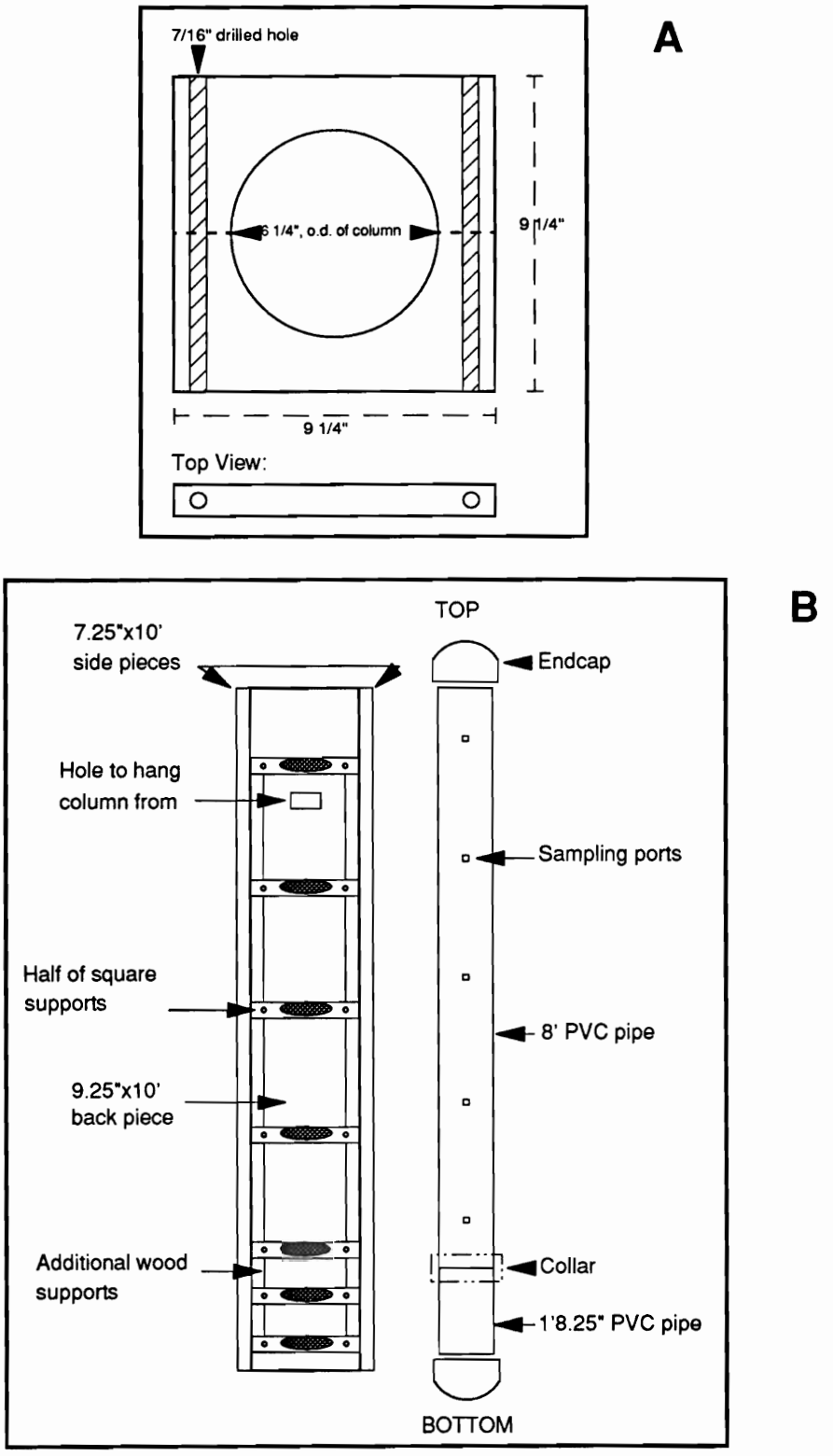

Figure E1 A) Square supports for column in plywood housing assembly. B) Housing assembly for columns.

by matching the wood supports with their mates, and connecting with all-thread. Attach washers and nuts to the all-thread and wrench them tightly with socket wrench. Hang the column vertically in its plywood housing on a hook attached to a building with the short PVC piece on the bottom (Fig. E1 B).

Add enough pea gravel into the column to fill the endcap. Put a 12' piece of 2" diameter PVC pipe down the column, and pour several shovel-fulls of field-moist sand down the 2" pipe. Lift the 2" pipe up-and-down to dislodge the sand and evenly fill the column. Continue adding sand in this manner until the column is full. Use smaller pieces of the 2" PVC pipe as the larger pieces become unwieldy. Use a vibrator to compact the sand in the column, and top the column off with additional sand. Attach a hose or other water source to the bottom of the column, and slowly imbibe water up the column at a rate of ~1 L/min until the water almost reaches the top of the column (determine this by unscrewing the sampling ports as the water rises). Continue to vibrate the column, and add more sand until the sand will no longer compact.

Place an endcap filled with pea gravel and capped with a screen onto the top of the column. The mesh side of the screen should face the sand, the side with holes should face the gravel. Caulk the endcap onto the top of the column, and screw it in place. Screw in 1/4" pipe thread to 1/8" Swagelok fitting onto the endcap using teflon tape. Continue adding water until water comes out the top of the column, then turn off the water, and let the column drain for 1 week.

Take the column assembly off the wall, invert the column, remove the collar, jam a thin piece of sheet metal between the PVC pieces and remove the short piece (this piece should contain the saturated portion of the column). Caulk and screw another endcap filled with gravel onto the exposed end of the column. Lower the column assembly to the ground, remove the column from the plywood housing, and hang the column horizontally onto prepared supports on the wall. Push needles through the sampling ports, swage them on, and plug the needles with teflon stoppers. Connect the column to gas tanks, humidifiers, and flow meters (Fig. E2).

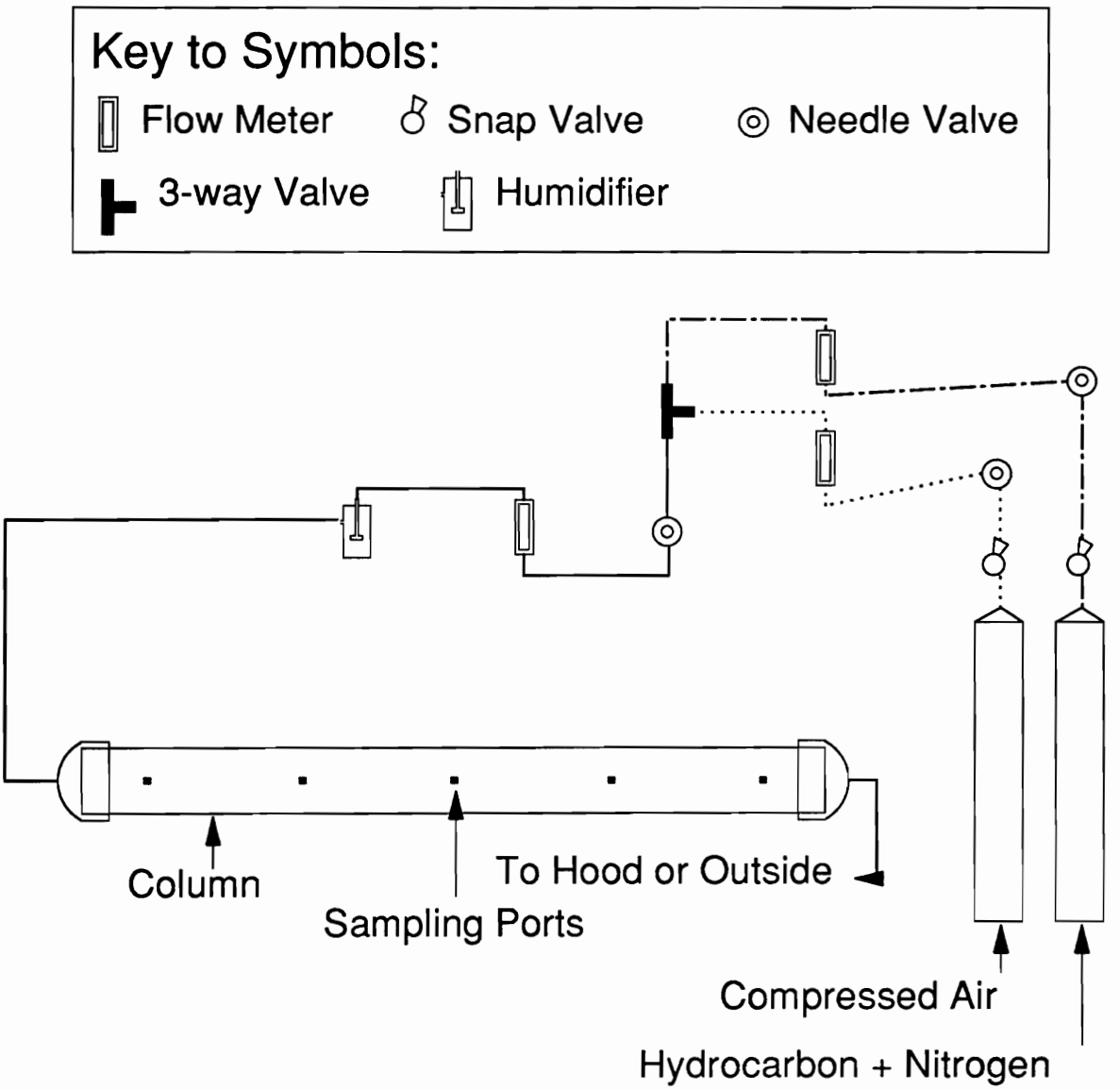

Figure E2 Schematic of completed column.

VITA

I was born in January, 1965 in Detroit Mich., and was raised in Utica, Mich. In 1983, I began my academic career at Oakland University in Rochester, Mich as a physical therapy major. My major changed several times, but I graduated in 1987 with a B.S. degree in Environmental Health, a specialization in Toxic Substance Control, and a minor in Chemistry. I graduated with departmental honors, and also received a Student Life Scholarship, an Upperclassman Achievement Scholarship, and an Undergraduate Research Award.

I began my professional work career during my junior year in college. My relevant professional experience is listed below in reverse chronological order:

Date	Employer	Job Title
1985 to 1986	Parke-Davis	Sterility Tester, Asst. Line Supervisor
1986 to 1987	Trinity Technologies	Chemist
1986 to 1987	Oakland University	Research Assistant
1987 to 1988	Chrysler Motors	Industrial Hygienist
1988 to 1992	Oregon Graduate Institute	Research Assistant

I left Chrysler Motors to begin graduate school at the Oregon Graduate Institute in Beaverton, Ore., where I received Research Assistantships and Tuition Scholarships. I will graduate in 1992 with a Ph.D. in Environmental Science and Engineering. My main area of interest was the clean-up of hydrocarbon-contaminated soils and groundwater by bioremediation, either alone or in conjunction with other clean-up technologies. The following is a list of my publications:

Toccalino, P. L., D. R. Boone, and R. L. Johnson. 1992. Stimulation of nitrogen fixation by alkane biodegradation in a sandy soil. Appl. Environ. Microbiol. Submitted for publication.

Harmon, K. M., P. L. Toccalino, and M. S. Janos. 1989. Hydrogen bonding. Part 29. Thermodynamics of dissociation and stoichiometric study of the lower hydrates of tetraethylammonium and tetrapropylammonium chlorides. J. Mol. Struct. **213:**193-200.

P. L. Toccalino, K. M. Harmon, and J. Harmon. 1988. Hydrogen bonding. Part 26. Thermodynamics of dissociation and infrared spectra-crystal structure correlations for betaine monohydrate and trimethylamine oxide dihydrate. J. Mol. Struct. **189:**373-382.

K. M. Harmon, J. E. Cross, and P. L. Toccalino. 1988. Hydrogen bonding. Part 25. The nature of the hydrogen bond in hydroxytropenylium chloride (tropone hydrochloride). J. Mol. Struct. **178:**141-145.