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# MICROBIAL ECOLOGY AND LONG-TERM PERSISTENCE OF CRUDE OIL IN A TAIGA SPRUCE FOREST

A

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Presented to the Faculty
of the University of Alaska Fairbanks
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By
Jon Eric Lindstrom, B.A., M.S.

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MICROBIAL ECOLOGY AND

LONG-TERM PERSISTENCE OF CRUDE OIL

IN A TAIGA SPRUCE FOREST

By

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# ABSTRACT

The microbial ecology of a 1976 experimental crude oil spill in an Alaskan taiga black spruce forest was investigated in this study. Substantial oil residue remained in the soil, and several microbial parameters showed evidence of long-term oiling effects. Overall, the data suggest that the surviving community in the oiled plot has shifted toward using oil C for growth. Numbers of hydrocarbon degrading microbes, and specific hydrocarbon mineralization potentials, were significantly elevated in the oiled (OIL) plot compared to an adjacent oil-free, reference (REF) plot. Glutamate mineralization potentials and soil C mineralization, on the other hand, were not different between treatments, suggesting that OIL plot heterotrophs were well-acclimated to the oil. Despite little difference between OIL and REF soils in total C mineralized *in vitro*, net N mineralized was lower and net nitrification was absent in OIL soils. Analysis of the residual oil indicated minimal amounts of N were added with the spilled oil. Biomasses of total fungi and bacteria, and numbers of protozoa, showed no consistent effects due to oiling, but metabolically active fungal and bacterial biomasses were uniformly lower in OIL samples. Community-level multiple substrate metabolism (Biolog) was assessed using a new technique for extracting kinetic data from the microplates. This analysis suggested that the microbial population diversity in the OIL soils was lower than in REF soils. Further, these data indicated that the surviving populations in the OIL plot may be considered metabolic generalists. Some evidence of crude oil biodegradation was seen in the chemistry data, but enrichment of the oil residue in higher molecular weight components, duration of contact with soil organic material, and slow rates of C mineralization indicate the crude oil will persist at this site for decades. Contamination of Alaskan taiga soil at this site has yielded observable long-term microbial community effects with larger-scale consequences for ecosystem function.

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I also thank Dr. Edward Brown at University of Northern Iowa for lending me a microplate reader and judiciously offering advice; much of the original work in this dissertation would not have been possible without this assistance. I thank my father and mother, Eugene and Joyce Lindstrom, and my sister, Meredith Goetz, for their loving support over the years. Finally, I will thank my wife, Janet Kidd, for "everything", since an exhaustive list would be prohibitively long.

# INTRODUCTION

In the 1970's, development of large petroleum reserves in the Canadian and Alaskan Arctic raised concerns that transport of crude oil across arctic and subarctic terrain would likely result in recurrent spills (Jenkins *et al.*, 1978). As there was little information about environmental impacts from terrestrial crude oil spills in northern regions, several groups of researchers implemented studies identifying the effects of such spills on local ecosystems (e.g., Hutchinson and Freedman, 1978) and the natural mechanisms likely responsible for recovery of affected areas (e.g., Jobson *et al.*, 1974). Following the decision to construct the Trans-Alaskan Pipeline System, one group of investigators conducted experimental crude oil spills designed to mimic the effects of an oil leak resulting from a pipeline failure in permafrost terrain (Johnson *et al.*, 1980). In 1976 two 7570 liter spills (one in February and one in July) of hot (57 °C) Prudhoe Bay crude oil were conducted in an open black spruce (*Picea mariana*) forest at the Caribou-Poker Creeks Research Watershed (CPCRW). There were four overall objectives of the study (Johnson *et al.*, 1980): i) to determine the physical effects of the oil on the forest, with attention to contaminant transport, area of impact over time, and permafrost effects; ii) to determine the fate of petroleum in subarctic terrestrial systems; iii) to determine the effect of crude oil on soil microbial populations; and iv) to determine the effects of oil on subarctic vegetation. Spill-related effects were evaluated for two years following the spill (Sparrow *et al.*, 1978; Jenkins *et al.*, 1978), and two additional follow-up studies were conducted after 10 (Sparrow and Sparrow, 1988; microbiology and chemistry) and 15 years (Collins *et al.*, 1994; chemistry, and physical and vegetation effects).

During the period from spring thaw through autumn freeze-up in 1994 and 1995, I re-visited the 1976 spill site at CPCRW. A substantial quantity (ca. 0.3 g · g dry soil$^{-1}$) of crude oil remained in the soil at the site 18 years after it was spilled. Given the long-term presence of the oil in this system, two interrelated questions were raised.

First, what was the effect of the crude oil on the microbial community in the affected soil? Second, were soil microbes breaking down the oil? I wished to know if the oil eliminated components of the microbial community essential for "proper" functioning of the soil system. Among the challenges was the search for measurements of the microbial community that would discriminate between oiled and oil-free soils. Further, I sought to resolve questions regarding community structure; in other words, was the diversity of the oiled microbial community increased or decreased relative to that of nearby, oil-free soils? Biodegradation of organic compounds like crude oil represents one of the lowest impact cleansing mechanisms available as it relies on natural element-cycling processes (Madsen, 1991; Dixon, 1996). I wished to understand the reasons for the crude oil's persistence and whether soil microbial potentials extant in the spill-affected community ultimately will be sufficient for biotic destruction of the residual oil.

One of the tasks facing soil ecologists is to assess the behavior of the soil community and relate the community's structure to its function (Schimel, 1995). No single tool currently available allows definitive assessment of the microbial community. Rather, the approach typically entails evaluating multiple lines of evidence to infer characteristics about the behavior of the system (Madsen, 1991). In this effort new tools offering a window into microbial community behavior are always welcome. Garland and Mills (1991) presented a promising new tool (Biolog multiple substrate microplates) to characterize microbial communities by their response to an array of carbon sources, generating a community-level "metabolic fingerprint." My attempts to use Biolog plates were beset by several problems with the published methodology. These included issues regarding how to objectively quantify the community response to the substrates, and the problem of varying inoculum densities affecting substrate response rates. Chapter One of this dissertation provides my solution to problems with Biolog, wherein I suggest that estimating parameters describing the kinetic response of

communities to each substrate offered provides objective criteria for assessing community-level integrated metabolisms.

Freed from concerns of how to quantify community-level responses to multiple substrates, I wished to move beyond generating community metabolic fingerprints. I applied my new kinetic approach to assessing microbial community structure. Other investigators have demonstrated that communities' metabolic responses to multiple substrates can discriminate communities according to environmental treatments (e.g., Garland and Mills, 1991; Zak *et al.*, 1994; Bossio and Scow, 1995), but evidence of changes in community diversity and structure due to these treatments has remained elusive (Haack *et al.*, 1995). Chapter Two of this dissertation addresses measuring the relative functional diversity of microbial communities by coupling my kinetic approach to quantifying community-level substrate responses with common sample dilution techniques. Using kinetic parameters estimated for each substrate used by soil communities inoculated into the assay microplates, I evaluate changes in community self-similarity across dilutions and demonstrate how information contained in these microplate responses can be extracted and used.

Despite the ongoing search for new techniques in microbial ecology, however, traditional microbiological techniques are still useful for assessing microbial community behavior and structure. As suggested by Mills and Wassel (1980, p. 586), "Diversity, measured by any procedure, will never become a total answer to the determination of low-level stress effects, but when coupled with methods such as activity measurements, biomass evaluations, and other descriptors of community structure, the property may become a valuable addition to the arsenal of ecological tools held by the microbial ecologist and may be used to further understand the world." Chapter Three of this dissertation contains an assessment of a variety of physical, chemical and microbiological soil parameters at my study site. Microbial biomass, mineralization potentials, and other data pertaining to the soil populations at

the site are examined in an effort to determine the microbiological factors involved in the persistence of crude oil in these soils almost twenty years after it was spilled.

The results of this research provide a view of microbial life in taiga soil subject to the stresses associated with heavy oil contamination. Assessments of microbial biomass and activities suggest that, after decades of exposure to oil, microbial communities have adapted to the oil but the associated population shift has disrupted mineral N cycling in these soils. Substrate-specific mineralization potentials and assays for specific functional populations provide evidence of microbial communities with diminished diversity, metabolically focused by petroleum hydrocarbons. Community acclimation to hydrocarbons has led to C mineralization rate potentials that are virtually indistinguishable from oil-free soils. Despite enrichment of the oiled soils in favor of hydrocarbon-acclimated populations, the shift in substrate use from soil organic matter to crude oil has resulted in diminished net N mineralization. This, coupled with the increasing recalcitrance of residual oil with time, may be responsible for the slow removal of crude oil from this site. Accordingly, it is anticipated that the spilled oil will persist as a feature of this taiga site for decades.

# MICROBIAL COMMUNITY ANALYSIS: A KINETIC APPROACH TO CONSTRUCTING POTENTIAL C SOURCE UTILIZATION PATTERNS[†]

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## SUMMARY

The analysis of multiple substrate metabolism by assemblages of bacterial strains may be used to differentiate inocula from environmental samples. Biolog plates, 96-well microtiter plates containing nutrients, a single carbon test substrate in each well and a tetrazolium redox dye to monitor substrate oxidation, have been used for this purpose. One of the difficulties faced by users of this technique is determining which substrates have been metabolized. Reliance on single-time-point absorbance data for each well is problematic due to variably non-linear rates of color development for each well. Previous efforts to use color-normalized single plate readings have been successful in discriminating between environmental sample types, but substrate-use contributions to sample classifications vary depending on duration of the plate incubation period. We present a model based on the logistic equation for density-dependent population growth providing a good (low $\chi^2$) fit to the sigmoidal kinetics of color development data. The kinetic parameters generated by the model can be used as surrogates for single-time-point data in constructing carbon source utilization patterns, and contribution of substrate use to sample classification does not depend on incubation time. This technique obviates the need to arbitrarily choose the time following inoculation to read the plate absorbance data and also provides two kinetic

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parameters that are invariant with respect to inoculum density. We provide a comparison of community potential substrate use analyses using single-time-point microplate data and parameters from our kinetic model.

## INTRODUCTION

Patterns of carbon substrate metabolism recently have been used to characterize microbial communities from environmental samples (e.g., Garland and Mills, 1991; Winding, 1994; Zak *et al.*, 1994; Bossio and Scow, 1995; Garland, 1996). Most of these efforts have involved use of commercially available microtiter plates containing 95 carbon test substrates and a tetrazolium redox dye (e.g., Biolog GN Microplates; Biolog, Inc., Hayward, CA). While these plates originally were designed to classify isolates based on their pattern of substrate use, community-level analysis entails inoculating the plates with whole environmental samples (e.g., soil suspensions, hydroponic solutions, etc.). Substrate use patterns are then quantified by comparing tetrazolium dye color development in each of the 95 wells of the plate with a control well containing no test substrate. Substrate metabolism typically is reported either by establishing minimum threshold values of color density to generate binary data (presence or absence of metabolism in a given well; Zak *et al.*, 1994) or by comparing color densities of wells after subtracting the measured density of the control well (Zak *et al.*, 1994; Bossio and Scow, 1995). Whichever method is used, the data are then subjected to multivariate analysis (e.g., principal components analysis) to both discriminate communities based on their substrate use patterns and reduce the dimensionality of the data set generated from the 95 variables (substrates) measured.

Analysis of community level metabolic diversity, as reflected by microplate C source use patterns, is restricted by the ability of microbes to metabolize substrates in the microplate environment. It cannot, therefore, be seen as a tool to directly assess total microbial functional diversity in environmental samples. However, functional diversity of the organisms able to metabolize microplate substrates *in vitro* can be a

useful tool to differentiate environmental samples if care is taken to protect against biases introduced in the analysis of microplate responses. When these biases are removed, it may be possible to associate *in vitro* metabolic diversity to phenomena *in situ*.

One problem faced by researchers using this technique is that the rate of color development in wells is a non-linear process depending on both time and inoculum density. As a result, color densities and/or binary data regarding substrate use patterns vary depending on the time of incubation following inoculation (Haack *et al.*, 1995). One way around this problem has been to read optical density (OD) of the microplate wells at a set time following inoculation or onset of color development in the microplate (Garland and Mills, 1991; Bossio and Scow, 1995). However, this is problematic as inoculum density variably affects the lag time from inoculation to onset of color development in any given well. Garland (1996) showed that the time to achieve a given average microplate well color development (AWCD) value was negatively correlated with inoculum density. Among the data analysis options he suggested were comparing samples' substrate use patterns when the plates had equivalent AWCD values, and normalizing each substrate's color density datum at a given time by dividing by that sample's AWCD at that time. While each technique was successful in discriminating his rhizosphere samples, he pointed out that the specific substrates contributing to the separation of sample types varied depending on duration of the incubation period prior to reading the plate. Faster developing wells were shown to be more influential in classifications based on plate readings after short incubations, and more slowly developing wells contributed more to sample separations after longer incubations. Haack *et al.* (1995) suggested that the kinetics of color development, rather than degree of development at a given time, could be used as data to assess the functional diversity of the organisms growing in the microplate.

We have used Biolog GN plates to detect differences in whole environmental samples from different field treatments and in axenic and mixed laboratory cultures.

Our approach avoided some of the pitfalls associated with single-time-point determinations by repeatedly measuring color density in each well over the period of several days. Our technique also allowed us to compare samples with different initial population levels by relying on analysis of kinetic parameters which are invariant with respect to inoculum density. We modeled the course of color development using a modified logistic equation and fit the data to the three parameters in this model. Patterns of potential C substrate metabolism were then analyzed using the kinetic parameters of the model, rather than the single-time-point color absorbance data commonly used. Each parameter provides an alternative to single-time-point optical density data for analysis by multivariate techniques. To allow comparison of our approach to previous methods, we analyzed our microplate data using the kinetic approach, AWCD-normalized single-time-point OD, and the method of comparing samples' ODs at equal AWCDs.

## MATERIALS AND METHODS

We used both well-characterized isolate cultures and soil suspensions as microplate inocula in the various experiments of this study. The first experiments used soil suspensions in time course measurements of test well OD to produce the color development curves for our kinetic model. A second set of experiments also used a soil suspension to determine cell growth in the Biolog microplate during color development. A third group of experiments used laboratory-grown isolates axenically or as a mixture to evaluate the effect of mixed cultures and inoculum density on our kinetic model's parameters.

### Microplate Data

All experiments reported here used Biolog GN microplates (Biolog, Inc., Hayward, CA) incubated at 21° C to assess multiple substrate metabolism. Absorbance data were collected at 595 nm using a Bio-Rad Model 3550-UV

microplate reader (Bio-Rad Laboratories, Hercules, CA). This wavelength was selected because it provides maximum absorbance for tetrazolium violet; however, turbidity from cell growth may also contribute to OD at this wavelength. To resuspend any cells or precipitate which had settled to the bottom of the well, each plate was shaken for 5 seconds 3 times prior to reading. Plates were read within one hour following inoculation and again repeatedly over the course of a week or two (in 8 to 24 h intervals depending on the experiment) to generate color development curves.

Environmental sampling

The soil samples used in the development of our kinetic model were collected from the Caribou-Poker Creeks Research Watershed (referred to as the "field site") located 48 km northeast of Fairbanks, Alaska. The sampling area, an open black spruce (*Picea mariana*) forest, is the site of an experimental crude oil spill which took place in February 1976 (Collins *et al.*, 1994). Samples were collected as soil cores in both the oiled area (OIL) and an adjacent, oil-free area (REF) and placed in coolers immediately following collection. The O horizon was separated from the A horizon (Brady, 1990), homogenized by sieving (2 mm mesh) twice, placed in sterile plastic bags, and refrigerated until further processing. Microplates were inoculated with these O horizon samples within 48 h of sampling.

Microplate inocula

Ten 5 g organic horizon soil samples from the study area were diluted 1000-fold in sterile saline solution (Ringer solution; Collins *et al.*, 1989). Thus tetrazolium dye reduction (color development) could be monitored with minimal interference from suspended and dissolved soil components. The original soil samples used as inocula had initial total bacterial populations (determined by direct count at Oregon State University's Soil Microbial Biomass Services Laboratory) ranging from $7x10^7$ to $7x10^8$ cells · g dry soil$^{-1}$. Final inoculum density was ca. $10^5$-$10^6$ cells · ml$^{-1}$. These samples

were used as microplate inocula in the first time course studies to produce the kinetic curves used to generate our model. To determine cell growth during color development in the microplates, we used a soil suspension from a single REF field sample ($9.05 \times 10^7$ cells $\cdot$ ml$^{-1}$ inoculum) in the second set of experiments.

Cultures of *Eschericia coli* (ATCC #25922; Difco, Detroit, MI) and *Enterobacter aerogenes* (ATCC #13048; Difco, Detroit, MI) were used as Biolog plate inocula to evaluate single isolate contributions to microplate well OD kinetics in the third group of experiments. These organisms were chosen for their rapid growth rates and because both enteric organisms would likely use many of the same substrates on the microplates. This would allow us to compare the kinetic parameters for each substrate metabolized by both isolates. Overnight flask cultures (nutrient broth; Difco, Detroit, MI) were harvested by centrifugation and resuspended in saline twice to wash residual nutrients from the cultures. These suspensions were used either as pure cultures or as a mixture of the two cultures as inocula in the Biolog plates. Inoculum density of axenic cultures and of the mixture was adjusted so that the total cell numbers were approximately equal for all treatments (ca. $4 \times 10^7$ cells $\cdot$ ml$^{-1}$).

To assess the effect of inoculum density on the parameters of the kinetic model, we used four densities of *E. coli* as inocula. After washing an overnight culture of this organism as described above, three dilutions of the final suspension were made. This provided inocula representing four orders of magnitude (ca. $2 \times 10^4$ - $10^7$ cells $\cdot$ ml$^{-1}$) of cell densities.

Kinetic analysis and model development

To evaluate dye reduction and color development following inoculation of the microplates, we used suspensions from five samples from each field site (OIL and REF) for the first set of experiments. These plates were read at approximately 8 hour intervals for one week following inoculation. From the time course change of each used substrate's OD at 595 nm, kinetic curves were determined to be sigmoidal in

shape, suggesting a density-dependent logistic growth curve. Using these data, a model based on a modified form of the logistic equation was developed (see Results).

Cell growth and color development

To determine the relationship between changing OD values in microplate wells and microbial densities, we enumerated populations of microorganisms growing on two of the substrates (β-hydroxy butyrate and L-glutamate) in the GN microplates over time in a second experiment. β-hydroxy butyrate and glutamate were selected as substrates representing two groups of compounds, a common microbial storage product and an amino acid. Glutamate has previously been shown to be a good representative substrate for measuring heterotrophic microbial activity (Griffiths *et al.*, 1977). Using a single REF soil suspension from the study site diluted 1000-fold, we inoculated 15 GN microplates. The plates were read at 24 hour intervals for 5 days following inoculation. After each reading, 100 μl of culture was removed from the β-hydroxy butyrate and L-glutamate wells in three of the plates not yet sampled, and placed in 900 μl of filtered (0.45 μm) formalin (1.8% v/v; Sigma Chemical Co., St. Louis, MO). Three 100 μl aliquots of the inoculum were collected and preserved in a similar manner prior to inoculating the plates to determine initial cell density.

The formalin-preserved samples were enumerated by acridine orange direct count epifluorescent microscopy (Hobbie *et al.*, 1977) as modified by Braddock *et al.* (1984). A minimum of ten fields per filter were counted unless fewer than 30 cells per field were observed, in which case more fields were counted until 300 cells were enumerated.

Optical density of every well in each of the plates in this experiment was determined at 24 hour intervals with the exception of the two wells in those plates which had already been sampled for microbial enumeration. Due to this destructive sampling, the mean OD for these two substrates was calculated using three fewer data

points for each successive sampling period (i.e., n=15 for initial and 24-h readings, n=12 for 48-h readings, etc.).

Evaluation of kinetic model

The reproducibility of the kinetic model parameters fit to the color development time course data was evaluated using laboratory-grown isolates in a third group of experiments. First, Biolog GN plates were inoculated in triplicate using equivalent cell densities of *E. coli*, *E. aerogenes* or mixtures of the two organisms in a 1:1 ratio. Inocula were prepared as described above. The plates were read immediately following inoculation and at eight time points over a period of 120 hours. Production of polysaccharides by *Enterobacter* species that may yield false positive microplate responses was checked by observing that the control well did not develop color (as recommended by the manufacturer; Biolog, 1992).

Another experiment used *E. coli* at several cell concentrations to evaluate the effect of inoculum density on the model parameters. We inoculated triplicate Biolog plates with four concentrations of inoculum and read the twelve plates immediately after inoculation and repeatedly over the course of the next 330 hours.

Data analysis

Raw OD data at a given reading time for every test well were corrected by subtracting that plate's blank well OD. Initial OD values for all wells in the plate ranged from ca. 0.250 to 0.450. Substrates with a final corrected OD less than 0.200 were omitted from the data set to be fit with the model. The corrected data for each substrate showing color development were fit to our kinetic model, and its parameters were estimated with the personal computer-based data analysis program, Origin (version 3.5; Microcal Software, Inc., Northampton, MA). This software uses the Levenberg-Marquardt algorithm and the simplex method for nonlinear least-squares curve fitting. A simple macro written in Origin script language was used to fit the time

course OD data set for each well in the plate and produced estimates of the model parameters and their standard errors along with an estimate of the goodness of fit ($\chi^2$). Using a Pentium processor-based personal computer, data for 95 test wells from a given microplate could quickly be fit to our model (ca. 3 minutes per plate). Curve parameters with standard errors larger than the parameter value were taken as evidence of a bad fit and the kinetic data for these substrates were not used. Only about 5% of the curves generated for the environmental samples (45 of 824) were excluded from further analysis. All parameters used in the analysis came from fit curves with $\chi^2$ values less than 0.01. Each kinetic parameter was then used to construct a data matrix for calculation of principal components (PCs). Thus a kinetic parameter from the model fit to the time course data for each substrate metabolized was used in place of the single-time-point datum commonly used in evaluating substrate use patterns. Separate sample analyses were performed using each of the three model parameters as data.

To compare our kinetic data analysis results with the single-time-point approach typically used in community-level substrate metabolism studies, we used two other methods to prepare our microplate data for analysis. Average well color development (AWCD; Garland, 1996) data were calculated for each sample at each reading time, and time points providing equivalent microplate AWCDs for each sample were selected from the data sets for laboratory culture and environmental inocula. The first comparative analysis used blank-corrected OD data from sample microplates measured at equivalent AWCDs. Negative corrected OD data were set to zero for all single-time-point data analyses. Sample AWCDs at the selected reading times were determined to be not different ($p > 0.10$) by testing for significant differences among samples with a one-way analysis of variance using each plate's 95 substrates' OD data as input. The second comparative data treatment involved normalizing each sample's single-time-point OD datum by dividing it by the sample's AWCD at that time. Both

OD data types were then subjected to the same multivariate analysis as the kinetic parameter data.

Principal components analysis (PCA) of both the single-time-point OD and kinetic data's correlation matrices and other statistical analyses were performed using Systat software (version 5.05; SPSS, Inc., Chicago, IL).

## RESULTS

Kinetic model

The first set of experiments showed test well dye reduction was non-linear in microplates inoculated with the soil samples, and the shape of the color development curve was generally sigmoidal (Fig. 1-1). Test well OD values over time suggested a kinetic model based on the density-dependent logistic growth equation of the general form

$$N(t) = \frac{K}{1 + be^{-rt}} \qquad \text{(Equation 1; Ricklefs, 1990)}$$

to describe a population of individuals, N(t), at time *t* exhibiting density-dependent growth under conditions of environmental constraint. In this equation, the exponential rate of population change is determined by the exponent *r* and is expressed in units of reciprocal time. *K* represents the "carrying capacity" of the system, or asymptote approached by the curve. The unitless coefficient *b* affects the horizontal displacement of the curve.

We modified Equation 1 to a form providing parameters which could be more readily interpreted with respect to the shape of the OD kinetic curve and to the underlying microbiological behavior driving its shape (Equation 2).

$$y = OD_{595\ nm} = \frac{K}{(1 + e^{-r(t-s)})} \qquad \text{(Equation 2)}$$

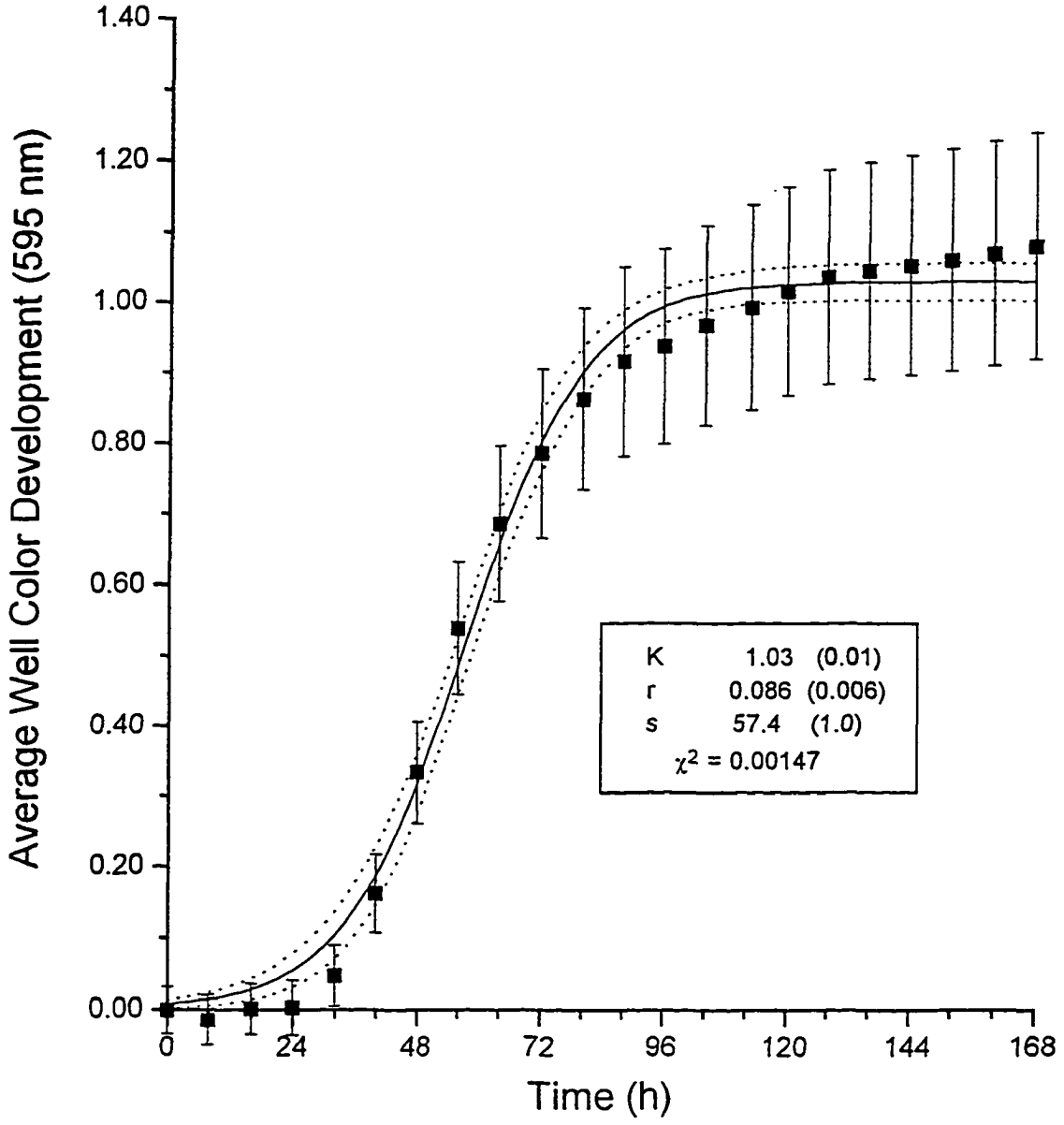


Fig. 1-1. Kinetics of average well color development for an oil-free (REF) environmental sample. Absorbance values (■) are the mean of 95 substrate well absorbances corrected by subtracting reference well absorbance at each time point. Error bars are standard errors. The solid line (——) is a plot of the equation fit to the mean absorbance data and the dotted lines (·····) represent upper and lower 95% confidence limits for the fit equation. Kinetic model *K*, *r* and *s* parameter data for the fit equation are presented (standard errors in parentheses) along with the $\chi^2$ value of the fit to the absorbance data.

In this equation, $K$ represents the asymptote ($y = K$) that the test well OD curve approaches, $r$ determines the exponential rate of OD change, and $t$ is the time following inoculation of the microplate. We have included Equation 1's coefficient $b$ as the exponential parameter $s$ in the denominator of Equation 2. The parameter $s$ is the time to the midpoint of the exponential portion of the curve (when $y = K/2$). The value of $s$ is expressed in units of time and is related to $b$ in Equation 1 as

$$s = \frac{\ln b}{r}, \text{ or } b = e^{rs} \qquad \text{(Equation 3).}$$

Cell growth and color development

Microbial growth during OD change in two microplate substrate wells (β-hydroxybutyric acid and L-glutamic acid) for the second experiment is presented in Figure 1-2. Cell numbers changed linearly with OD ($r^2$=0.70 for β-hydroxybutyric acid and $r^2$=0.90 for L-glutamic acid; n=12) from the initial values at inoculation (9.05 x $10^7$ cells · $ml^{-1}$) through the third sampling (i.e., prior to decrease in rate of color change). Beyond the time when OD change began to level off, little change in microbial population was observed. The kinetic model fit to the OD data for the two substrate wells yielded $r$ parameter values of 0.135 and 0.172, respectively, for β-hydroxybutyric acid and L-glutamic acid. When we fit our kinetic model to the direct count data, the $r$ parameter values were lower than those derived from OD data ($r$ = 0.069 and 0.139 for β-hydroxybutyric acid and L-glutamic acid, respectively).

Effect of inoculum density on kinetic model parameters

The triplicate plates inoculated with washed cells of *E. coli* at cell concentrations ranging over four orders of magnitude demonstrated the stability of the model's $K$ and $r$ parameters with changing inoculum density in the third experiment. The values for

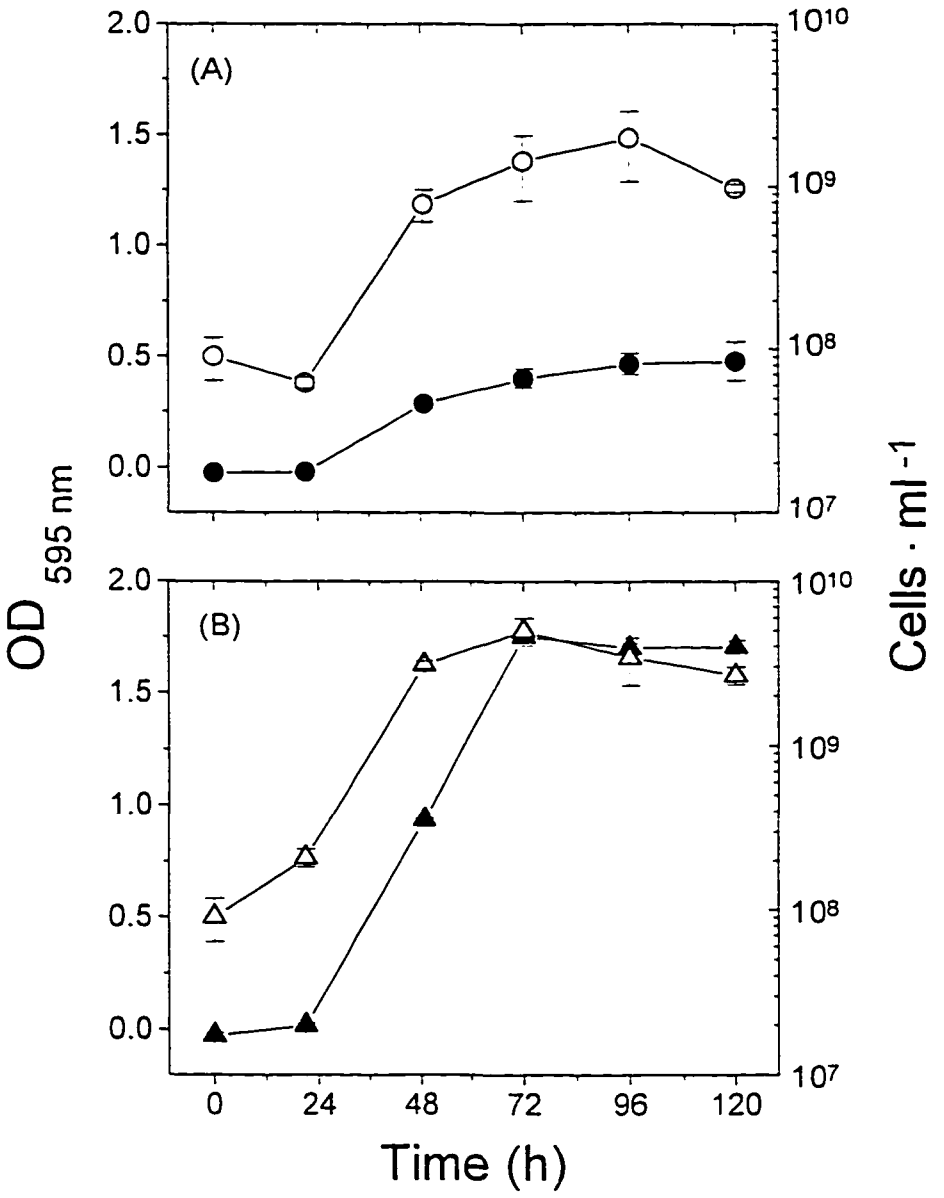


Fig. 1-2. Kinetics of color change ($OD_{595}$) and population increase (cells · $ml^{-1}$) in two Biolog GN microplate substrate wells. Inoculum biomass was 9.05 x $10^7$ cells · $ml^{-1}$. OD data for (A) β-hydroxybutyric acid (●) and (B) L-glutamic acid (▲) are presented as mean corrected absorbance of replicate plates not yet sampled for cell enumeration (n=15 at 0 h and 24 h, n=12 at 48 h, n=9 at 72 h, n=6 at 96 h, and n=3 at 120 h) in this experiment. Cell number data (O - β-hydroxybutyric acid; Δ - L-glutamic acid) are presented as the mean of replicate samples (n=3) removed from the plates at each time indicated. Error bars are standard errors.

the *s* parameter generally decreased with increasing inoculum density. The general behavior of the kinetic model's parameters with changing inoculum density can be seen in plots of average well color development (AWCD) over time for the four sets of plates (Fig. 1-3). Each plot in Figure 1-3 presents the mean OD change over time in the 285 blank-corrected test wells of the three replicate plates. Similar results were seen in the OD kinetic data for each individual substrate used. The values of *K* and *r* for each metabolized substrate and their coefficients of variation (CV) were calculated for all twelve plates in the experiment to assess the invariance of these parameters with changing inoculum density. The mean CV (± s.e.) for all substrates across four orders of magnitude of inoculum density was 0.11 (± 0.01) for *K* data and 0.20 (± 0.02) for *r* data.

Spearman rank correlation coefficients ($r_S$; Zar, 1984) were calculated to determine the relationship between inoculum density and the value of the kinetic parameters for each metabolized substrate. Correlation coefficients ($r_S$) for *K* values appeared to be randomly distributed among the substrate variables, ranging from -0.605 to 0.777 with a mean $r_S$ (± s.e.) of 0.217 (±0.054); $p > 0.5$. The *r* parameter correlation values ranged from -0.648 to 0.820 with a mean $r_S$ (± s.e.) of 0.000 (±0.068; $p > 0.5$), also indicating no correlation with inoculum density. The value of the model's *s* parameter, on the other hand, was negatively correlated with the initial cell number in the inoculum. All but two substrates had *s* values negatively correlated with initial cell density. The range of correlations for *s* with inoculum density was from 0.259 to -0.972, with a mean Spearman rank correlation coefficient (± s.e.) of -0.747 (±0.043); $p < 0.001$.

Data ordination using single-time-point OD data or kinetic parameters

Principal components analysis was used to detect differences between sample types in the pure culture experiments as well as with the field site samples. Single-time-point OD data from plates at equivalent AWCD values, AWCD-normalized OD

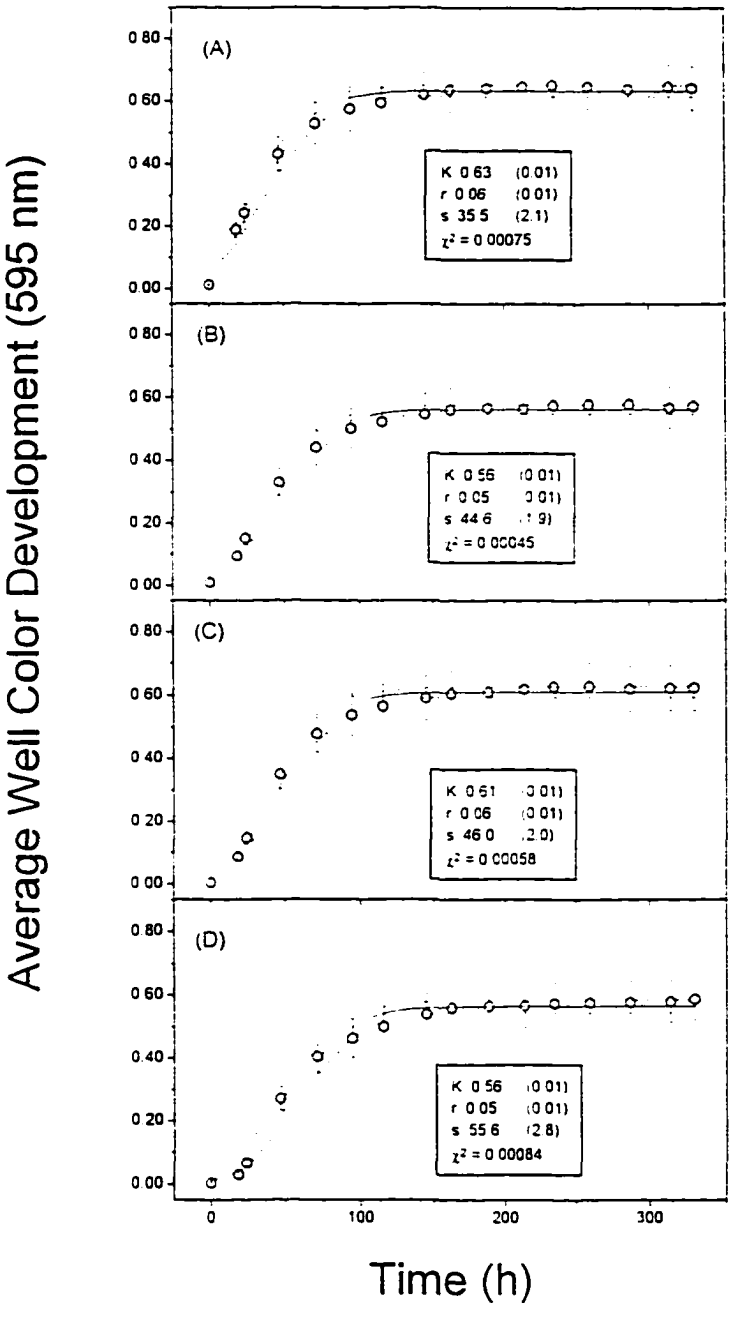


Fig. 1-3. Kinetics of average well color development (O ) for suspensions of *E. coli* at four inoculum densities: (A) 2 x $10^{7}$ cells · $ml^{-1}$, (B) 2 x $10^{6}$ cells · $ml^{-1}$, (C) 2 x $10^{5}$ cells · $ml^{-1}$, and (D) 2 x $10^{4}$ cells · $ml^{-1}$. The solid line (———) is a plot of the equation fit to the mean absorbance data and the dotted lines (·····) represent upper and lower 95% confidence limits for the fit equation. Kinetic model *K*, *r* and *s* parameter data for the fit equation are presented (standard errors in parentheses) along with the $\chi^2$ value of the fit to the absorbance data for each curve.

data at various incubation times, and kinetic parameter values from our model fit to the OD time-course data were used in this analysis. Each substrate (variable) from a microplate generated three kinetic variables ($K$, $r$ and $s$; see Equations 2 and 3) which were used individually to calculate principal components.

Principal component plots derived from the pure culture experiment OD data are shown in Figure 1-4. The component scores calculated using single-time-point OD data from plates with equivalent AWCDs (0.2 and 0.3), using AWCD-normalized OD data at 24, 48 and 72 h, and using each kinetic parameter ($K$, $r$ and $s$) for the laboratory-grown cultures are plotted for the first and second PCs in this figure. In all analyses using the laboratory cultures, the first two PCs explain at least 80% of the data variance. The percentage of variance explained by each PC plotted is displayed on each plot's axes. Plates with AWCD values of 0.2 and 0.3 generated component scores providing separation of pure cultures along the first principal axis (Fig. 1-4A and B). At AWCD of 0.2, separation of the *E. aerogenes* data from the data for the 1:1 mix of cultures was achieved along the second component axis (Fig. 1-4A), while this did not occur at AWCD of 0.3 (Fig. 1-4B). Ordination of the AWCD-normalized single-time-point OD data (Fig. 1-4C, D and E) also separated the pure cultures along the first principal component axis, though only the 48 and 72-hour normalized data sets discriminated the pure culture samples from the mixed cultures. The $K$, $r$ and $s$ kinetic parameter data (Fig. 1-4F, G and H) all resulted in separation of the axenic cultures of *E. coli* and *E. aerogenes* from each other as well as from the 1:1 mix.

A similar analysis was applied to the microplate data generated by soil suspensions from the field site. Single-time-point OD data at a microplate AWCD value of 0.8, AWCD-normalized data at 48, 72 and 96 h, and the three kinetic model parameters were used to compute PCs for the environmental samples. In general, the first two PCs explained much less of the variance in these data than for the laboratory culture experiments presented in Figure 1-4. Data ordination plots of the first two principal components for the various data treatments are presented in Figure 1-5 along

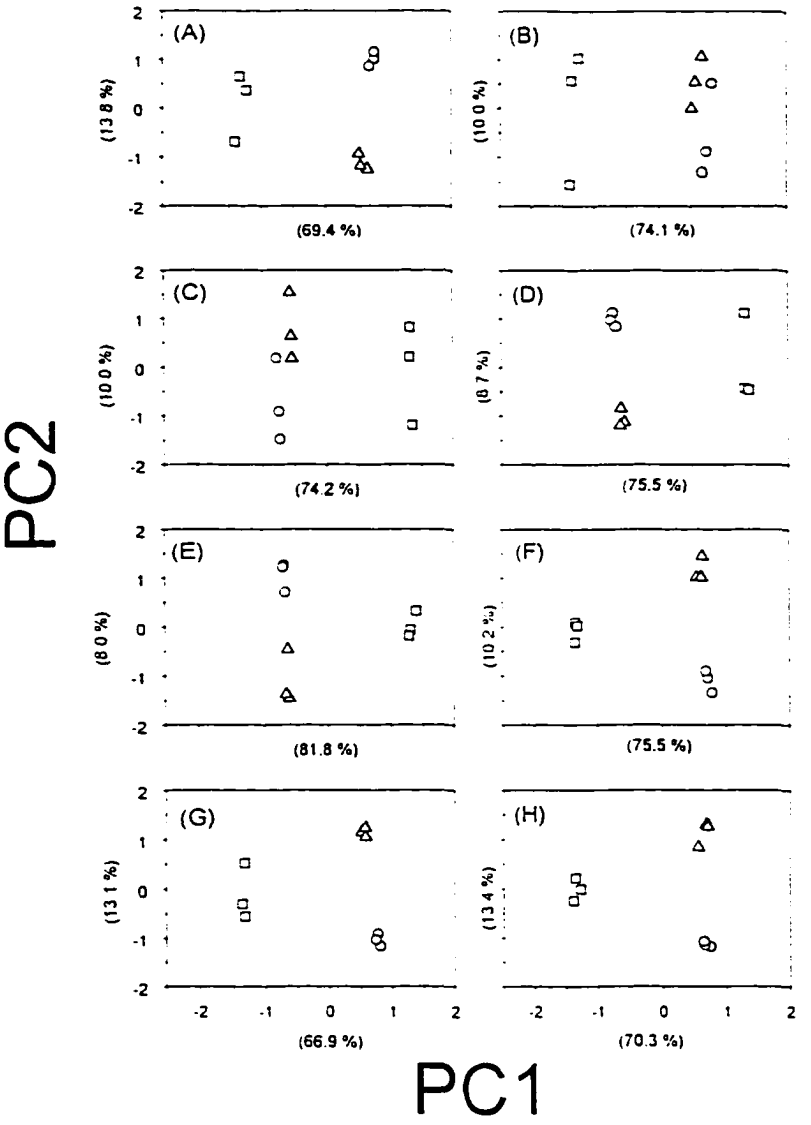


Fig. 1-4. Ordination produced from laboratory culture microplate OD data using single-time-point OD values or kinetic parameters from time course OD data fit to the logistic model. Component scores for *E. coli* (□ ), *E. aerogenes* ( O ), and a 1:1 mix of the two cultures ( Δ ) are plotted for first two principle components calculated using correlation matrices constructed from each metabolized substrate's datum. Percent variance explained by each PC axis is given in parentheses. (A) principal component (PC) scores determined for OD data when microplate AWCD = 0.2. (B) scores for OD data when AWCD = 0.3. (C) scores for 24 h AWCD-normalized OD data. (D) scores for 48 h AWCD-normalized OD data. (E) scores for 72 h AWCD-normalized OD data. (F) principal component scores calculated from logistic model $K$ parameter values. (G) scores calculated from model $r$ parameter values. (H) scores calculated from model $s$ parameter values.

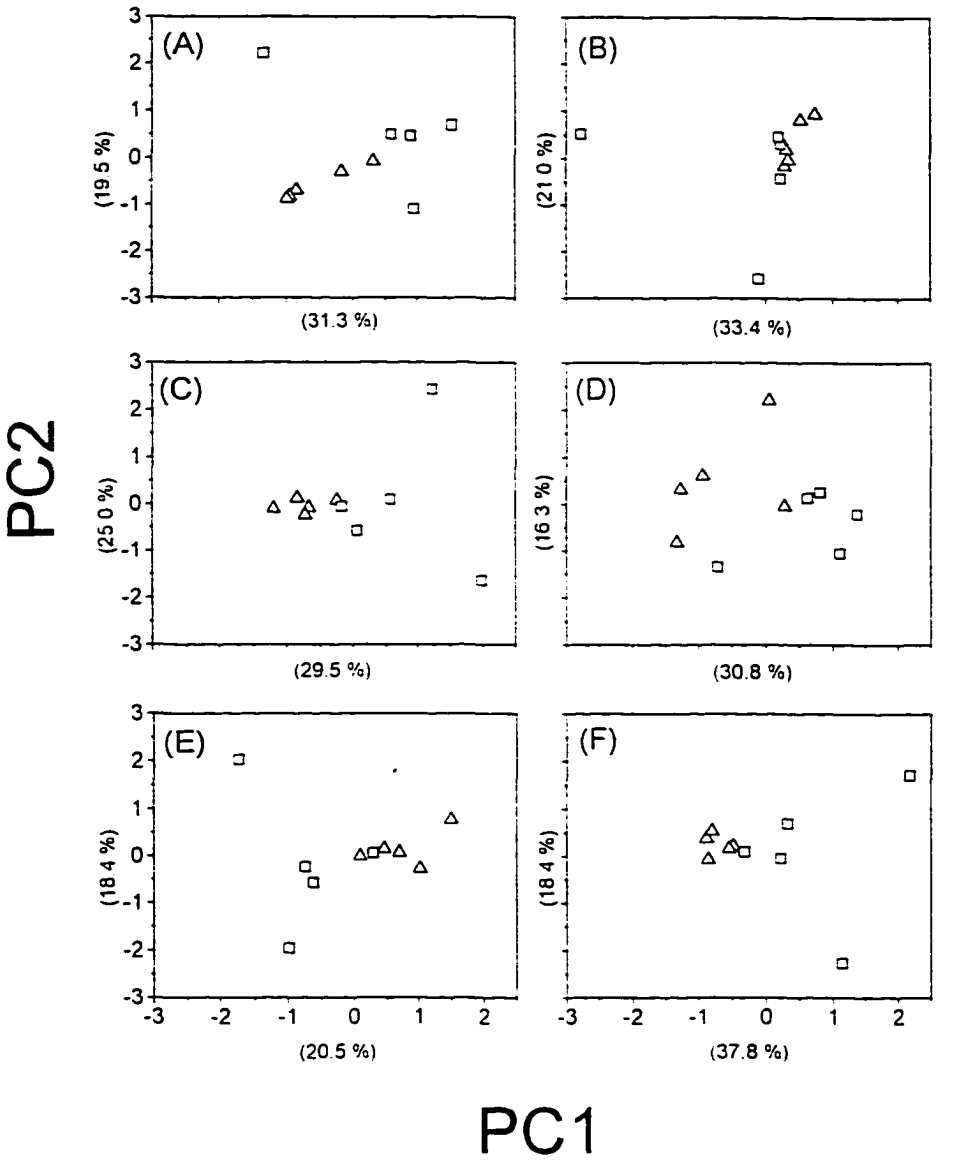


Fig. 1-5. Ordination produced from field site soil suspension microplate OD data using single-time-point OD values or kinetic parameters from time course OD data fit to the logistic model. Component scores for oiled (OIL, □ ) and oil-free (REF,Δ) environmental sample soil suspensions are plotted for first two principal components calculated from correlation matrices constructed from each metabolized substrate's datum. Percent variance explained by each PC axis is given in parentheses. (A) component scores determined for OD data when microplate AWCD = 0.8, (B) scores for 72 h AWCD-normalized OD data, (C) scores for 96 h AWCD-normalized data, (D) scores calculated from logistic model *K* parameter values, (E) scores calculated from model *r* parameter values, (F) scores calculated from model *s* parameter values.

with the percent variance explained by each PC. Separation of oiled (OIL) and oil-free, reference (REF) samples along the first PC axis was generally successful for the OD data set taken at an AWCD of 0.8, with the exception of a single OIL sample (Fig. 1-5A). Normalizing the OD data through division by AWCD failed to discriminate OIL and REF samples at 48 h and 72 h (72 h AWCD-normalized OD data shown in (Fig. 1-5B). PCA of 96 h AWCD-normalized data resulted in separation of OIL and REF samples along the first PC axis (Fig. 1-5C). PCA of kinetic parameter $K$ and $r$ data was generally successful at separating oiled and oil-free, reference samples with the exception of a single OIL sample in each case (Fig. 1-5D and E). Use of the $s$ parameter data separated OIL and REF samples along the first PC (Fig. 1-5F).

## DISCUSSION

The use of multiple substrate microtiter plates to evaluate community functional diversity has until now relied upon determination of substrate well color development at a single time point. The decision regarding the proper time to make this measurement in various studies has been based on differing criteria. These include achievement of a given color development threshold relative to the well with maximum absorbance on the plate (Haack *et al.*, 1995) and reading at a set time following onset of color development (Bossio and Scow, 1995). Efforts to normalize the single-time-point OD data using AWCD values appear to succeed in correcting for biomass differences to the extent that the rate of reaching a given AWCD correlates with biomass (Garland, 1996). However, the relative contribution of any substrate to a pattern of potential C source use characteristic of a given sample depends on the time chosen to evaluate substrate use. Differences among rapidly metabolized substrates dominate principal component scores generated after short incubation times, while their contribution to principal components is eclipsed by differences in more slowly metabolized substrates after longer incubation periods. For example, at 48 h and 72 h, AWCD-normalized data failed to discriminate OIL and REF samples, while 96 h

normalized data succeeded (see Fig. 1-5), suggesting that AWCD-normalized substrate use patterns of the sample types diverged after 72 hours.

Concerns over the effect of inoculum density on rate of color development are founded on the observation that a denser inoculum typically produces a positive well response more quickly than a less dense culture. Garland (1996) demonstrated that the time following inoculation to achieve a given AWCD value was inversely proportional to the inoculum density. Using our kinetic technique to fit the time course of OD change showed that this is an artifact of a shorter "lag" period in test well color development for denser inocula. The average rate of color change from inoculation to reading time includes this lag period, causing less dense cultures to exhibit an apparently slower rate of dye reduction. Thus, the rate of OD change from the time of inoculation to a given AWCD (as opposed to the rate from an OD of zero to AWCD), and therefore the AWCD-normalized substrate use pattern, depends on the inoculum biomass and not its functional metabolic diversity or "taxonomic richness".

Fitting the kinetic model to the color development data removed the effect of the lag period, and the actual rate of color change (from zero absorbance to $K$) was determined. This technique yielded color development rates that were insensitive to inoculum density in a range up to four orders of magnitude lower than suggested in the microplate manufacturer's standard protocol (Biolog, 1992) and that were sensitive to the community composition in the well. Two of the kinetic parameters ($K$ and $r$) do not vary with inoculum density and, subject to the limitations discussed below, reflect the composition of the microbial assemblage in each test well. Because the kinetic approach uses all the time course OD data, rather than individual sampling times (either equivalent AWCD set points or AWCD-normalized data), the relative contributions of substrates (variables) to characteristic potential C source utilization patterns do not vary with sampling time or inoculum density. The model parameters provide at least three data options (parameters $K$, $r$ and $s$) for inclusion in substrate-use analysis schemes.

The parameter $K$ in our model is most similar to the data currently used in multiple substrate metabolism analysis as both are measures of optical density. It represents the asymptote that the modeled absorbance curve approaches and may be viewed as an estimate of the maximum potential extent of dye reduction seen in a given test well for a particular inoculum. The parameter $r$, an estimate of the exponential rate of color development, provides information about a different aspect of the same community in the test well, i.e., how rapidly reducing power is generated in the well. The inverse relationship of $s$ with inoculum density suggests that, in general, this may not be a useful descriptive parameter for environmental samples since the inoculum biomass is likely to vary among samples. The fact that $s$ increases with decreasing inoculum density while the actual rate of color change (from zero absorbance to $K$) remains constant (see Fig. 1-3) is consistent with Garland's (1996) observation that time to a midpoint in the AWCD for his rhizosphere samples was negatively correlated to biomass. This implies there is a minimum active biomass threshold necessary for dye reduction to be detected. Thus growth rate and induction of the necessary catabolic enzymes likely play roles in the lag period prior to onset of color development in the wells.

$K$ and $r$ appear to be constant over a range of inoculum densities, but their values for a metabolized substrate may depend both on abiotic and biological factors. One abiotic factor that could affect $K$ is complete reduction of the tetrazolium redox dye prior to nutrient limitation. In the absence of abiotic limits to $K$, the traditional biological interpretation of $K$ as "carrying capacity" is possible. Considering each microplate well as a 150 µl batch culture, $K$ represents a biologically imposed limit on dye reduction in the wells. Such biological factors as efficiency of reducing power production (affecting reduced tetrazolium yield per cell) or cell growth, exhaustion of substrate or mineral nutrients, temperature, oxygen limitation, accumulation of waste products, or production of secondary metabolites may affect the values of $K$ and $r$. Metabolite production may allow growth of organisms unable to grow on the well's

designated C source, or may inhibit organisms otherwise able to grow. Thus, $K$ and $r$ will be affected by synergistic or antagonistic effects due to the mix and relative abundance of culturable organisms in the test wells. In other words, $K$ and $r$ are constants at a fixed temperature that depend on the taxonomic richness (metabolic diversity) of microplate-culturable organisms and their relative abundance, not on inoculum density *per se*. Evidence of this is seen in the separation of laboratory-grown cultures (Fig. 1-4) and, to a lesser extent, environmental samples based on $K$ and $r$ parameter data (Fig. 1-5).

Our curve fitting technique fits closely the time course OD data for microplate test wells and is useful for extracting kinetic parameter data that reflect the response of culturable organisms in the microplate inoculum. The resemblance of the model to a batch culture growth curve, however, does not imply that strict physiological interpretations may be ascribed to its parameters. For example, Figure 1-2 displays field sample OD and population kinetics for two microplate substrates whose microbial growth rate constants were substantially lower than $r$ values calculated for the OD change in the wells. This may be due to differences in cell yields of the dominant organisms selected by the substrate in each test well. Also, since color change depends on production of reducing power in the microplate well, microbial energy spilling reactions not tied to ATP production, cell growth or maintenance (Tempest and Neijssel, 1987) may provide dye reduction rates ($r$) or extents ($K$) above those directly attributable to microbial growth.

Our experience with environmental samples and laboratory cultures suggests that plates' ODs should be read repeatedly for as long as one week following inoculation. This allows more slowly developing test wells the opportunity to achieve their ultimate OD ($K$ value). Longer incubation periods may be useful for less dense inocula than were used in this study (i.e., less than ca. $10^4$ cells $\cdot$ $ml^{-1}$), but care should be taken to inhibit evaporation of liquid from the wells causing increased OD values. Frequent

plate readings (i.e., at intervals less than 24 h) tend to provide data yielding better fits (lower $\chi^2$ values) when model parameters are estimated.

The primary drawback of our approach is that the construction and analysis of data matrices based on our model's kinetic parameters entails more data collection work than the single-time-point data approach. Frequent readings over the course of several days need to be compiled to provide time course OD data for each substrate. The kinetic model needs to be fit to the data and model parameters estimated. The kinetic parameter data then need to be screened for the quality of their fit to the time course OD data (based on standard errors of estimates and $\chi^2$ values). Only after these several steps are the data ready to be analyzed by multivariate techniques. Despite the extra work involved, however, we believe the benefits of this approach outweigh the drawbacks. This method removes concerns regarding variable inoculum densities in environmental samples. Our technique provides objective data parameters for community analysis and frees investigators from the need to choose the AWCD values for comparing microplates or the time at which single-time-point data are normalized. Thus, the contribution of test substrates to a sample's C source metabolism pattern is no longer an artifact of the incubation period or inoculum density. The kinetic approach provides a method for gathering data from multiple substrate microtiter plates which is reproducible and amenable to data analysis techniques currently used in multiple substrate metabolism studies. Future work using kinetic data analysis may illuminate further the precise relationship between population dynamics, microbial physiology and dye reduction in these microplates.

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# REDUCED POPULATION DIVERSITY IN SOIL MICROBIAL COMMUNITIES 19 YEARS AFTER A CRUDE OIL SPILL‡

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## SUMMARY

Terrestrial petroleum spills commonly have been shown to increase the proportion of heterotrophic microbes in the soil community able to metabolize hydrocarbons. Previous studies have demonstrated the potential utility of multiple substrate metabolism analysis to fingerprint microbial communities, and recent methodological improvements have provided new options for extracting and analyzing microplate data. Assessing microbial community diversity has remained an elusive goal, however, as complex population interactions occur in microplate wells. This has precluded evaluation of community population diversity based on substrate use patterns alone since these patterns may arise from a variety of population interactions in the microplate. We report here evidence of diminished population diversity in soil communities at a taiga site exposed to crude oil for 19 years based on observed changes with dilution in microplate kinetic response patterns. The microplate data also suggest that surviving populations in the oiled soils may be considered physiological generalists.

‡ Prepared for submission to *Soil Biology and Biochemistry*

# INTRODUCTION

Microbes frequently have been used as indicator organisms to examine environmental conditions otherwise difficult to assess (e.g., fecal coliforms indicating fecal contamination, APHA 1989; petroleum-oxidizing bacteria indicating exposure to hydrocarbons, Braddock *et al.*, 1996). In addition to measuring densities of indicator microbial taxa, evaluating microbial diversity and community structure, rather than numbers of organisms *per se*, may be used to assess environmental perturbation or stress (e.g., Atlas, 1984b; Hood *et al.*, 1975). Microbial diversity may be considered from a variety of perspectives. As stated by DeLong (1996), "trophic, physiological or functional diversity, intraspecific genetic diversity, or phylogenetic diversity of species or higher taxa" are all levels of diversity of concern to the microbial ecologist. Observed community-level functional diversity may be due to the activity of a diverse assemblage of specialized populations or that of a few populations possessing a broad array of capabilities. Kawanabe (1996) proposed that the diversity of ecological relationships among life forms (e.g., competition, cooperation, etc.) is a more important part of biodiversity than "simply the diversities among creatures." This suggests that synergistic and competitive interactions are a critical feature of community functional diversity (Atlas, 1984b). Microbial community structure changes and diversity generally decreases as a response to chemical (e.g., pollution; Mills and Wassel, 1980; Atlas, 1984a; Atlas *et al.*, 1991; Fritze and Bååth, 1993) and physical stresses (Holder-Franklin *et al.*, 1978; Bell *et al.*, 1982). Changes in relative abundances of organisms due to pollution should yield altered interactions among community member populations and be reflected in a shift in the community functional diversity (Atlas, 1984a).

The role soil microbial diversity may play in ecosystem function depends on the scale (i.e., microbial, process, landscape, or global) at which the ecosystem is examined, as well as whether the function examined is considered as a "broad" process (e.g., C mineralization) or a more specialized "narrow" process (e.g., cellulolytic

activity; Schimel, 1995). For processes requiring specialized physiologies, reduced microbial functional diversity may have significant consequences for an ecosystem's behavior. In the context of hydrocarbon pollution, for example, Wünsche *et al.* (1995) suggested that successful bioremediation depends in part on the composition of microbial communities and their degradative potential. Salonius *et al.* (1970) pointed out that populations of soil microbes have been shown capable of synergistic decomposition of soil substrates, but capable of much less activity when acting individually, emphasizing the role of integrated metabolisms in processing soil matter. Even ecosystem functions not considered to be specialized (e.g., litter decomposition) may suffer the effects of decreased microbial diversity due to the absence of key taxa that represent substrate processing "bottlenecks" (Clein and Schimel, 1994; Schimel, 1995). Salonius (1981) showed that forest soil populations of artificially reduced diversity were "considerably diminished" in metabolic capability (respiration measured as $O_2$ uptake) compared to full soil populations. This was attributed to a reduction in the variety of enzymes generated by a community of reduced species diversity.

The response of a soil microbial community to crude oil pollution represents the integration of the reactions of individual organisms and populations. The addition of oil into the soil system often enhances the hydrocarbon-oxidizing potential of the community (Atlas, 1981). This community adaptation for hydrocarbon mineralization may be due to induction or repression of specific enzymes, genetic changes yielding new metabolic capacities, and/or selective enrichment of component populations able to transform the compound of interest (Leahy and Colwell, 1990). The various chemical components of the oil may inhibit, enhance or have no effects on growth or metabolism of different community member populations (Pfaender and Buckley, 1984). Individual organisms can metabolize only a limited range of hydrocarbon substrates and biodegradation of the complex chemical mixture found in oil appears to require assemblages of mixed populations together possessing broad enzymatic abilities (Leahy and Colwell, 1990).

While there have been several studies documenting an observed increase in hydrocarbon-degrader populations (e.g., Sexstone and Atlas, 1977) and hydrocarbon mineralization potentials (e.g., Jobson *et al.*, 1974) following petroleum soil pollution, fewer studies have investigated the broader effects on population structure and community-level metabolism. Walker *et al.* (1975) found that sediments contaminated by crude and refined oils exhibited shifts in functionally-defined (e.g., proteolytic, lipolytic, chitinolytic) populations. In a study of community characteristics in petroleum-contaminated and pristine aquifer soils, exposure to petroleum altered microbial community structure (Long *et al.*, 1995). These investigators found that petroleum contaminants exert toxic effects on the active soil microbial community at high concentrations, while enrichment of specific degraders occurred at lower concentrations of dissolved contaminants. Chemical pollutants have been implicated in reduction of microbial taxonomic and genetic diversity in environmental samples, with evidence that surviving populations had enhanced physiological tolerances and substrate use capabilities (Atlas *et al.*, 1991). Wünsche *et al.* (1995) found that hydrocarbon addition to soils resulted in shifts in substrate use patterns, as well as changes in the occurrence of specific bacterial groups in the soils. Little information exists regarding community-level oil pollution effects and the relationship between organismal and community functional diversity.

Microtiter plates (Biolog) have been used to measure community multiple substrate utilization profiles at a set time following inoculation to characterize or "fingerprint" soil microbial communities (e.g., Winding, 1994; Haack *et al.*, 1995; Wünsche *et al.*, 1995). Despite the fact that this technique relies on growth of microbes from environmental inocula and suffers some of the biases of other culture-based methods, it has been used to document changes in profiles correlated with environmental influences (Zak *et al.*, 1994; Bossio and Scow, 1995). We recently have developed a method for evaluating substrate use kinetics over time in these microtiter plates that yields profiles reflecting the integrated metabolisms of community member

populations inoculated into the microplate without unduly weighting individual substrates based on how quickly they are metabolized following inoculation (Lindstrom *et al.* in press; see Chapter 1). It has been suggested that using microplates to determine community-level multiple substrate use patterns provides data that "are rich in information about functional biodiversity of bacteria" (Zak *et al.*, 1994). While this may be true, microplate substrate use patterns determined at a single time and inoculum density do not allow assessment of the influence of a soil community's structure on its functional diversity. As suggested above, acclimation of a community exposed to hydrocarbons may be the result of changes in the genetic expression of individual populations, of selective enrichment of community member populations, or of some combination of these factors.

In this study of a nearly 20-year-old experimental oil spill, we wished to determine if oiled taiga soils exhibited distinctive substrate use patterns compared with pristine reference soils, and whether differences in community substrate use patterns could be related to differences in diversity of bacterial types. This entailed the use of multivariate analysis of multiple substrate microplate kinetic data coupled with serial dilution of environmental samples to compare substrate use patterns of oiled and pristine taiga soils at various dilutions and across the growing season. We present here evidence of decreased organismal diversity associated with changes in community-level substrate use in oil-polluted soils.

## MATERIALS AND METHODS

### Site Description

The soil samples used for microplate inocula in this study were collected from the Caribou-Poker Creeks Research Watershed located 48 km northeast of Fairbanks, Alaska. The sampling area included the site of a 7570 L experimental crude oil spill that occurred in February 1976 (Johnson *et al.*, 1980). The vegetation at the site is an open black spruce (*Picea mariana*) forest (total tree canopy cover less than 60%) with

a shrub understory of Labrador tea (*Ledum decumbens*), blueberry (*Vaccinium uliginosum*), resin birch (*Betula glandulosa*) and willow (*Salix* spp.). The ground surface is covered with mosses and fruticose lichens with scattered cotton grass tussocks (*Eriophorum vaginatum*; Johnson *et al.*, 1980). Spilled oil generally flowed between the tussocks, killing the moss and lichen surface cover. The area is underlain by permafrost, with an active layer thickness of 40-60 cm. A typical soil profile in the unoiled area consists of a 5 cm moss and lichen surface layer above a 15 cm undecomposed peat horizon (O1), a 5 cm decomposed dark brown peat horizon (O2), a 5 cm dark grayish-brown silt loam horizon (A1), and a grayish-brown silt loam mineral soil (C; Collins *et al.*, 1994). Oiled area soil samples contained substantial residual oil (Collins *et al.* 1994; average concentration of 0.285 g oil · g dry soil$^{-1}$, see Chapter 3) and had no living plant matter on the surface. All organic material above the C horizon in these samples was used in the study.

Sample Collection and Preparation

Sampling for this study took place on three visits to the site in 1995: in early July when soil temperatures were above freezing and vegetation at the site was growing; in early September after senescence of local vegetation, but before the first hard frost; and in mid-October, after several days of below freezing air temperatures and with shallow subsurface (10 cm) soil temperatures near freezing. Twenty-eight samples were collected as soil cores from an oiled area (OIL, N=13; n = 3 for July, n = 5 for September, and n = 5 for October) approximately 10 m by 16 m, and from an adjacent, oil-free reference area (REF, N=15; n = 5 for each sampling visit) approximately 10 m by 20 m. Samples were collected through the moss/lichen layer, between *E. vaginatum* tussocks, meters apart at randomly selected locations within the sampling areas and placed in coolers immediately following collection. The O2/A1 horizon was separated from the C horizon in the laboratory, homogenized by sieving (2 mm mesh) twice,

placed in sterile plastic bags, and refrigerated until further processing. Microplates were inoculated with the samples within 48 h of sampling.

Five g soil samples from the study area were serially diluted $10^3$-, $10^4$-, and $10^5$-fold (w/v) in sterile saline solution (Ringer solution; Collins *et al.*, 1989). The original soil samples used as inocula had initial total bacterial populations (determined by direct count at Oregon State University's Soil Microbial Biomass Services Laboratory) ranging from ca. $8x10^7$ to $6x10^8$ cells · g dry $soil^{-1}$. Final inoculum density was therefore ca. $10^5$ - $10^6$ cells · $ml^{-1}$ for the $10^{-3}$ dilution.

Hydrocarbon Degrading Microbes

Numbers of hydrocarbon degrading microbes in the soil samples were estimated by the most probable number (MPN) "Sheen Screen" technique of Brown and Braddock (1990). Samples were diluted in C-free mineral nutrient broth and sterile crude oil was added to provide a carbon source for microbial growth. After incubation for three weeks at 21°C, oil degrader presence or absence in each replicate tube of the MPN array was determined by observing whether or not emulsification of the added oil had occurred.

Microplate Data

We used Biolog GN microplates (Biolog, Inc., Hayward, CA) incubated at 21° C to evaluate the kinetic responses of our soil suspensions to the substrates presented. Raw absorbance (OD) data were collected at 595 nm using a Bio-Rad Model 3550-UV microplate reader (Bio-Rad Laboratories, Hercules, CA). Plates were read within one hour following inoculation and again repeatedly over the course of 7 to 10 days (in 8 to 24 h intervals) to generate color development curves for each microplate substrate. Raw OD data at a given reading time for every substrate well were corrected by subtracting that plate's blank well OD. Initial OD values for all wells in the plate ranged from ca. 0.250 to 0.450. Color change greater than 0.2 OD units was

never observed in the control well of any microplate. Substrates with a final corrected OD less than 0.200 were omitted from the kinetic data set, as this represented a color change value less than twice that ever seen in the control well. The corrected data for each substrate showing color development were fit to a logistic equation (Equation 1) as described previously (Lindstrom *et al.* in press; see Chapter 1), and the three kinetic parameters (*K*, *r* and *s*) were estimated for each substrate.

$$OD_{595} = \frac{K}{1 + e^{-r(t-s)}} \qquad \text{(Equation 1)}$$

Two of the parameters (*K*, representing extent of dye reduction in OD units, and *r*, representing the exponential rate of color change per unit time, $e^r$, following the lag after inoculation) are insensitive to inoculum density in the range of inocula used in this study (Lindstrom *et al.* in press; see Chapter 1). Curve parameters with standard errors larger than the parameter value were taken as evidence of a bad fit and the kinetic data for these substrates were not used. All parameters used in the analysis came from fit curves with $\chi^2$ values less than 0.01.

Correlation matrices constructed from the *K* or *r* parameter data for all samples at all dilutions were used to calculate principal components (PCs). Principal components analysis (PCA) provides a technique for succinctly expressing the variance in samples' kinetic responses to the 95 substrates contained in the microplate. By including all dilutions in the analysis, differences among sample types' (i.e., whether OIL or REF, and season samples were collected) microplate responses could be evaluated for their sensitivity to dilution. Samples exhibiting similar responses to microplate substrates will tend to have similar PC scores, while differing PC scores indicate samples with dissimilar substrate responses. Principal components analysis (PCA) of both *K* and *r* parameter data correlation matrices and various univariate analyses were performed using Systat software (version 5.05; SPSS, Inc., Chicago, IL).

Samples' responses to microplate substrates also were examined as binary data (presence or absence of substrate metabolism) by observing which substrates had kinetic parameters estimated. Substrates were defined as "positive" for community metabolism if their color development curves met the criteria described above for kinetic analysis and were defined as "negative" otherwise.

## RESULTS

### Total Bacteria and Hydrocarbon Degraders

Mean total bacterial populations varied with season, but no obvious difference due to oiling was seen (Table 2-1). Numbers of hydrocarbon degrading microbes were ca. three orders of magnitude higher in OIL than in REF samples for all seasons sampled (Table 2-2). Hydrocarbon degrader estimates represented between one and three percent of total bacterial cells in OIL samples (Table 2-2), indicating microbial community acclimation to crude oil in these samples. Hydrocarbon degrader populations also were present in REF samples, but represented at most 0.005% of total bacteria in these samples (Table 2-2).

### Principal Components

The environmental samples' kinetic data ($r$ and $K$ values) at all three dilutions were analyzed together by PCA to assess variance of kinetic responses (rate or extent of dye reduction) to Biolog microplate substrates within and among treatments and sampling dates, and across dilution factors. The results of PCA for $r$ parameter (exponential rate constant for dye reduction) data are plotted as mean PC scores and standard errors of OIL and REF samples (by season) for the first two PC axes in Figure 2-1, which explain 23.8% and 5.5% of the data variation, respectively. This figure plots samples' scores at each dilution factor and generally shows separation of OIL and REF samples along the first PC axis, though at the $10^{-5}$ dilution (Fig. 2-1C) the separation of treatment types is less clear due to the scores for July REF samples.

Table 2-1. Mean (± S.E.) bacterial populations in each soil sample type. Data are based on n = 5 soil samples of each type, except for July OIL samples with n = 3.

| Month | Treatment | Bacteria (cells · g dry soil$^{-1}$) |
|---|---|---|
| July | REF | 1.4E+08 (±8.7E+06) |
| July | OIL | 5.7E+08 (±9.3E+07) |
| September | REF | 2.5E+08 (±1.8E+07) |
| September | OIL | 2.0E+08 (±1.5E+07) |
| October | REF | 1.1E+08 (±1.9E+06) |
| October | OIL | 7.9E+07 (±3.5E+06) |

Table 2-2. Mean (± S.E.) numbers of hydrocarbon (HC) degraders estimated by Sheen Screen MPN enumeration and percent of total bacterial cells in the soil these degraders represent. Data are based on n= 5 soil samples of each type, except for July OIL samples with n = 3.

| Month | Treatment | HC degraders (cells · g dry soil$^{-1}$) | Percent of Total Bacteria (%) |
|---|---|---|---|
| July | REF | 7.1E+03 (±5.4E+03) | 0.0052 |
| July | OIL | 1.0E+07 (±6.7E+06) | 1.8321 |
| September | REF | 7.9E+02 (±3.2E+02) | 0.0003 |
| September | OIL | 2.1E+06 (±6.2E+05) | 1.0154 |
| October | REF | 9.3E+02 (±7.1E+02) | 0.0009 |
| October | OIL | 1.8E+06 (±8.4E+05) | 2.3353 |

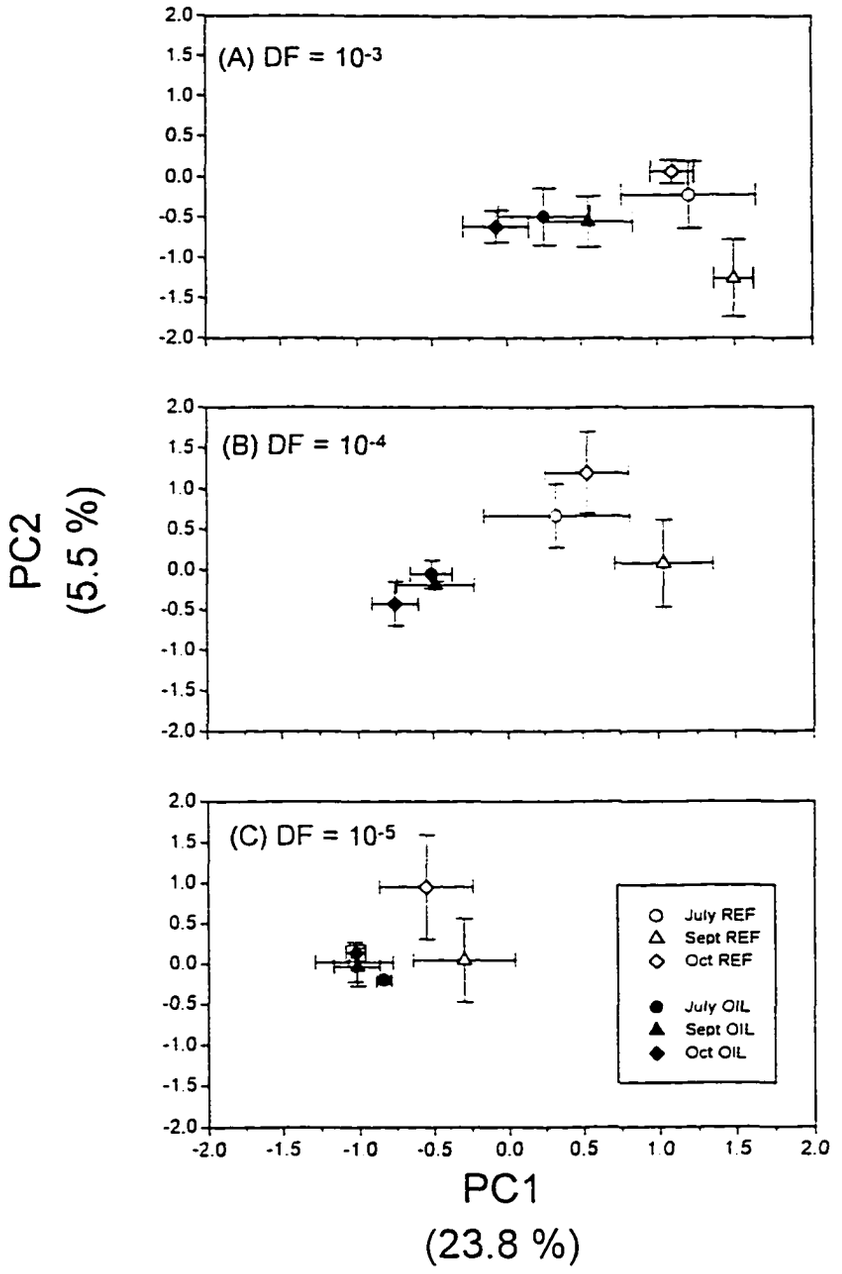


Fig. 2-1. Principal component (PC) scores plotted for the first two components determined from *r* kinetic parameter data. Mean PC data for each season's OIL (solid symbols) and REF (open symbols) samples are plotted. Error bars are standard errors of the means. Percent of data variance explained by each PC axis is given in parentheses. Scores were calculated from a data correlation matrix constructed using all sample dilutions, but each dilution factor (DF) is plotted separately for clarity. (A) mean component scores for $10^{-3}$ dilution, (B) mean scores for $10^{-4}$ dilution, and (C) mean scores for $10^{-5}$ dilution.

Clear separation of OIL and REF samples by season within a given dilution can also be seen. Inspection of the principal components for the *r* value data shows that, for those substrates loading most strongly on the first PC (41 substrates with component loadings greater than 0.500), most were amino acids or amines (25 substrates) with the remainder being carbohydrates (9 substrates) or carboxylic acids (7 substrates; data not shown). Figure 2-1 also shows that the OIL samples were generally more tightly clustered (lower diversity in kinetic responses to microplate substrates) in PC space than were REF samples at any given dilution. Evidence of this is seen by comparing standard errors for the mean PC values calculated for OIL and REF samples across all sampling seasons (Table 2-3). Standard errors for PCs at each dilution were generally smaller for OIL than for REF samples (except at the $10^{-3}$ dilution for PC1). Further, in general the OIL samples' microplate responses varied less with increasing dilution, while the REF samples' responses varied more with increasing dilution (Fig. 2-1, Table 2-3). Principal component scores also varied less across dilutions for OIL samples than for REF samples (Fig. 2-1A, B and C) as reflected by their lower standard errors for each sampling season (Table 2-4). Mean *r* value PC scores and standard errors for all samples by season, treatment and dilution factor are presented in Table 2-5.

A similar analysis was performed for *K* parameter (extent of dye reduction) data. Mean PC scores and standard errors are plotted for the first two PC axes (accounting for 18.7% and 11.1% of data variation, respectively) in Figure 2-2. As with component scores for *r* value data, samples' PC scores for *K* value data generally plotted OIL samples as discrete from REF samples at any given dilution, an exception only occurring for the July REF samples at the $10^{-4}$ dilution (Fig. 2-2B). All samples also were clearly separated by treatment (OIL vs. REF) at each season of sampling within a given dilution (Fig. 2-2). The OIL samples were generally more tightly clustered (lower standard errors) along the first PC axis than REF samples within a given dilution, though this trend was not seen for the second PC (Table 2-6). No clear trend in variation across all dilutions was observed for OIL and REF samples' *K*

Table 2-3. Means and standard errors (S.E.) of principal component scores using kinetic *r* values for oiled soil (OIL; n = 13 for each dilution) and oil-free soil (REF; n = 15 for each dilution) for all sampling seasons. Standard errors for PCs at each dilution are generally smaller for OIL than for REF samples, and decrease with increasing dilution for OIL samples' PCs while they generally increase for REF samples.

| Sample and Dilution | Mean PC1 | S.E. PC1 | Mean PC2 | S.E. PC2 |
|---|---|---|---|---|
| OIL DF = $10^{-3}$ | 0.231 | 0.168 | -0.561 | 0.156 |
| OIL DF = $10^{-4}$ | -0.616 | 0.114 | -0.269 | 0.131 |
| OIL DF = $10^{-5}$ | -0.987 | 0.059 | 0.011 | 0.093 |
| REF DF = $10^{-3}$ | 1.244 | 0.141 | -0.400 | 0.230 |
| REF DF = $10^{-4}$ | 0.617 | 0.204 | 0.714 | 0.292 |
| REF DF = $10^{-5}$ | -0.622 | 0.183 | 0.412 | 0.318 |
| OIL All DFs (N=39) | -0.468 | 0.100 | -0.274 | 0.079 |
| REF All DFs (N=45) | 0.413 | 0.149 | 0.242 | 0.173 |

Table 2-4. Means and standard errors (S.E.) for *r* value principal components for each sampling season and treatment calculated across all dilutions. There were n = 15 samples for each season and treatment, except for July OIL which had n = 9. Each season's samples' microplate responses are more similar (lower S.E.) across dilutions for OIL than for REF treatments.

| Season and Treatment | Mean PC1 | S.E. PC1 | Mean PC2 | S.E. PC2 |
|---|---|---|---|---|
| July OIL | -0.367 | 0.190 | -0.248 | 0.130 |
| July REF | 0.163 | 0.328 | 0.158 | 0.217 |
| September OIL | -0.320 | 0.216 | -0.257 | 0.135 |
| September REF | 0.740 | 0.252 | -0.376 | 0.320 |
| October OIL | -0.618 | 0.125 | -0.298 | 0.132 |
| October REF | 0.358 | 0.207 | 0.743 | 0.283 |

Table 2-5. Mean *r* value PC scores and standard errors (S.E.) for each sample type (season, treatment and dilution); n = 3 for July OIL samples and n = 5 for all other samples. Excepting July REF samples, the REF samples' PC scores generally increase in variability with increasing dilution while OIL samples show the opposite trend.

| Season and Treatment | Dilution | Mean PC1 | S.E. PC1 | Mean PC2 | S.E. PC2 |
|---|---|---|---|---|---|
| July REF | $10^{-3}$ | 1.203 | 0.436 | -0.214 | 0.417 |
| | $10^{-4}$ | 0.324 | 0.489 | 0.668 | 0.394 |
| | $10^{-5}$ | -1.036 | 0.256 | 0.020 | 0.248 |
| July OIL | $10^{-3}$ | 0.253 | 0.310 | -0.488 | 0.352 |
| | $10^{-4}$ | -0.513 | 0.141 | -0.054 | 0.170 |
| | $10^{-5}$ | -0.840 | 0.052 | -0.201 | 0.045 |
| September REF | $10^{-3}$ | 1.492 | 0.130 | -1.254 | 0.482 |
| | $10^{-4}$ | 1.031 | 0.318 | 0.078 | 0.539 |
| | $10^{-5}$ | -0.302 | 0.339 | 0.047 | 0.516 |
| September OIL | $10^{-3}$ | 0.545 | 0.293 | -0.547 | 0.315 |
| | $10^{-4}$ | -0.485 | 0.257 | -0.188 | 0.043 |
| | $10^{-5}$ | -1.019 | 0.153 | -0.038 | 0.235 |
| October REF | $10^{-3}$ | 1.097 | 0.139 | 0.077 | 0.144 |
| | $10^{-4}$ | 0.531 | 0.275 | 1.200 | 0.501 |
| | $10^{-5}$ | -0.554 | 0.313 | 0.953 | 0.644 |
| October OIL | $10^{-3}$ | -0.073 | 0.217 | -0.610 | 0.198 |
| | $10^{-4}$ | -0.754 | 0.156 | -0.420 | 0.271 |
| | $10^{-5}$ | -1.028 | 0.065 | 0.137 | 0.102 |

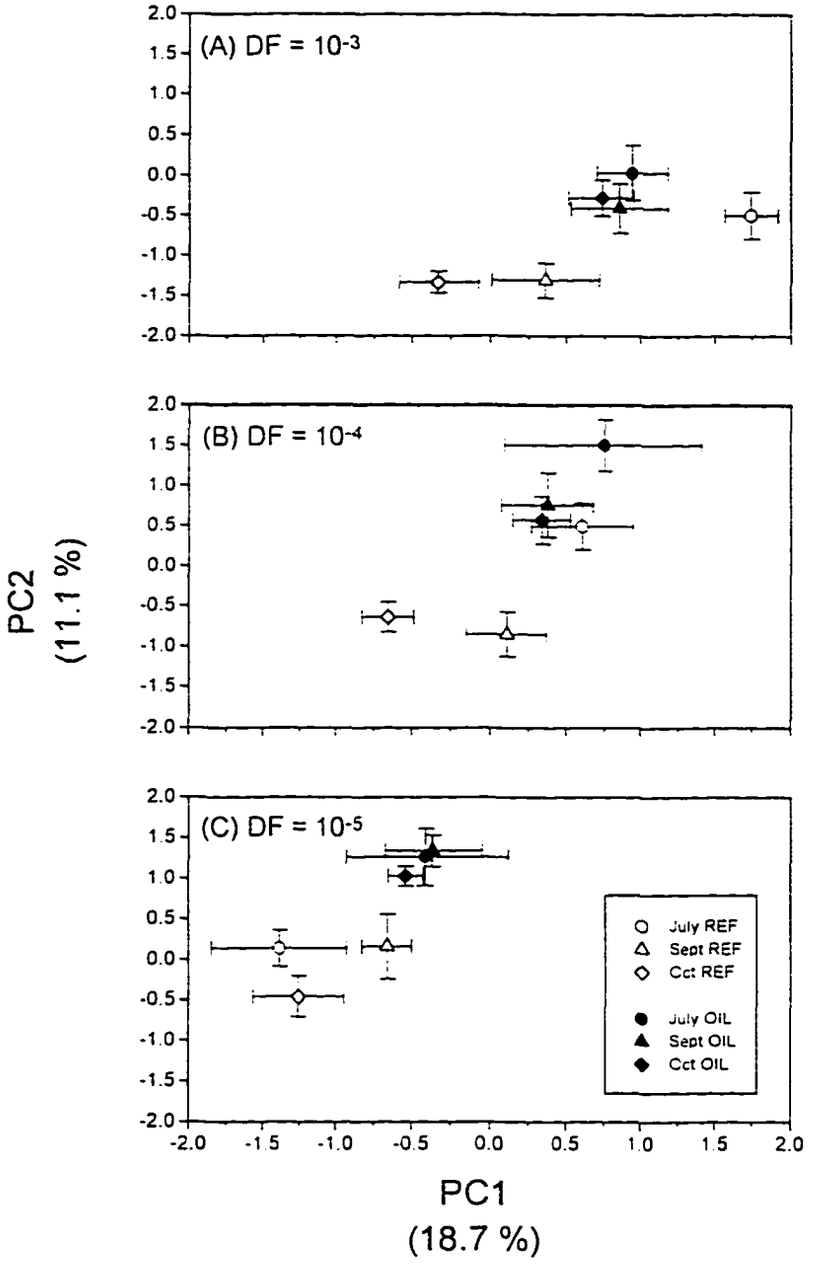


Fig. 2-2. Principal component (PC) scores plotted for the first two components determined from *K* kinetic parameter data. Mean PC data for each season's OIL (solid symbols) and REF (open symbols) samples are plotted. Error bars are standard errors of the means. Percent of data variance explained by each PC axis is given in parentheses. Scores were calculated from a data correlation matrix constructed using all sample dilutions. Each dilution factor (DF) is plotted separately. (A) mean component scores for $10^{-3}$ dilution factor, (B) mean scores for $10^{-4}$ dilution, and (C) mean scores for $10^{-5}$ dilution.

Table 2-6. Means and standard errors (S.E.) of principal component scores using kinetic *K* values for each treatment type for all sampling seasons. Mean scores for each dilution factor (DF) are given along with mean scores for all dilutions together.

| Sample and Dilution | | Mean PC1 | S.E. PC1 | Mean PC2 | S.E. PC2 |
|---|---|---|---|---|---|
| OIL | DF = $10^{-3}$ | 0.894 | 0.136 | -0.255 | 0.166 |
| OIL | DF = $10^{-4}$ | 0.432 | 0.174 | 0.822 | 0.212 |
| OIL | DF = $10^{-5}$ | -0.454 | 0.143 | 1.179 | 0.107 |
| REF | DF = $10^{-3}$ | 0.482 | 0.260 | -1.076 | 0.146 |
| REF | DF = $10^{-4}$ | -0.060 | 0.189 | -0.364 | 0.192 |
| REF | DF = $10^{-5}$ | -1.121 | 0.194 | -0.099 | 0.174 |
| OIL | All DFs | 0.265 | 0.119 | 0.582 | 0.130 |
| REF | All DFs | -0.233 | 0.155 | -0.513 | 0.113 |

values (Table 2-7). Mean *K* value PC scores and standard errors for all samples by season, treatment and dilution factor are presented in Table 2-8.

Substrate Use By Treatment

An alternative to analysis of kinetic data by PCA is to determine which substrates had been used by all samples in a given season, treatment and dilution. This entails transforming kinetic data into binary data (presence or absence of substrate use) according to a dye reduction threshold (see Methods). Table 2-9 presents the number of substrates used by all samples of a given type according to dilution. Each sample type exhibited universal use of fewer substrates with increasing dilution and, at the highest dilution ($10^{-5}$), REF samples used fewer substrates than OIL samples, particularly in July and October. At this dilution, substrates most represented in OIL samples were amino acids or amines (14 substrates in July, 13 in September and 10 in October) and carboxylic acids (18 substrates in July, 13 in September and 12 in October). Amino acids or amines were used by all REF samples at this dilution rarely in July (1 substrate) and October (3 substrates), but more in September (9 substrates). For REF samples at this dilution, carboxylic acids were used by all samples only in July (3 substrates) and September (10 substrates).

Table 2-7. Means and standard errors (S.E.) for *K* value principal components for each sampling season and treatment calculated across all dilutions.

| Season and Treatment | Mean PC1 | S.E. PC1 | Mean PC2 | S.E. PC2 |
|---|---|---|---|---|
| July OIL | 0.429 | 0.329 | 0.935 | 0.283 |
| July REF | 0.320 | 0.392 | 0.046 | 0.182 |
| September OIL | 0.289 | 0.215 | 0.565 | 0.256 |
| September REF | -0.065 | 0.185 | -0.664 | 0.231 |
| October OIL | 0.177 | 0.155 | 0.442 | 0.173 |
| October REF | -0.749 | 0.162 | -0.806 | 0.136 |

Table 2-8. Mean *K* value PC scores and standard errors (S.E.) for each sample type (season, treatment and dilution); n = 3 for July OIL samples and n = 5 for all other samples.

| Season and Treatment | Dilution | Mean PC1 | S.E. PC1 | Mean PC2 | S.E. PC2 |
|---|---|---|---|---|---|
| July REF | $10^{-3}$ | 1.742 | 0.176 | -0.495 | 0.295 |
| | $10^{-4}$ | 0.607 | 0.341 | 0.494 | 0.292 |
| | $10^{-5}$ | -1.389 | 0.460 | 0.140 | 0.224 |
| July OIL | $10^{-3}$ | 0.943 | 0.241 | 0.037 | 0.340 |
| | $10^{-4}$ | 0.753 | 0.658 | 1.506 | 0.319 |
| | $10^{-5}$ | -0.408 | 0.525 | 1.263 | 0.351 |
| September REF | $10^{-3}$ | 0.359 | 0.352 | -1.304 | 0.215 |
| | $10^{-4}$ | 0.108 | 0.259 | -0.845 | 0.274 |
| | $10^{-5}$ | -0.662 | 0.161 | 0.158 | 0.398 |
| September OIL | $10^{-3}$ | 0.856 | 0.326 | -0.404 | 0.310 |
| | $10^{-4}$ | 0.375 | 0.302 | 0.760 | 0.400 |
| | $10^{-5}$ | -0.364 | 0.311 | 1.340 | 0.194 |
| October REF | $10^{-3}$ | -0.330 | 0.255 | -1.329 | 0.132 |
| | $10^{-4}$ | -0.657 | 0.170 | -0.635 | 0.185 |
| | $10^{-5}$ | -1.258 | 0.308 | -0.454 | 0.248 |
| October OIL | $10^{-3}$ | 0.732 | 0.222 | -0.275 | 0.221 |
| | $10^{-4}$ | 0.335 | 0.191 | 0.572 | 0.298 |
| | $10^{-5}$ | -0.538 | 0.117 | 1.029 | 0.121 |

Table 2-9. Number of substrates used by every sample of a given type by season and treatment at each dilution factor (DF); n = 3 for July OIL samples and n = 5 for all others. Chemical classes of substrates used by all samples of a given type at the $10^{-5}$ dilution are given in parentheses.

| | | Number of Substrates Used by All Samples | | |
|---|---|---|---|---|
| Season and Treatment | | DF = $10^{-3}$ | DF = $10^{-4}$ | DF = $10^{-5}$ |
| July | OIL | 65 | 54 | 49 (14A, 18CA, 8OH, 7CH, 2P)[a] |
| July | REF | 61 | 39 | 4 (1A, 3 CA) |
| September | OIL | 46 | 40 | 38 (13A, 13CA, 5OH, 6CH, 1P) |
| September | REF | 59 | 50 | 30 (9A, 10CA, 5OH, 4CH, 2P) |
| October | OIL | 53 | 46 | 38 (10A, 12CA, 6OH, 8CH, 2P) |
| October | REF | 55 | 40 | 4 (3A, 1 OH) |

[a] A = amino acid/amine, CA = carboxylic acid, OH = alcohol, CH = carbohydrate, P = polymer.

# DISCUSSION

The continued presence of crude oil at our study site since 1976 (Collins *et al.*, 1994) has led to detectable differences between OIL and REF soils in the collective physiology of their microbial communities, as revealed by PCA of kinetic microplate data. The technique of collecting kinetic dye reduction data allowed discrimination of OIL and REF community responses by treatment, and also generally according to season of sampling, with more homogeneous responses within and across dilutions for the OIL samples. Similarities among OIL samples' substrate metabolism patterns irrespective of season indicate these soils' communities exhibited little seasonal variation in physiology compared to those in REF soils. Moreover, we suggest that homogeneity of substrate response patterns across dilutions implies that OIL soil communities were composed of fewer distinct functional populations than were those in REF soils.

The use of multiple substrate metabolism analysis to evaluate community structure is complicated by the fact that color development in a given well of the microplate is a consequence of many unknown interacting factors. Bacterial growth is known to occur in the microplate wells (Garland and Mills, 1991; Lindstrom *et al.* in press; see Chapter 1), but rate and extent of color development are not strictly correlated to the wells' population densities (Haack *et al.*, 1995). Rather, the color response of a given microplate substrate well to a particular inoculum is the result of the integrated metabolisms of the mixture of microbes present (Lindstrom *et al.* in press; see Chapter 1).

Attempts to use microplates to evaluate community diversity have typically relied on assessing numbers and types of substrates used at a selected plate reading time (e.g., Zak *et al.*, 1994). However, since the average rate of color development following inoculation depends on inoculum density (Haack *et al.*, 1995) as well as the mix of microbes in the inoculum, analysis of microplate substrate responses based on OD at a single time will reveal little information regarding relative functional diversity

of microbial communities from samples of differing total bacterial biomass. Even with samples of similar bacterial densities, substrate use patterns determined at a single time vary depending on duration of incubation following inoculation (Bossio and Scow, 1995). In addition, measurable dye reduction eventually will occur in most substrate wells in the microplate if enough populations are included in the inoculum to allow for synergistic interactions.

To circumvent the problems described above, we examined the change in kinetic patterns as individual populations were removed from the community through dilutions. By determining dye reduction kinetic parameters not sensitive to inoculum density and observing how they change with dilution, we may infer differences in community structure and functional diversity that depend on the relative contribution of member populations. The presence in a community of a large number of metabolically distinct microbial types or "species" (high diversity) allows for numerous inter-specific relationships (Atlas, 1984a). Conversely, a community of low diversity (low species richness) has fewer potential opportunities for unique combinations of interactions. A microbial community of high population diversity will have larger numbers of rare populations than one of low diversity with a similar total microbial biomass. Given two communities of similar total population densities but of differing microbial diversity, the numbers and types of interactions, and hence the kinetic responses to microplate substrates, will be more greatly affected by dilution in the more diverse community because member populations that are relatively rare will be more quickly diluted to extinction. Thus a community of lower diversity will vary less in microplate response across a range of dilutions than a community of relatively higher population diversity. Focusing on changes in substrate use patterns that occur with dilution at the community level necessarily ignores physiological diversity that may exist within a population. However, it does allow one to link changes in community physiology to organismal diversity within the community.

The dilution response of OIL and REF samples to microplate substrates, as measured by the *r* parameter (rate of dye reduction from no color development to a maximum), provided evidence of decreased population diversity in the OIL sample communities. Principal components of *r* data for diluted samples from a given season showed greater variation across dilutions for REF than for OIL samples (Table 2-4). Also, OIL samples within a given dilution showed similar responses to substrates (Fig. 2-1, Table 2-3) whether the samples were collected during summer (July), autumn (September) or early winter (October), while the REF samples exhibited greater seasonal variation. This is in spite of the fact that the OIL samples varied more in total bacterial population with season compared to the relatively stable seasonal biomass estimates for REF samples (see Table 2-1). Despite the greater population fluctuation in OIL samples, their responses to microplate substrates (based on dye reduction rate) appeared more similar over time than did REF samples.

The *K* parameter data did not exhibit the strong trend with dilution seen for the *r* parameter data. Principal component scores for *K* data did discriminate OIL from REF samples but, unlike the *r* data, the standard errors for the OIL PC scores were not uniformly smaller than those for REF samples (Tables 2-3 and 2-6, and Tables 2-4 and 2-7). The different meanings of the kinetic parameters may explain why the trend seen in the *r* parameter data was not seen for *K* data. The extent of dye reduction (*K*) seen in a microplate well represents the sum of reducing power generated in that well before limitation occurs due to exhaustion of metabolic resources or redox dye, or buildup of toxic metabolites (Lindstrom *et al.* in press; Chapter 1). On the other hand, rate of dye reduction ($e^r$) represents the maximum rate of reduction by one or several populations in the well. Production of metabolites by one population in a well may produce a usable substrate for another population in the well not able to grow at the expense of that well's designated substrate, or may produce metabolic inhibitors for populations that could otherwise grow (Bouma and Mills, 1994). Thus the extent of dye reduction (*K*) could be higher or lower than might occur in the absence of

metabolic intermediates from the community in the well (i.e., if the only C source present were the well's designated substrate). However, the *rate* of reduction in the well would still be limited by the metabolic rate of the population growing at the expense of the well's designated substrate, as other populations could not be affected by the metabolite (not originally present in the well) faster than it was produced. Therefore, one can consider the $K$ parameter as the sum of a collection of metabolisms in a microplate well, while $r$ represents the metabolic rate of the population (or populations) able to most rapidly turn over the substrate presented as the well's sole C source. $K$ may be thought of as the maximum possible reducing power generated from the carbon available in the well (designated C source plus metabolites) and $r$ represents the rate limiting step in substrate processing specifically related to the well's designated substrate. To the extent that community-level population interactions affect dye reduction in the microplate well, these interactions are expected to yield greater variation in $K$ than in $r$ parameter data.

The role of population interactions within communities inoculated into the microplate is evident when one examines the number of substrates used by all samples of a given type (Table 2-9). At the low dilutions ($10^3$-fold dilution), where populations of relatively lower abundance in the community are present, all samples (OIL or REF) used many substrates irrespective of season. With increasing dilution, however, fewer substrates were used by all samples of a given type. The number of substrates used by all samples diminished faster with dilution for REF than for OIL samples and, at the highest dilution, all REF samples used only ca. 10% of the total number of substrates used by OIL samples in July and October. Less difference between OIL and REF was observed in substrates used in September samples.

Compared to REF soils, the selective pressure provided by hydrocarbon contaminants enriched the microbial community in hydrocarbon-oxidizing populations at our OIL site (Table 2-2). This enrichment is in concert with findings from other petroleum contamination studies (e.g., Hood *et al.*, 1975; Wünsche *et al.*, 1995). The

associated diminished population diversity implied by such enrichment in our study is also consistent with observations made of other hydrocarbon-polluted samples (e.g., Sayler *et al.*, 1983).

Our data suggest not only that the population composition of the OIL soil community is less diverse than that of REF soils, but also the populations surviving the oiling exhibit enhanced nutritional versatility. Atlas *et al.* (1991) found that communities exposed to petroleum hydrocarbons exhibited diminished genetic diversity relative to undisturbed reference communities, but displayed increased physiological tolerances and substrate utilization capabilities. In other words, the populations surviving the hydrocarbon disturbance were characterized as "generalists", not physiological "specialists." Ecologically generalist populations have broad niches and tend to sacrifice efficiency in using a narrow range of resources for the ability to survive using a broad range of resources (Smith, 1992). By contrast, specialists are more narrowly focused in ability to exploit niche resources. Crude oil contamination at our OIL site represents a perturbation of the soil environment with respect to changes in temperature and perhaps oxygen tension (Collins *et al.*, 1994), as well as additions of organic carbon as variously toxic, degradable or inert petroleum components (Leahy and Colwell, 1990). Altering the soil environment in this way may have eliminated many specialized niches actively exploited in REF soils, thereby excluding from the OIL soil communities several specialized populations. Eliminating metabolically efficient specialist populations from competition in the OIL soil community may have provided opportunities for the less efficient generalists to exploit niche space previously unavailable ("competitive release"; Smith, 1992).

Bacterial populations exposed to crude oil have been shown to demonstrate enhanced abilities to use carboxylic acids and hydrocarbons (Atlas *et al.*, 1991), and several studies have shown that additions of petroleum hydrocarbons to a soil community can have significant long-term effects on the metabolic activity of the community (see Pfaender and Buckley, 1984). Our data appear to confirm this

observation. OIL samples' kinetic responses (*r* values) to substrates were more consistent across dilutions and seasons compared with REF samples (implying lower diversity; Tables 2-3 and 2-4), and OIL samples consistently used more and a greater variety of substrates (notably amines and carboxylic acids) than REF samples when diluted $10^5$-fold (Table 2-9). This increased nutritional versatility cannot be attributed solely to higher numbers and varieties of bacterial types in OIL samples, as the relative densities of bacteria vary from greater than REF in July to less than REF in October (Table 2-1).

Evaluation of our soil samples' community-level kinetic responses to serial dilutions suggests that the microbial communities from OIL soil samples exhibit reduced population diversity compared to REF soils. Increasing the dilution factor or varying the season of sampling had a more pronounced effect on REF samples, reflecting the greater site heterogeneity and likelihood of a higher variety of microbial interactions compared to OIL samples. Despite the fact that our soils were collected in spatially (across meters) and temporally (summer, early autumn, late autumn) dispersed samplings, the OIL soils' microplate behaviors exhibited relatively little variation with respect to dilution, location or season.

Our data appear to be consistent with those from other oil spill studies showing decreased diversity, but enrichment favoring metabolic generalists, in oiled soils (Atlas *et al.*, 1991). A substantial amount of residual oil remained at the study site nearly 20 years after the experimental crude oil spill (Collins *et al.*, 1994). This disturbance of the soil system may have caused diverse specialist populations to be displaced by fewer populations of generalists. To the extent that substrate processing (either soil organic matter or added oil) depends on key taxa of specialists now absent from these soils, this reduced diversity may have larger scale consequences for long-term site recovery.

# MICROBIAL ECOLOGY AND LONG-TERM PERSISTENCE OF CRUDE OIL IN A TAIGA SPRUCE FOREST

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## SUMMARY

Crude oil spilled in terrestrial impact experiments in 1976 was abundant at the taiga spill site through 1995. Measurements of physical, chemical and microbiological parameters were made to assess the effect of the oil on the site's soil microbial community and to examine the reasons for the oil's persistence. Fungi dominated the microbial biomass in both oiled (OIL) and oil-free, reference (REF) soils. Total fungal and bacterial biomass, as well as protozoal abundance and soil C respiration measurements, showed no differences between OIL and REF soils. Metabolically active fungal and bacterial biomasses, however, always were depressed in OIL soils. Net N mineralization was lower and net nitrification was never observed in OIL samples. Numbers of hydrocarbon degraders and specific hydrocarbon mineralization potentials indicated that oil-acclimated microbial populations were present in OIL soil samples, and some evidence of hydrocarbon oxidation was seen in the petroleum chemistry data. These data indicate a shift in substrate use in favor of oil has occurred in the microbial communities of the OIL plot. Changes in microbial community function associated with this shift are likely responsible for a disruption in normal nutrient cycling in the OIL plot. This has implications for revegetation and site recovery as the crude oil residue is expected to persist for decades.

# INTRODUCTION

Microbiological activity in soils directly influences soil productivity and plant health in the natural environment (Brady, 1990). "Soil microbial biomass has been called the eye of the needle through which all the natural organic material that enters the soil must pass" (van Veen *et al.*, 1984, p. 257), and microbial biomass serves as the source of labile nutrients available for plant roots and soil microbes (Coleman and Crossley, 1996). All living organisms participate to some extent in the biogeochemical cycling of materials, but microorganisms are the major players in driving these cycles due to their ubiquity, diverse metabolic capabilities, and high enzymatic activity (Atlas and Bartha, 1992). Thus, the vitality and structure of soil and plant communities are affected by the capabilities of the microbial participants in the soil community.

Environmental stress of any sort will have an impact on the soil microbial community and, by extension, soil productivity. "Stress" may be defined as anything that increases the maintenance requirements of an organism above its usual ("non-stressed") condition (Atlas, 1984). Stress to specific soil populations will alter the natural organization of the microbial community. Due to these selective pressures, the microbial diversity of a soil under stress may be lower than otherwise (Atlas, 1984). Despite the common reduction in microbial diversity due to pollutants (Alexander, 1994), the effects of stress may not be visible in all soil functions. Studies on the effects of toxic chemicals on nutrient cycling have yielded a variety of responses, ranging from reduced C and N mineralization to diminished enzyme activity to increased rates of respiration, depending on the chemicals' concentrations and target organisms (see Hendricks, 1997).

Terrestrial crude oil spills provide a complex source of hydrocarbons able to both destroy and enrich various components of a soil microbial community (Leahy and Colwell, 1990). Organic matter processing that depends on this community ultimately determines the fate of the oil and the ability of the ecosystem to recover functions lost as a result of the spill (Baker, 1994). As suggested above, the stress to a soil

community from hydrocarbons may yield a variety of responses; petroleum spills in Alaska are no exception. Crude oil addition has been found to either stimulate heterotrophic microbial populations in northern soils (Sexstone *et al.*, 1978b) or to depress them (Sparrow and Sparrow, 1988). These discrepancies may be due to differing methods among studies, time following the spill, or site-specific soil or contaminant conditions. Whatever the response of the soil community to spilled petroleum, understanding the microbial ecology of the system in question is essential to any effort to enhance biological removal of the contaminant.

We examined microbiological, physical, and chemical parameters at the site of a nearly 20-year old terrestrial crude oil spill in the subarctic. Our objective was to study the spill's effects on various populations of the microbial community and their activities, and to determine the factors causing long-term persistence of the oil.

## MATERIALS AND METHODS

### Site Description

The study area was located in the Caribou-Poker Creeks Research Watershed 48 km northeast of Fairbanks, Alaska. The sampling areas included the site of a 7570 L experimental crude oil spill conducted in 1976 to investigate the long-term effects of crude oil contamination on subarctic taiga (see Collins *et al.*, 1994). For the original 1976 study the oil was applied at the top of a plot established on a west-facing, low angle slope and allowed to flow downslope. Soil samples were collected from both the oiled site (OIL) and an adjacent oil-free, reference site (REF) located just upslope from the spill area.

Local vegetation at the site is open black spruce (*Picea mariana*) forest (total tree canopy cover less than 60%) with a shrub understory of blueberry (*Vaccinium uliginosum*), Labrador tea (*Ledum decumbens*), resin birch (*Betula glandulosa*) and willow (*Salix* spp.). The ground surface is covered by mosses and lichens with scattered (10% of cover) cotton grass (*Eriophorum vaginatum*) tussocks (Collins *et*

*al.*, 1994). The area is underlain by permafrost, with an active layer thickness of 40-60 cm, and the soil is classified as a histic pergelic cryaquept (Jenkins *et al.*, 1978). A typical soil profile consists of a 5 cm moss and lichen surface layer above a 15 cm undecomposed peat horizon (O1), a 5 cm decomposed dark brown peat horizon (O2), a 5 cm dark grayish-brown silt loam horizon (A1), and a grayish-brown silt loam mineral soil (C; Collins *et al.*, 1994).

In the OIL plot, most of the oil flowed between *Eriophorum* tussocks. As a result, almost all mosses, lichens and shrubs in the upper 10 to 15 m below the source of the spill were killed by the oil. In 1995, moss and lichen surface vegetation was generally dead and incorporated into the O2 and A1 horizons. With few exceptions the only surviving vegetation in this area of the OIL plot was scattered *E. vaginatum* tussocks, some of which apparently increased "dramatically in size" since the spill (Collins *et al.*, 1994).

Sampling

In both OIL and REF areas soil cores were collected at randomly selected locations between the *Eriophorum* tussocks, with OIL sample cores collected only in the upper 10 m just downslope from the source of the oil spill. Samples were collected with a 2.5 cm diameter stainless steel coring device fitted with plastic sleeves for holding sample cores (Ben Meadows Co., Atlanta, GA). Prior to sampling, the plastic sleeves were washed with soap and water, rinsed three times with distilled water, then disinfected by rinsing with a 3% solution of hydrogen peroxide. To collect a sufficient amount of soil for the many analyses to be performed, six cores were taken at each location within the site to be combined later into a single sample for analysis. The sample cores were placed in coolers immediately following collection at the site and kept at ca. 4°C until processing the next day in the laboratory.

Upon return to the laboratory, samples were removed from the plastic core sleeves and their horizons separated. After removing the surface layer of live and/or

undecomposed dead plant material, the cores were separated into organic (O2 plus A1) and mineral (C) horizons. OIL samples had little or no surface plant material present and all surface organic material was used for the organic fraction. Following segregation of the organic and mineral fractions, the separate horizons from the six cores were mixed and homogenized by sieving (2 mm mesh) twice. The samples were then placed in sterile plastic bags and refrigerated until further processing for microbiological assays the following day. At that time a sample of approximately 10 g (wet weight) of the homogenized soil was placed in pre-combusted glass jars with dichloromethane (DCM)-rinsed lids and frozen for later hydrocarbon extraction and analysis.

Sampling took place on 13 July, 11 August, 20 September, and 11 October 1994, and on 5 July, 5 September and 17 October 1995. The sampling dates represented seasonally distinct times of the year with the July and August sampling occurring during the middle and late parts of the growing season, respectively, September sampling occurring while the dominant vegetation in the area was senescing, and October sampling following several days of freezing weather.

Physical and Chemical Parameters

Soil temperatures at 10 cm depth were determined at each core sampling location in the study area. Field moisture in each homogenized soil sample was determined gravimetrically. Soil saturation or water holding capacity (WHC) also was determined for field-moist homogenized soil (Forster, 1995). The degree of saturation upon sample collection (% WHC) was calculated from field moisture and WHC data. Soil pH was determined in soil slurries (1 part soil to 2 parts distilled water) for the homogenized organic soil fractions.

Soil total C and total N were determined for each homogenized organic horizon sample after oven-drying and grinding. Ground samples' C and N were measured in triplicate (1994 samples) or duplicate (1995 samples) using a C-N-S analyzer (Leco

2000 CNS Analyzer; Leco Inc., St. Joseph, MI). Carbon and nitrogen content of material extracted from the soil with DCM (i.e., "petroleum", see below), also was determined using the CNS analyzer after evaporating off the DCM. Organic matter content was determined for each organic horizon sample by loss on ignition (450°C).

Due to the small quantity of soil remaining in each sample following the many analyses performed, only selected samples were analyzed for inorganic nitrogen ($NO_3^-$-N and $NH_4^+$-N) and extractable phosphorus. Previously frozen soil samples were extracted with 2M KCl and the extracts analyzed by the University of Alaska Fairbanks Agricultural and Forestry Experiment Station. Some of these samples (seven REF and eleven OIL) also were assayed for inorganic nitrogen after a 30-day respiration assay to determine net N mineralization.

Petroleum Hydrocarbons

The mass of petroleum hydrocarbons in soil samples was determined by extracting each sample with DCM. Frozen samples were thawed, placed in pre-combusted glass beakers, and mixed for drying with an equivalent mass of pre-combusted $Na_2SO_4$. This mixture was then placed in a new or DCM-extracted Soxhlet extraction thimble containing ca. one g of infusorial earth ("filter aid"). A DCM-extracted cotton wool plug was placed on top of the soil/sodium sulfate mixture to prevent loss of solid material during the extraction process. Extractions were performed on a Soxhlet extraction apparatus modified for rapid extractions (Soxtec System HT 1043 Extraction Unit; Tecator AB, Höganäs, Sweden) allowing sample extraction times to be reduced to 2 h per sample. Each sample thimble was placed on the apparatus and extracted by boiling in 50 ml DCM at 85°C for one hour. Thimbles were next rinsed for one hour with the solvent, and the rinsate containing extracted solute was collected. After evaporating away the DCM, the extracted residue was precisely weighed and its mass recorded. Data were recorded as g DCM-extractable material · g dry soil$^{-1}$ after correcting for soil moisture. Following residue mass

determination, the residue was returned to solution by adding 5 ml fresh DCM. Four ml of the DCM-residue solution was then placed in pre-combusted 4 ml amber glass vials with DCM-rinsed, Teflon-lined silicone septum caps. These solutions were stored in a freezer (-20°C) for future chemical analyses.

Efficiencies of hydrocarbon extraction were measured using uncontaminated soils spiked with fresh Prudhoe Bay crude (PBC) oil and $^{14}C$-labeled hexadecane, naphthalene and phenanthrene. Gravimetric residue recoveries for nine spiked samples ranged from 78% to 89% of added fresh oil mass. Hexadecane recovery was 96%; phenanthrene recovery was 92%, and naphthalene recovery was 52%. Based on these data we concluded that most of the weathered oil residue in the soil samples would be recovered by this extraction method, though oil fractions boiling below 85°C or exhibiting appreciable volatility at this temperature would not be recovered quantitatively.

DCM extracts were characterized according to gross chemical gravimetric composition by a series of extractions coupled with preparatory chromatography. Some extract fractions were further characterized by gas chromatographic analysis. Raw DCM extracts were first treated to precipitate large, high molecular weight compounds ("asphaltene" fraction) by placing one ml extract in 9 ml pentane and centrifuging the solution (5000 x g for 10 min.). Two ml of supernatant was removed, solvent evaporated and residue mass determined to obtain "total pentane-soluble" mass fraction. Remaining supernatant was treated to remove any residual polar material by solid phase extraction (SPE; silica solid phase; Extract-Clean Silica 1000 mg tubes, Alltech Associates, Inc., Deerfield, IL). SPE tubes were pre-conditioned by adding 5 ml pentane, allowing the pentane to drain off and leaving the silica bed "wet." The SPE tube was next loaded with 5 ml of the pentane-soluble sample and eluted with two additional aliquots of 5 ml pentane. Fifteen ml of eluate was collected for each sample (see below). Five ml eluate was removed and its solvent evaporated to determine the residue mass (pentane-soluble, non-polar fraction). Remaining eluate

was placed in amber glass septum vials and frozen (-20°C) for later gas chromatographic (GC) analysis.

To determine whether our SPE preparatory process allowed complete elution of non-polar alkane and aromatic hydrocarbons, recovery of model alkane and aromatic compounds in SPE pentane eluate was assessed. We added $^{14}C$-labeled hexadecane (50 µl, 2 µg · µl$^{-1}$ in acetone, ca. 740 Bq) or phenanthrene (50 µl, 2 µg · µl$^{-1}$ in acetone, ca. 1455 Bq) to one ml of raw DCM extract from an OIL sample, and proceeded with the protocol as described above. The material was sequentially eluted from the tube with four 5 ml aliquots of pentane. Each eluted aliquot was collected separately and its radioactivity determined by liquid scintillation counting, as described above. Based on this procedure, it was determined that greater than 95% of added radioactivity for both hexadecane and phenanthrene was recovered in the first two aliquots of pentane. Thus, we were assured that the pentane-soluble, non-polar hydrocarbon fraction containing alkanes and aromatics present in our soil extracts would be effectively recovered after the SPE step.

The pentane-soluble, non-polar fractions of DCM soil extracts recovered from the SPE were further characterized by a contract laboratory (Boreochem Mobile Laboratory, Fairbanks, AK) using a gas chromatograph equipped with a flame ionization detector (GC/FID; Model 9300B GC, SRI Instruments, Inc., Torrance, CA) and a splitless injector. Five µl aliquots of each extract were analyzed using a 30 m, 0.53 mm i.d. Silcosteel (TM) column (MXT-5 stationary phase; Restek, Inc., Bellefonte, PA), with $H_2$ carrier gas at a flow rate of 25 ml · min$^{-1}$ on a temperature programmed run (5 min @ 40°C, ramp @ 10°C · min$^{-1}$ to 350°C, 10 min @ 350°C; total run time of 46 min).

Chemical composition and degree of weathering compared to fresh 1976 PBC oil were assessed by evaluating relative amounts of various chromatographic fractions (based on relative GC retention times) using integrated areas calculated from baseline to chromatogram peak valley. Weathering also was evaluated by comparing "resolved"

peak areas to total integrated area in a manner similar to that of Sexstone *et al.* (1978a); "resolved" peak area was defined by re-drawing the chromatogram baseline from valley to valley for every peak in the chromatogram.

Microbiological Parameters

Homogenized soil samples (stored at 4°C) were assayed for microbial populations within one week of sample collection. All organic horizon samples were examined for abundance of bacterial cells and fungal hyphae, and for "active" fungi and bacteria. Most probable number (MPN) assays were performed to enumerate hydrocarbon oxidizing microbes and protozoa. Soil respiration was measured in each sample and radiorespirometric assays were performed on samples collected in 1994.

Microbial biomass content of organic soil fractions was determined by microscopic examination of diluted samples by the Microbial Biomass Services Laboratory (Oregon State University, Corvallis OR). Bacterial biomass was estimated by the direct microscopy technique of Babiuk and Paul (1970). Total fungal biomass, as well as metabolically active fungal and bacterial biomass, was estimated using an agar film technique (Bottomley, 1994) with fluorescein diacetate (FDA) as an activity-indicating stain. Biomasses were calculated from biovolumes using an average bacterial density of 0.33 $g \cdot cm^{-3}$ and an average fungal hyphae density of 0.41 $g \cdot cm^{-3}$ (Van Veen and Paul, 1979).

Numbers of hydrocarbon degraders were estimated in organic soil fractions using the method of Brown and Braddock (1990). Soil samples were decimally diluted in carbon-free Bushnell-Haas broth (Atlas, 1993) in a 24-well microtiter plate, and five replicates of each dilution were provided one drop of sterile Prudhoe Bay crude oil as sole carbon source. Plates were incubated at 21°C for three weeks. Inoculated wells in the plate were scored positive for microbial growth on crude oil if the oil sheen on the broth surface exhibited emulsification. All plates were read by one person and positive wells were checked by comparison with a sterile, uninoculated microtiter plate treated

as an environmental sample. Numbers of hydrocarbon degraders were determined by an MPN algorithm (Koch, 1994) and recorded as cells · g dry soil$^{-1}$.

Protozoa also were enumerated in organic horizon samples using a MPN technique as described by Ingham (1994) using REF sample organic horizon soil extract agar and Ringer solution (Collins *et al.*, 1989) as growth medium. Enumeration plates were incubated at 21°C for 10 days to 2 weeks. Presence of protozoa in each microplate well was determined by microscopic examination. Presence of general morphological class was noted for flagellate, amoeboid and ciliated protozoa.

Microbial potentials for specific glutamate, hexadecane, and phenanthrene substrate use were assayed radiorespirometrically by the method of Brown *et al.* (1991) in July, August and September 1994 samples. Time course data were collected to determine optimum incubation durations for all substrates in nutrient amended (Bushnell-Haas broth, BH; Atlas, 1993) slurries. Nutrient amendments were used to evaluate the potential activity of the extant microbial populations irrespective of the nutrient status of each soil sample assayed. Samples were diluted 10-fold (wet weight basis) in BH, 10 ml slurry placed in 40-ml glass septum vials, and each vial spiked with 50 µl of a 2 µg · µl$^{-1}$ solution of a radiolabeled substrate (UL-$^{14}$C L- glutamic acid in water, ca. 830 Bq; 1-$^{14}$C n-hexadecane in acetone, ca. 680 Bq; or 9-$^{14}$C phenanthrene in acetone, ca. 1230 Bq). Samples were incubated at 21°C until soil respiration was stopped by addition of 1 ml 10N NaOH to the vial. Glutamate sample time courses were incubated 0, 6, 12, 18, and 24 h, hexadecane time courses for 0, 24, 47 and 72 h, and phenanthrene time courses for 0, 47, 72, and 97 h. Based on the time course assays, samples were incubated 18 h for glutamate mineralization potential determinations and 67 h for hexadecane and phenanthrene. Final mineralization potentials were recorded as µg substrate C mineralized · g dry soil$^{-1}$ · d$^{-1}$.

Total soil C mineralization potentials were measured for all organic horizon samples in 1994 and 1995. Ten g homogenized soil samples were placed in half-pint canning jars (Mason jars; Alltrista Corp., Muncie, IN) equipped with sealable lids

fitted with butyl rubber septa for headspace gas sampling. Soil moisture was adjusted to 60% WHC, and samples were allowed to equilibrate for a few (ca. 2 to 4) days. Jars were then sealed and incubated at 15°C. Soil C mineralization was determined by measuring $CO_2$ evolved in the jar headspace by gas chromatography (Shimadzu GC-14A gas chromatograph; Shimadzu Corp., Kyoto, Japan) at ca. one week intervals. Jars were vented to the atmosphere after each measurement to maintain aerobic conditions in the jar. Headspace $CO_2$ concentration was converted to μg C mineralized and soil C mineralization rate calculated from incubation durations.

Community-level substrate utilization in 1995 samples was evaluated using Biolog GN microtiter plates (Biolog Inc., Hayward, CA) and the kinetic data extraction technique of Lindstrom *et al.* (in press; see Chapter 1). Each sample was diluted $10^3$-, $10^4$- and $10^5$-fold before being inoculated into the microplates. Plates were incubated at 21°C for 7 to 10 days. Dye reduction rates due to substrate oxidation in each microplate well were measured (595 nm) on a microplate reader (Model 3550-UV, Bio-Rad Laboratories, Hercules, CA) over the course of the incubation period. The technique has been used elsewhere in this study to assess community population diversity by multivariate analysis of substrate metabolism patterns (see Chapter 2). Community-level substrate use rates for individual substrates are reported here to evaluate soil communities' N mineralization potentials.

Data Treatment

To express the microbiologically relevant data per unit mass of original soil matrix, we wished to correct for added crude oil mass in each OIL sample. However, attempts to correct for the contribution of oil C to soil total C were not successful. Total C and N analysis of DCM-extractable material (N = 28) from several OIL samples (n = 17) indicated a mean value (± s.e.) of 85.9% (± 0.45%) carbon and 0.31% (±0.02%) nitrogen in the residue. The mass of DCM-extractable residue for each sample was multiplied by the average residue C content (i.e., 0.859 g C · g DCM

extract$^{-1}$), and this value was then subtracted from the total soil C value obtained for each sample. The "corrected" mean soil C (± s.e.) was 0.123 (±0.015) g C · g dry soil$^{-1}$ for REF samples and 0.046 (± 0.006) g C · g dry soil$^{-1}$ for OIL samples. This suggested that OIL sample soil organic C not attributable to crude oil was ca. 37% that of REF samples, a finding not supported by other studies at this site (e.g., Sparrow and Sparrow, 1988). A similar correction procedure also was used in an unsuccessful effort to correct the organic matter (as mass loss on combustion) data by subtracting the DCM-extract mass from organic matter.

Because we were unable to correct for added crude oil C or organic matter mass in the soil samples, all microbiological data expressed as mass or number per g soil were transformed from a g dry soil basis to a g inorganic matter basis. Physical and chemical data were transformed the same way when necessary for correlation to microbiological data. This was accomplished by dividing the dry soil datum by g inorganic matter · g dry soil$^{-1}$ for that sample. As organic matter content was significantly higher in OIL than in REF samples, normalizing data per unit inorganic matter was considered the most reliable way to compare samples without unduly biasing the data as a result of oiling treatment. By expressing values per g inorganic matter, the data are expressed in units that likely have not changed as a result of crude oil treatment.

Some microbiological data also were transformed to a g organic matter basis. Data were considered per g organic matter to expose microbiological effects due to substrate quality (see Discussion). These data were transformed in a manner similar to the inorganic matter data.

Student's t-tests, analyses of variance and various other statistical analyses were performed with Systat for Windows (version 5.05; SPSS, Inc. Evanston, IL), and best-fit linear regressions and all graphics were accomplished using Microcal Origin data analysis and graphing software (version 4.00; Microcal Software, Inc., Northampton,

MA). Where multiple comparisons were used, appropriate Bonferroni-adjusted probabilities are reported for the number of comparisons made (Zar, 1984).

## RESULTS

### Physical and Chemical Parameters

Soil temperatures were higher in the OIL plot than in the REF plot at each sampling ($p < 0.001$), with the exception of the October samplings for each year ($p > 0.5$; Table 3-1). Evidence of hydrophobic oil in the soil matrix of OIL samples can be seen in the generally reduced soil moisture and WHC for OIL samples compared to the values for REF samples on the same sampling date (see Table 3-2). On a dry soil basis, DCM-extractable material (residual "oil") was negatively correlated with water holding capacity (Spearman rank; $r_s = -0.53$) and soil moisture ($r_s = -0.63$) in the soil samples ($n = 74$). Soil moisture was similar between the two sample types in summer of 1994 ($p = 1$) but was generally higher in REF samples later that season and in 1995 samples ($p < 0.05$; except September 1995, $p = 1$). Mean values for WHC varied less among samples that were oiled (range of 1.42 to 2.40 g $H_2O \cdot$ g dry soil$^{-1}$) than among REF samples (range of 1.52 to 4.36 g $H_2O \cdot$ g dry soil$^{-1}$). Despite the overall greater moisture per g dry soil mass in REF samples however, degree of saturation at time of sample collection was similar for both treatments due to the relatively higher WHC of REF samples (Table 3-2). In September 1994 REF samples were more saturated than OIL ($p = 0.001$) and in October 1995 OIL samples were more saturated than REF ($p = 0.011$); there was no difference ($p > 0.2$) in soil saturation between treatments on all other sampling dates.

Soil pH for both REF and OIL organic horizon samples was quite low and ranged from 4.10 to 4.95. Mean (± s.e.) pH was 4.58 (± 0.03) for REF samples and 4.47 (± 0.03) for OIL samples, indicating little influence of oiling on the natural pH of the horizon. Soil pH did not vary with season or with year of sampling ($p > 0.2$).

Table 3-1. Soil temperatures for each sampling date in 1994 and 1995. Mean temperatures for all samples are presented and standard errors of means are given in parentheses. Except for October samplings, OIL plot soil temperatures were always warmer ($p < 0.001$) than those in the REF plot.

| | REF Plot | | OIL Plot | |
|---|---|---|---|---|
| Date | n | Soil Temperature (°C) | n | Soil Temperature (°C) |
| 7/13/94 | 6 | 0.0 (0.0) | 9 | 5.7 (0.2) |
| 8/11/94 | 6 | 7.9 (0.7) | 9 | 13.1 (0.6) |
| 9/20/94 | 6 | 0.9 (0.2) | 9 | 4.1 (0.2) |
| 10/11/94 | 5 | 0.4 (0.1) | 5 | 0.7 (0.1) |
| 7/5/95 | 5 | 4.8 (0.6) | 3 | 11.5 (0.4) |
| 9/5/95 | 5 | 2.0 (0.1) | 5 | 6.7 (0.4) |
| 10/17/95 | 5 | 0.2 (0.4) | 5 | 1.3 (0.3) |

Table 3-2. Soil moisture data for each sampling date and sample type (OIL or REF samples). Mean field moisture, water holding capacity (WHC) and percent saturation data are presented as mean values for all samples collected. Standard errors of the mean values are given in parentheses. Sample sizes are the same as those given in Table 3-1 for each treatment.

| | Mean Field Moisture ($g\ H_2O \cdot g\ dry\ soil^{-1}$) | | Mean Soil WHC ($g\ H_2O \cdot g\ dry\ soil^{-1}$) | | Mean Soil Saturation (fraction of WHC) | |
|---|---|---|---|---|---|---|
| Date | REF | OIL | REF | OIL | REF | OIL |
| 7/13/94 | 0.80 (0.09) | 0.82 (0.07) | 1.64 (0.22) | 1.66 (0.15) | 0.499 (0.032) | 0.491 (0.013) |
| 8/11/94 | 0.77 (0.06) | 0.74 (0.06) | 1.52 (0.17) | 1.83 (0.13) | 0.514 (0.022) | 0.404 (0.021) |
| 9/20/94 | 1.20 (0.08) | 0.85 (0.03) | 1.82 (0.25) | 1.74 (0.14) | 0.694 (0.069) | 0.501 (0.029) |
| 10/11/94 | 1.98 (0.13) | 0.83 (0.06) | 4.36 (0.27) | 2.40 (0.18) | 0.454 (0.016) | 0.349 (0.023) |
| 7/5/95 | 1.36 (0.13) | 0.83 (0.12) | 2.78 (0.46) | 1.45 (0.14) | 0.518 (0.050) | 0.569 (0.047) |
| 9/5/95 | 1.48 (0.14) | 1.27 (0.15) | 2.19 (0.31) | 1.69 (0.18) | 0.693 (0.038) | 0.753 (0.030) |
| 10/17/95 | 2.12 (0.19) | 1.23 (0.11) | 3.24 (0.48) | 1.42 (0.19) | 0.697 (0.078) | 0.890 (0.049) |

The organic matter and total soil C content of the samples reflected their origins, with REF samples generally having lower values than OIL samples (Table 3-3). The two independent measures of soil carbon content were strongly correlated when all dates and treatments were included in the analysis, with a correlation coefficient of 0.779 ($p < 0.001$). With the exception of the October 1994 samples, total soil C was significantly higher ($p < 0.06$) in OIL than in REF samples. Overall, mean total C (± s.e.) was 0.169 (± 0.011) g · g dry soil$^{-1}$ for REF samples (n = 34) and 0.295 (± 0.011) g · g dry soil$^{-1}$ for OIL samples (n = 45). Organic matter also was generally higher in OIL than in REF samples, though the mean values for September and October 1994, and July 1995, were not significantly different ($p > 0.1$) between treatments.

Overall, soil total N on a soil dry mass basis was very similar between the OIL and REF treatments, with mean values (± s.e.) of 0.63 % (± 0.02 %) for OIL samples and 0.68 % (± 0.04 %) for REF samples. Soil total inorganic N (essentially all $NH_4^+$-N) was higher in REF samples (n = 7) than in OIL samples (n = 12; $p < 0.001$) examined, but averaged ca. 1000-fold lower than total soil N (data not shown), suggesting that almost all N present is organically bound. Mean $NO_3^-$-N concentrations in both OIL and REF samples were essentially zero (averaging <1 μg · g dry soil$^{-1}$) and showed no treatment effects ($p > 0.3$). Mean $NH_4^+$-N values were somewhat higher than $NO_3^-$-N, but still quite low, with a higher mean concentration in REF samples ($p < 0.001$; mean ± s.e. of 7.4 ± 0.6 μg · g dry soil$^{-1}$ ) than in OIL samples (1.0 ± 0.2 μg · g dry soil$^{-1}$). Extractable P was not different between the two treatments ($p > 0.5$) and averaged (± s.e.) 3.7 (± 1.0) μg · g dry soil$^{-1}$ for REF samples and 3.2 (±0.3) μg · g dry soil$^{-1}$ for OIL samples.

The ratio of soil total C to total N was quite constant per g inorganic matter across all sampling dates for each treatment, with OIL samples' ratios more than twice those seen in REF samples. Total C was strongly correlated with total N for both REF ($r^2 = 0.96$, $p < 0.001$) and OIL ($r^2 = 0.82$, $p < 0.001$) samples. The mean ratio (± s.e.)

Table 3-3. Mean soil organic matter and total soil C for OIL and REF samples. Sample sizes are the same as given in Table 3-1 for each treatment. Standard errors of the mean values are given in parentheses.

| | Organic Matter ($g \cdot g$ dry $soil^{-1}$) | | Total C ($g \cdot g$ dry $soil^{-1}$) | |
|---|---|---|---|---|
| Date | REF | OIL | REF | OIL |
| 7/13/94 | 0.279 (0.039) | 0.514 (0.041) | 0.107 (0.016) | 0.295 (0.024) |
| 8/11/94 | 0.118 (0.021) | 0.346 (0.016) | 0.107 (0.009) | 0.253 (0.011) |
| 9/20/94 | 0.201 (0.005) | 0.315 (0.033) | 0.163 (0.011) | 0.251 (0.020) |
| 10/11/94 | 0.357 (0.035) | 0.326 (0.051) | 0.267 (0.018) | 0.313 (0.034) |
| 7/5/95 | 0.300 (0.017) | 0.336 (0.045) | 0.197 (0.016) | 0.297 (0.039) |
| 9/5/95 | 0.203 (0.047) | 0.368 (0.039) | 0.167 (0.009) | 0.336 (0.017) |
| 10/17/95 | 0.400 (0.035) | 0.594 (0.052) | 0.204 (0.019) | 0.395 (0.038) |

of C to N was 23.0 (± 0.8) for REF samples and 52.7 (± 3.8) for OIL samples ($p < 0.001$).

Petroleum Hydrocarbons

Dichloromethane (DCM) -extractable material was present in much higher amounts in OIL than in REF samples ($p < 0.001$; Table 3-4). Concentrations of DCM-extractable material did not vary among seasons or between years of sampling for REF samples ($p = 1.0$). All OIL samples generally had uniform concentrations of this material irrespective of year or season of sampling (data not shown), with most samples showing no significant differences among seasons or between years ($p > 0.38$). October 1995 samples appeared to contain somewhat higher concentrations than other OIL samples, with a mean (± s.e.) value of 0.368 (± 0.053) g DCM-extractables · g dry soil$^{-1}$. Samples from this date ($n = 5$) had significantly higher extractable material than August ($n = 8$; $p = 0.048$) or September ($n = 8$; $p = 0.004$) 1994 samples.

Gravimetric analysis of DCM extracts showed that a significant fraction of the material in OIL samples was "asphaltene" (high molecular weight, pentane insoluble) in nature (Table 3-4). We were unable to detect asphaltene fractions in REF samples with our protocol (all less than 0.001 g · g dry soil$^{-1}$). The pentane-soluble extract components were amber to light green in color, with much higher levels in OIL samples, and low REF sample values almost equivalent to their total DCM-extractable amounts (Table 3-4). A large fraction of pentane-solubles was removed in the solid phase extraction (SPE) step, and all color in the original pentane solution was retained in the polar solid phase of the SPE tube. Mean concentrations of residue in the SPE eluate (Table 3-4) were ten-fold higher in OIL than in REF samples. This final fraction was subjected to analysis by GC/FID as described above. Samples from the REF site had too little of this fraction for GC analysis (i.e., analytes were below detection limits).

Table 3-4. Gravimetric hydrocarbon concentrations for OIL (n = 41) and REF (n = 11) soil samples. Data are mean values per g dry soil for all samples measured; standard errors are given in parentheses. Asphaltenes were not detected (ND) in REF samples.

| Gravimetric Datum | OIL | REF |
|---|---|---|
| DCM-extractable material | 0.285 (0.015) | 0.017 (0.001) |
| asphaltenes | 0.052 (0.007) | ND |
| pentane soluble material | 0.234 (0.015) | 0.016 (0.001) |
| SPE eluate | 0.041 (0.002) | 0.004 (0.001) |

Analysis of pentane-soluble, SPE eluate for the 32 OIL samples characterized by GC/FID showed that relatively little low molecular weight (MW) material was present and that heavier components of the mixture were present in abundance increasing with MW (Table 3-5). The GC conditions for this analysis allowed detection of material up to ca. C44 compounds (corresponding to MW of ca. 620). Comparison of "resolvable" GC/FID chromatographic fractions to total area integrated also showed that the residual hydrocarbons were weathered and appeared to provide evidence of biodegradation. The ratio of "resolved" peak to total area integrated for 32 OIL samples ranged from a minimum of 0.061 to a maximum of 0.221 (median of 0.115), with a mean value (± s.e.) of 0.117 (± 0.007). This ratio was not calculated for the fresh Prudhoe Bay crude (PBC) oil spilled at this site, but Sexstone *et al.* (1978b) calculated the value for both fresh and artificially weathered (by blowing sterile air over the oil for 96 h) PBC oil from 1976, the year PBC oil was applied to our study site. The ratio was 0.312 for fresh 1976 PBC oil and 0.206 for the artificially weathered material.

Microbiological Parameters

Fungal biomass was the predominant contribution to soil microbial biomass in both OIL and REF samples, averaging ca. $10^4$ $\mu g \cdot g$ soil inorganic matter$^{-1}$ for both soils (Fig. 3-1). No clear difference due to oiling was evident for fungal biomass; on some sampling dates fungal biomass was greater in the OIL samples and on others it was greater in REF samples. Rainfall events prior to the October 1994 sampling (see soil moisture data, Table 3-2) appear to have stimulated fungal growth in the REF samples (Fig. 3-1), but this stimulation was not seen in OIL samples from the same date. Active fungal biomass (Fig. 3-2), as determined by fluorescein diacetate staining, was orders of magnitude lower than the total fungal value for each treatment. Biomass of active fungi by this assay was typically 10- to 100-fold higher in REF samples than OIL samples for every sampling date. As a fraction of total fungal biomass, REF

Table 3-5. Composition of SPE eluate determined from gas chromatographic data for OIL samples (n = 32) and comparison to a Prudhoe Bay crude (PBC) oil sample (estimated from data in National Research Council, 1985). Approximate C chain length of each fraction was estimated from n-alkane retention time standards. Approximate molecular weights are for n-alkanes of the given chain length. Percent total area represents the mean percentage of the total chromatogram area for all samples analyzed. Standard errors of the mean are given in parentheses. Data for PBC represent approximate mass fraction percentages corresponding to boiling point ranges for each fraction listed.

| Carbon chain length | Molecular weight | % Total Area | PBC |
|---|---|---|---|
| <C12 | MW < ca. 170 | 1.3% (0.2%) | 30% |
| C12 to C16 | MW 170 to 226 | 13.7% (0.5%) | 28% |
| C16 to C24 | MW 226 to 340 | 40.8% (0.5%) | 24% |
| >C24 | MW > ca. 340 | 44.3% (0.9%) | 18% |

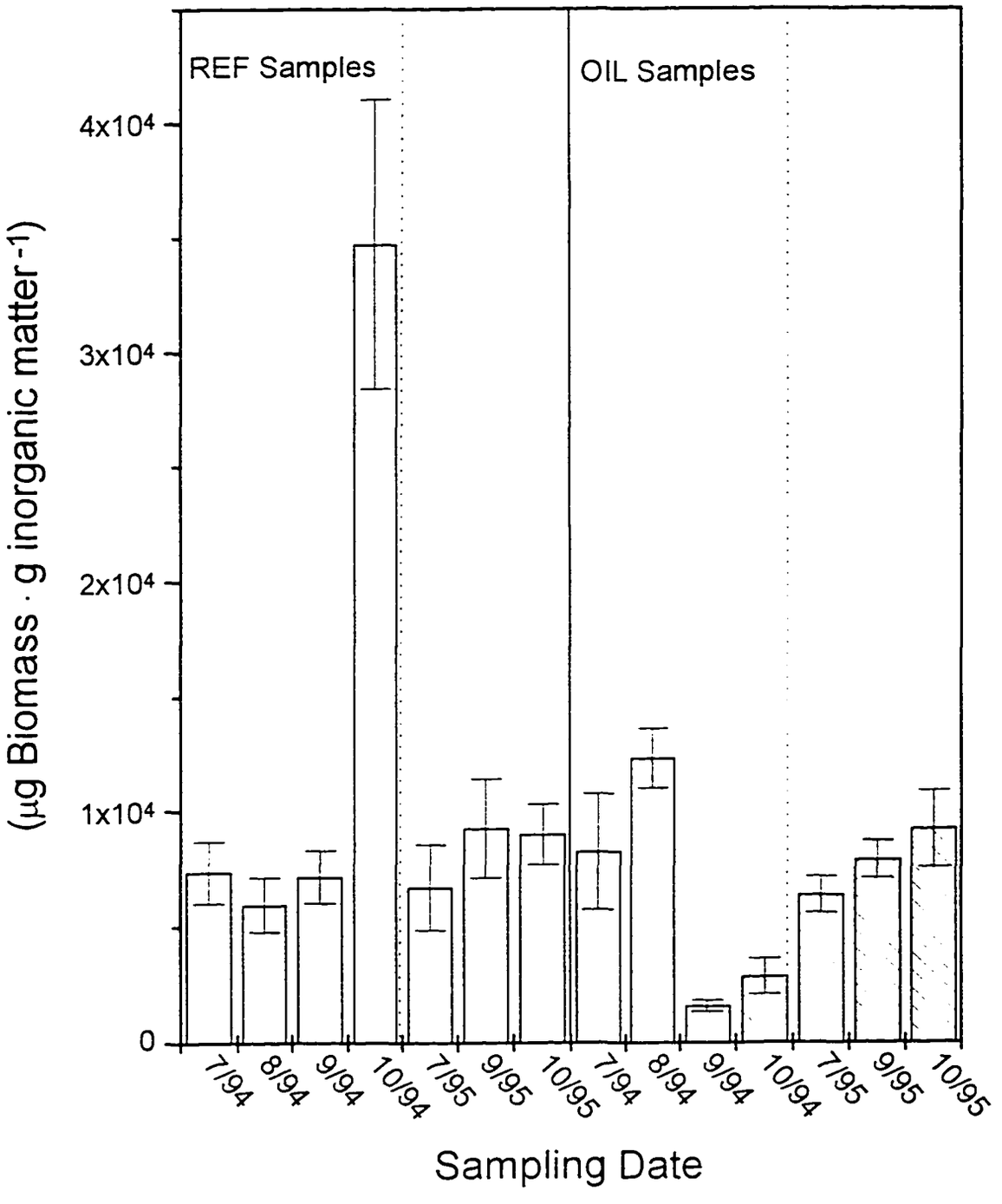


Fig. 3-1. Total fungal biomass per g inorganic matter in REF and OIL samples for all sampling dates. Column height represents mean value of all samples for that date; error bars are standard errors of the mean. Sample sizes are the same as given in Table 3-1 for each sampling date and sample type.

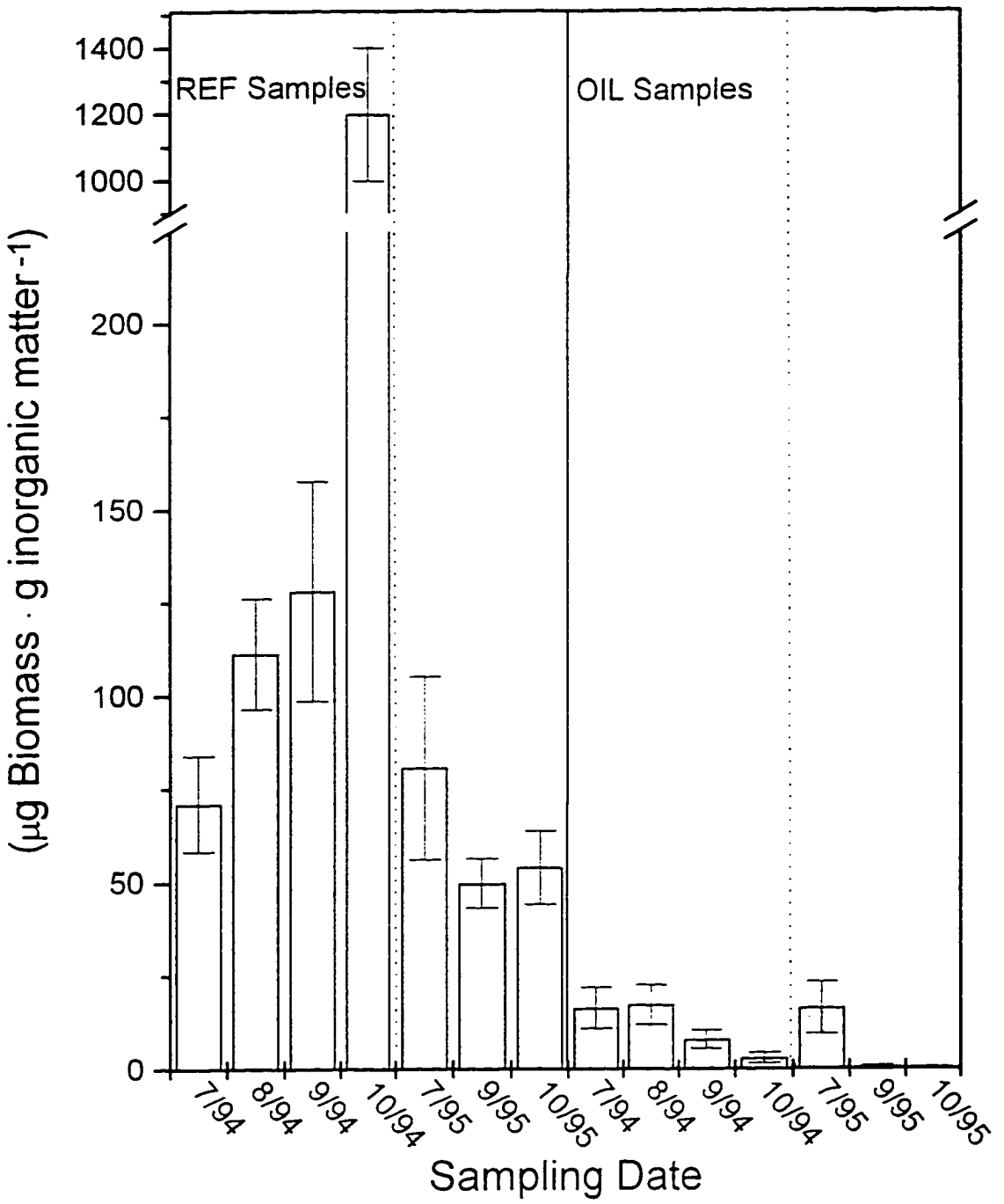


Fig. 3-2. Active fungal biomass per g inorganic matter in REF and OIL samples for all sampling dates. Column height represents mean value of all samples for that date; error bars are standard errors of the mean. Note broken scale on biomass axis allowed REF sample for October 1994 to be displayed on graph. Sample sizes are the same as given in Table 3-1 for each sampling date and sample type.

samples always had more active fungi, but this never represented more than ca. 5% of the total (Fig. 3-3A). The peak in REF total fungal biomass seen in Figure 3-1 for October 1994 also is seen for the REF active fraction on the same date (Fig. 3-2).

Total bacteria appeared to represent a minor fraction of the microbial biomass present in these soils, with values ranging from ca. 5 to 175 μg biomass (ca. $10^7$ to $10^9$ cells) · g soil inorganic matter$^{-1}$ (Fig. 3-4). OIL sample bacterial biomass values exhibited greater variation across seasons than REF sample values, but no clear trend was evident with respect to season or treatment. Active bacterial biomass always was higher in REF than in OIL samples (Fig. 3-5) but, as with the total bacterial biomass, no obvious seasonal trend was seen. The ratio of active to total bacterial biomass (Fig. 3-3B) was higher than the ratio of active to total fungal biomass (Fig. 3-3A) for both treatments, and active bacterial biomass was as high as ca. 60% of the total.

Most probable number (MPN) enumerations of hydrocarbon degrading microbes indicated that, compared to REF samples, OIL samples were enriched for organisms adapted to crude oil (Fig 3-6). Assuming this MPN assay primarily measures bacterial hydrocarbon degraders, OIL samples' bacterial populations were ca. 1% to 10% hydrocarbon degraders. REF samples also contained microbes able to emulsify crude oil, but MPN values for REF samples were uniformly two or three orders of magnitude lower than those for OIL samples (Fig. 3-6). Populations of hydrocarbon degraders per g inorganic matter essentially were constant across sampling dates in OIL samples, with no seasonal trend. Greater variation in numbers with respect to sampling date was exhibited in REF samples, but hydrocarbon degrader cell numbers were never more than 0.01% of total bacterial numbers.

Protozoan MPN data were quite variable both within and among sampling dates for both treatments, as can be seen for flagellate protozoa (Fig. 3-7). Flagellates were the most common protozoa observed in all soil samples at all dates (data not shown) and were used as an index of total protozoal abundance in these soils. Amoebae generally were present in numbers ca. 10- to 100-fold fewer than flagellates, and

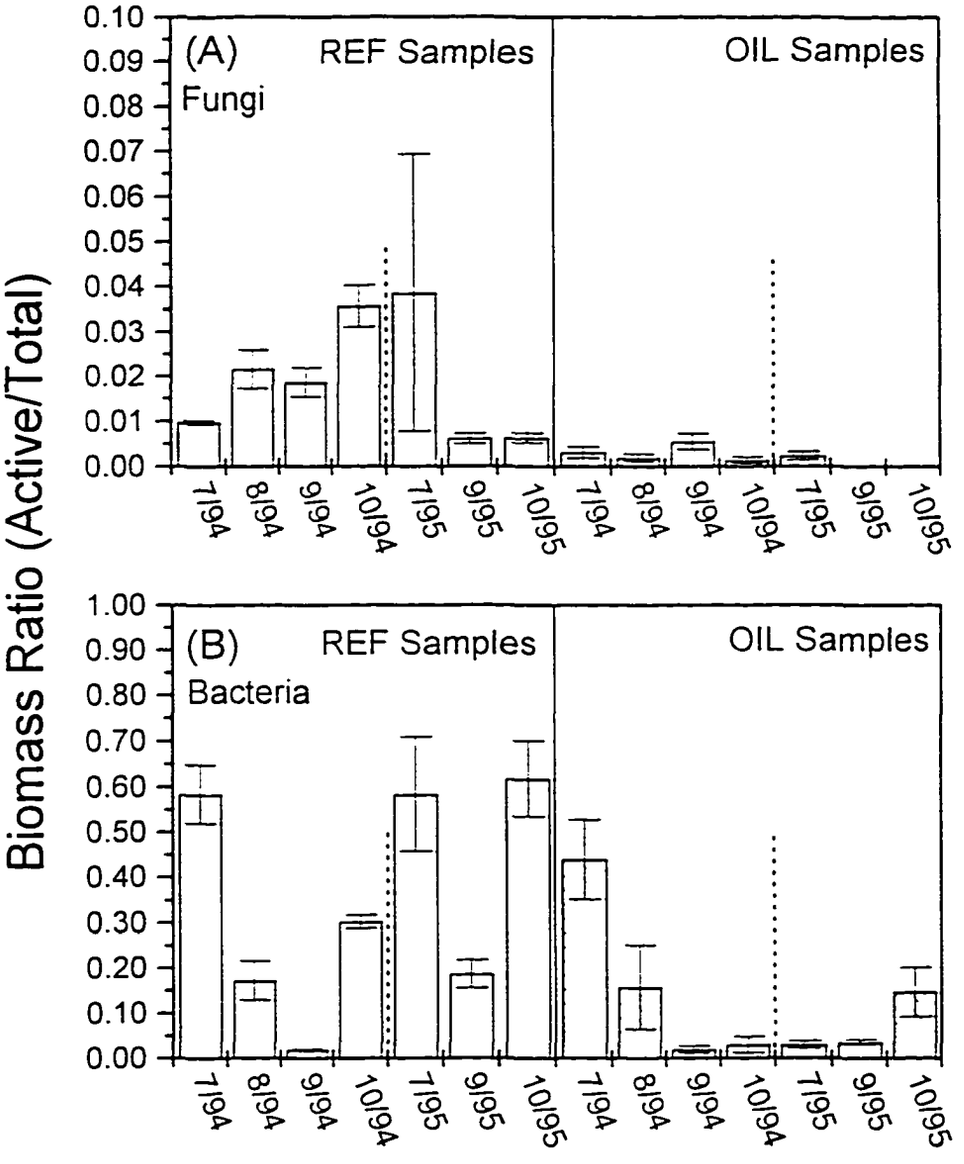


Fig. 3-3. Ratios of active to total biomass for (A) fungal and (B) bacterial biomass estimates. Column height represents proportion of REF and OIL samples showing FDA activity for that biomass estimate. Error bars are standard errors of the ratios presented. Sample sizes are the same as given in Table 3-1 for each sampling date and sample type.

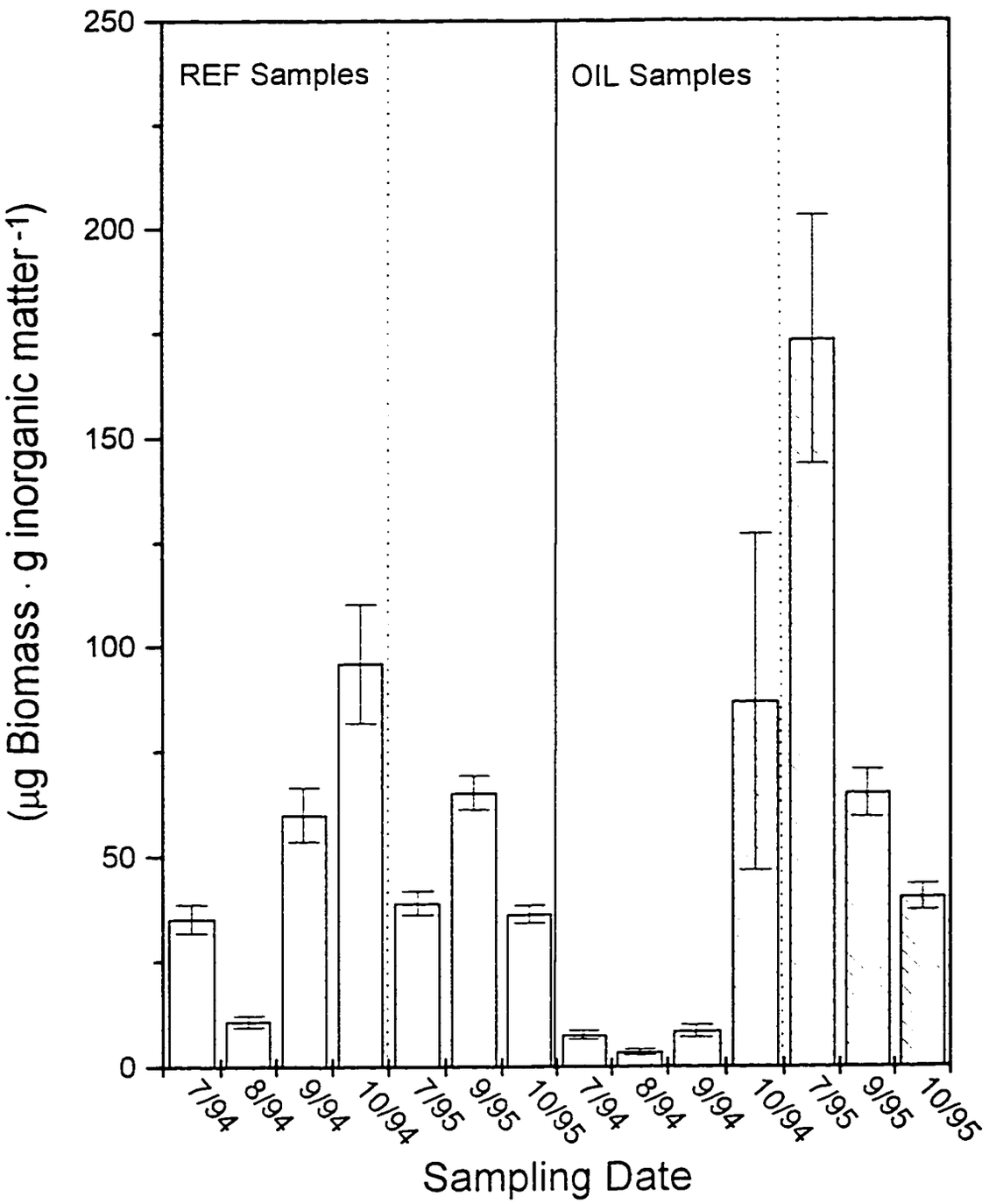


Fig. 3-4. Total bacterial biomass per g inorganic matter in REF and OIL samples for all sampling dates. Column height represents mean value of all samples for that date; error bars are standard errors of the mean. Sample sizes are the same as given in Table 3-1 for each sampling date and sample type.

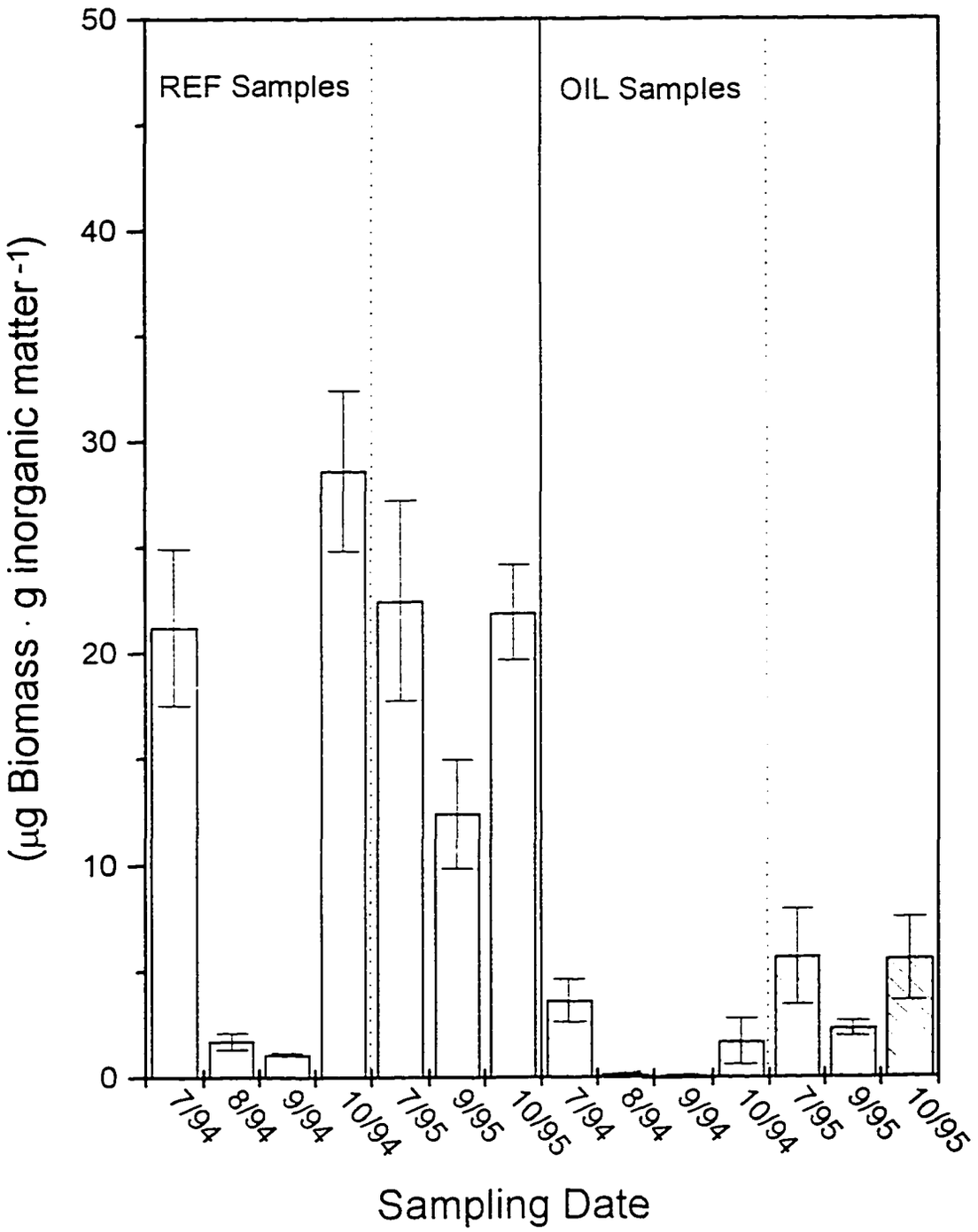


Fig. 3-5. Active bacterial biomass per g inorganic matter in REF and OIL samples for all sampling dates. Column height represents mean value of all samples for that date; error bars are standard errors of the mean. Sample sizes are the same as given in Table 3-1 for each sampling date and sample type.

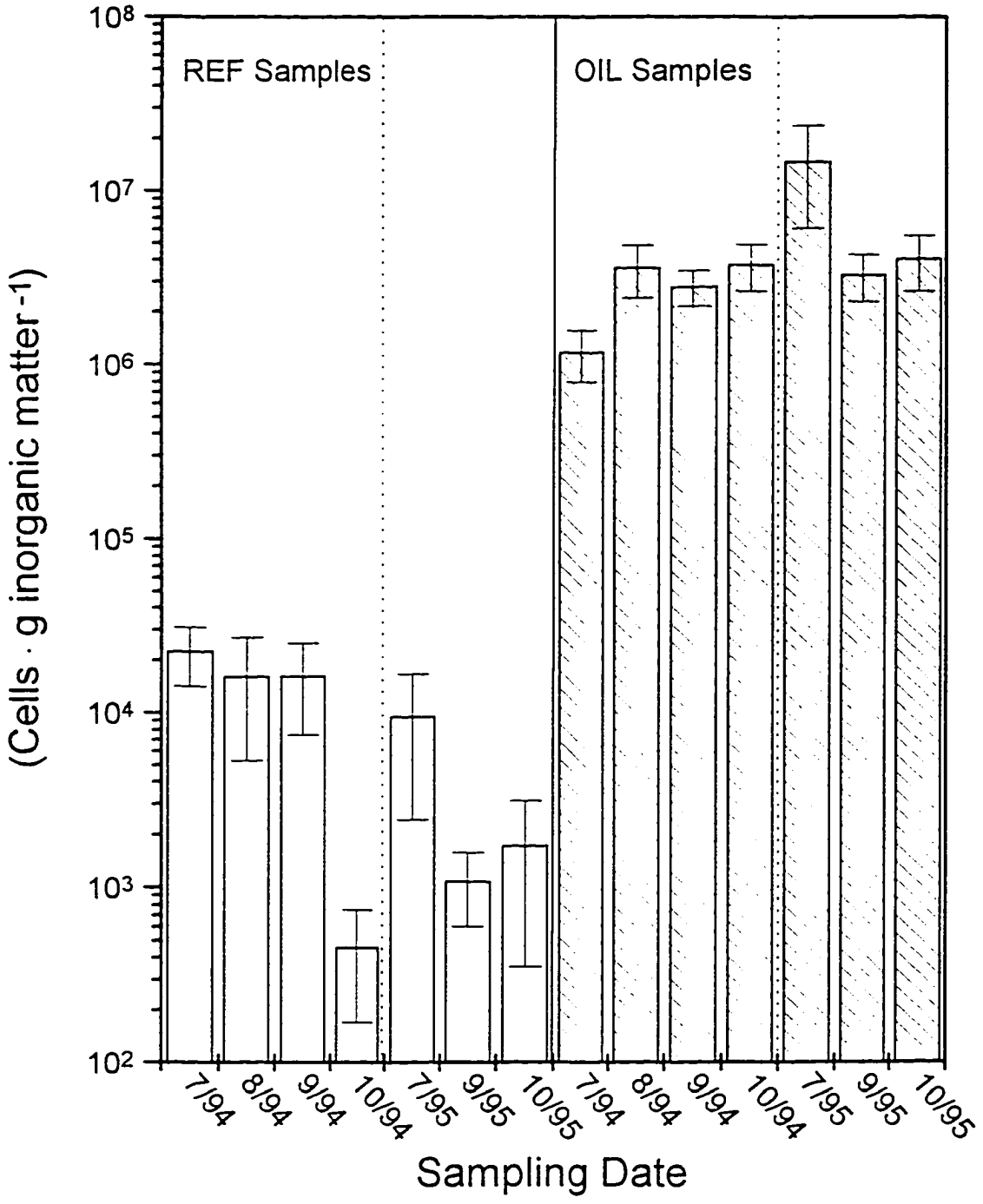


Fig. 3-6. Numbers of hydrocarbon degrading cells per g inorganic matter for REF and OIL samples. Scale is logarithmic with column height representing the mean of all samples for that sampling date. Error bars are standard errors of the mean. Sample sizes are the same as given in Table 3-1 for each sampling date and sample type.

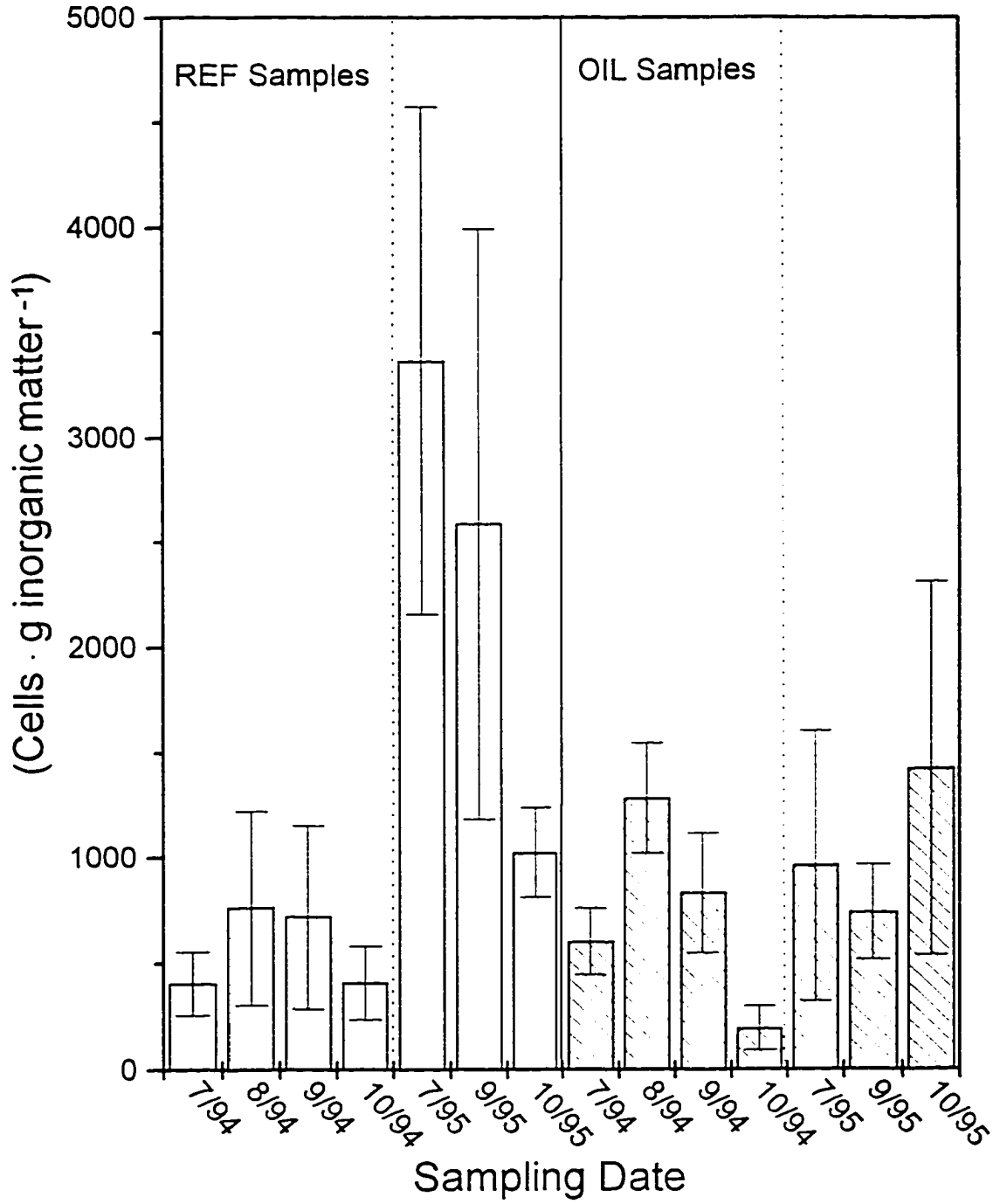


Fig. 3-7. Numbers of flagellate protozoa per g inorganic matter in REF and OIL samples. Column height represents mean value of all samples for that date; error bars are standard errors of the mean. Sample sizes are the same as given in Table 3-1 for each sampling date and sample type.

ciliates only were observed three times in two years. No seasonal or treatment trends were seen in these data, with relative abundance of flagellates in REF samples ranging from ca. 0.5 to 4 times that in OIL samples from the same date.

The general trends in biomass data expressed per g inorganic matter were also seen when the data were transformed to an organic matter basis. The abundant additional C associated with the crude oil tended to depress values expressed per unit organic matter for OIL samples relative to REF samples, and differences attributable to oiling seen per unit inorganic matter were more pronounced in these data. Total fungal biomass trends showed the greatest change on transformation to an organic matter basis, with every sampling date's REF mean higher than the corresponding OIL mean. Total bacterial biomass differences remained essentially the same on data transformation; the main effect appears to have been a reduction in the amount of variability shown by REF sample means across seasons. As with inorganic matter data, active fungal and bacterial biomasses per g organic matter were uniformly higher in REF than in OIL samples collected the same date. Hydrocarbon degraders were two to three orders of magnitude higher in OIL than in REF samples, but protozoa (as flagellates) were similar across treatments (data not shown).

Substrate-specific mineralization potentials determined by radiorespirometry for soil samples collected in July, August and September of 1994 demonstrated differences between treatments in the potential activities of hydrocarbon-degrading soil microbial populations. Glutamate time course data were similar for both REF and OIL soil samples assayed (Fig. 3-8A). Time course data for hexadecane (Fig. 3-8B) and phenanthrene (Fig. 3-8C) radiorespirometry assays showed differences in hydrocarbon acclimation associated with treatment. Hexadecane mineralization potential for the REF samples reached approximately the same level as the OIL samples after 72 h (Fig. 3-8B), but it took longer to achieve this level, indicating slower *in vitro* acclimation of hexadecane-degrading microbial populations in REF samples than OIL samples. Phenanthrene mineralization time courses showed that OIL soil samples were well-

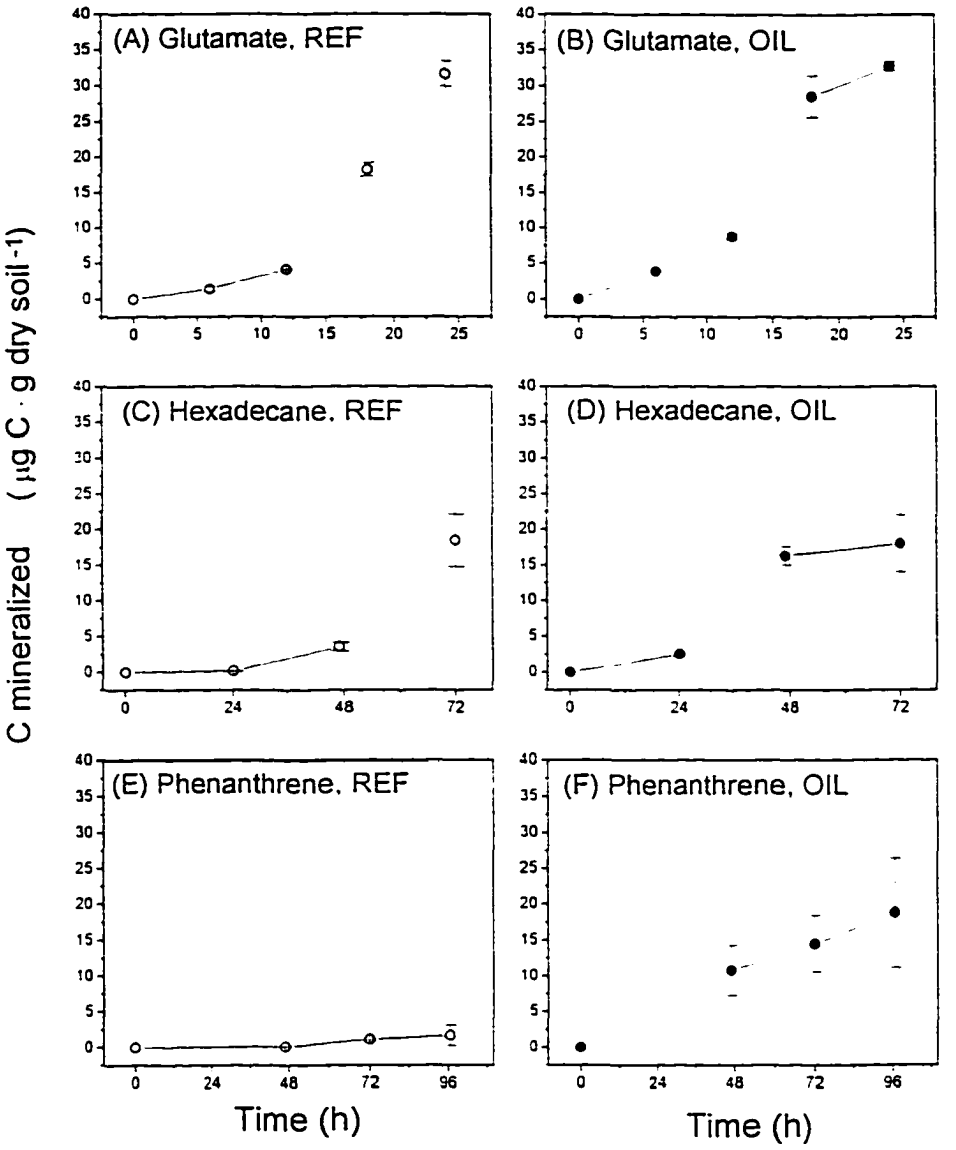


Fig. 3-8. Radiorespirometry time courses for the substrates glutamate (A, REF sample; B, OIL sample), hexadecane (C, REF sample; D, OIL sample), and phenanthrene (E, REF sample; F, OIL sample). Substrate C mineralization data are plotted per g dry soil for nutrient amended REF (O) and OIL (●) samples. Data points are mean values of triplicate analyses and error bars are standard errors of the mean.

adapted to phenanthrene (Fig. 3-8C). In contrast, REF sample time course data indicate little ability of the extant microbial population in the slurry to acclimate to the presence of phenanthrene after a 96 h incubation (Fig. 3-8C).

Organic horizon mineralization potentials per g inorganic matter for glutamate based on short-term (18 h) incubations were similar for OIL and REF samples (Fig. 3-9A and B); July and August 1994 REF samples were not different from OIL samples ($p > 0.5$), while in September 1994 they were slightly higher ($p < 0.01$). Organic horizon hexadecane (Fig. 3-9C and D) and phenanthrene (Fig. 3-9E and F) mineralization potentials per g inorganic matter, on the other hand, were significantly lower ($p < 0.001$) in REF than OIL samples for all months. OIL sample hexadecane (Fig. 3-9D) and phenanthrene (Fig. 3-9F) mineralization potentials were highest in July 1994 and decreased through September 1994. REF samples' hexadecane potentials (Fig. 3-9C) showed no seasonal trend and averaged ca. 20% of the OIL samples' rates, while phenanthrene mineralization potential rates were uniformly low for REF samples (Fig 3-9E) and averaged ca. 8% of the OIL samples' rates.

On transformation to an organic matter basis, glutamate mineralization potentials showed treatment differences, with REF samples' potentials at least twice those of the OIL samples (data not shown). The transformed data also indicate populations in the OIL plot were well-acclimated to hydrocarbon mineralization; OIL samples' mean potentials were higher for both hexadecane and phenanthrene substrates, with one exception. Hexadecane mineralized per g organic matter did not differ between OIL and REF samples in August 1994, suggesting REF populations from that date were likely acclimated to this substrate *in situ*.

Mean organic soil C mineralization rates essentially were constant over the course of the 30-day assay (data not shown) and the average daily rates per g inorganic matter are presented in Figure 3-10. Mean REF soil respiration rates generally were not significantly different ($p = 1$) from those seen in OIL samples from the same dates, with the possible exception of the July 1994 ($p = 0.07$) and October 1995 ($p < 0.001$)

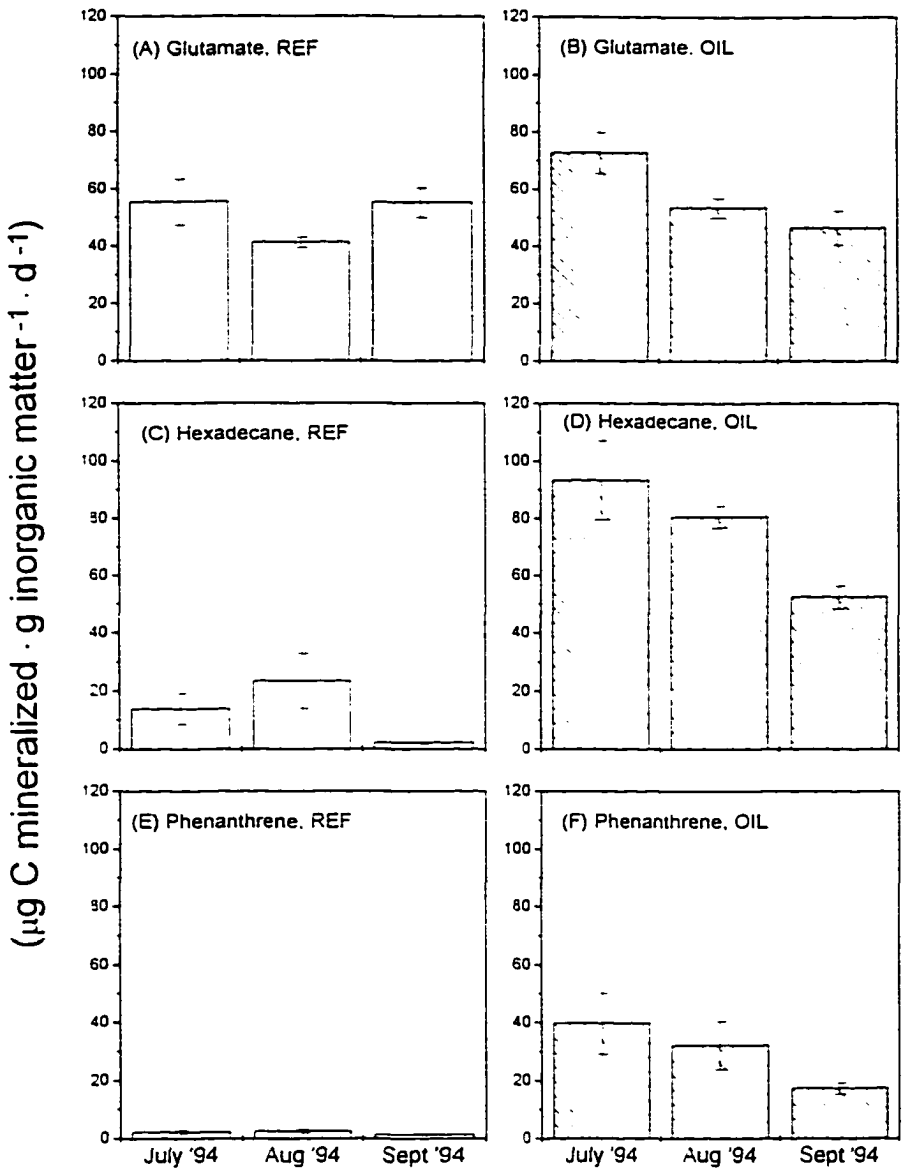


Fig. 3-9. Substrate C *in vitro* mineralization rates for glutamate (A, REF samples; B, OIL samples), hexadecane (C, REF samples; D, OIL samples), and phenanthrene (E, REF samples; F, OIL samples). Substrate C mineralization data are plotted per g inorganic matter for nutrient-amended soil slurries as described in the text. Column height represents the mean of all samples for that sampling date and sample type; error bars are standard errors of the mean. Sample sizes are the same as given in Table 3-1 for each sampling date and sample type.

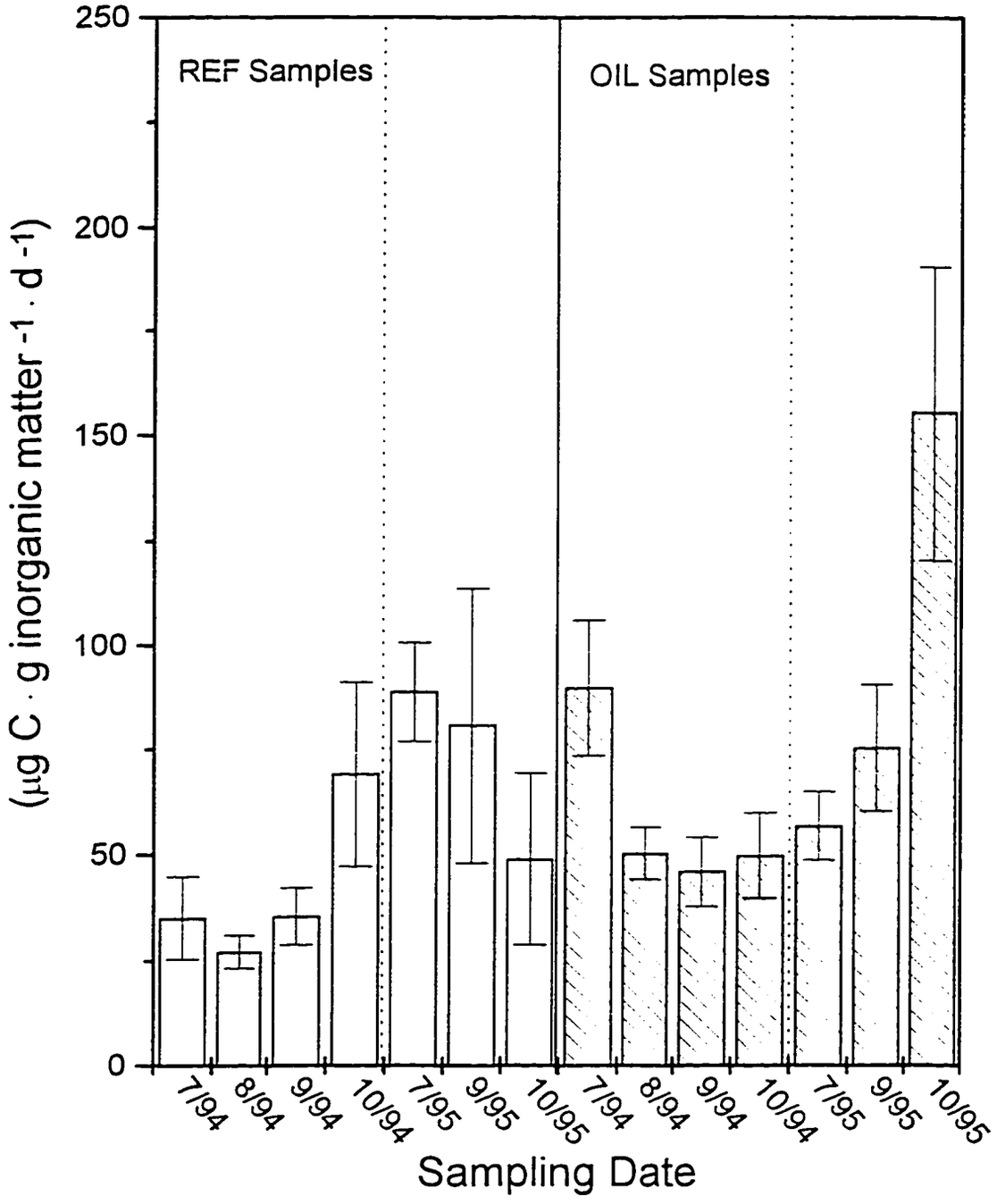


Fig. 3-10. Average daily soil C *in vitro* mineralization rates per g inorganic matter in REF and OIL samples for all sampling dates. Column heights represent mean values of C mineralization rates for all samples for that date; error bars are standard errors of the mean. Sample sizes are the same as given in Table 3-1 for each sampling date and sample type.

sampling dates. When the data were considered per g organic matter, REF samples' rates showed seasonal variation, while OIL samples' rates were essentially constant across seasons and years (Fig. 3-11). REF samples' rates were often higher than the OIL samples', though the differences were less pronounced in late season.

The few samples assessed for net N mineralization showed that, over the course of the 30-day assay, net nitrogen mineralization occurred in both REF ($n = 7$) and OIL ($n = 11$) samples. Net change in ammonium was not significantly different between the two treatments ($p = 0.444$). No net nitrification was observed in OIL samples, but REF samples' net nitrification was significantly higher ($p<0.1$). Differences seen between OIL and REF samples in N mineralization were not affected by data transformation. Per unit organic or inorganic matter, total net N mineralization was higher in the REF soils assayed ($p < 0.1$).

Dye reduction rate data collected from Biolog GN plates provides further information about N mineralization in OIL and REF samples. These microplates carry 95 carbon substrates, of which 34 contain nitrogen and 20 are amino acids. When data from all three dilutions ($10^3$-, $10^4$- and $10^5$-fold) for each treatment were considered together to calculate principal components (PCs), 41 substrates in the microplate had high loadings ($> 0.500$) on the first PC which accounted for ca. 24% of the total variance in the data set (see Chapter 2). Twenty-two of these 41 substrates contained N (carboxylic acids, amino acids and nucleosides), and all N-containing substrates in this group were metabolized faster (higher *r* values; Lindstrom *et al.* in press; Chapter 1) by REF samples ($p<0.001$). When all 34 N-containing substrates were examined at each dilution, REF samples used 19 substrates faster ($p < 0.05$) at the $10^3$ dilution, 22 substrates faster ($p < 0.05$) at the $10^4$ dilution, and 15 substrates faster ($p < 0.10$) at the $10^5$ dilution. Statistical theory would predict that fewer than four substrates would be significantly different by random chance at the 90% significance level. No N-containing substrates were used faster by OIL samples.

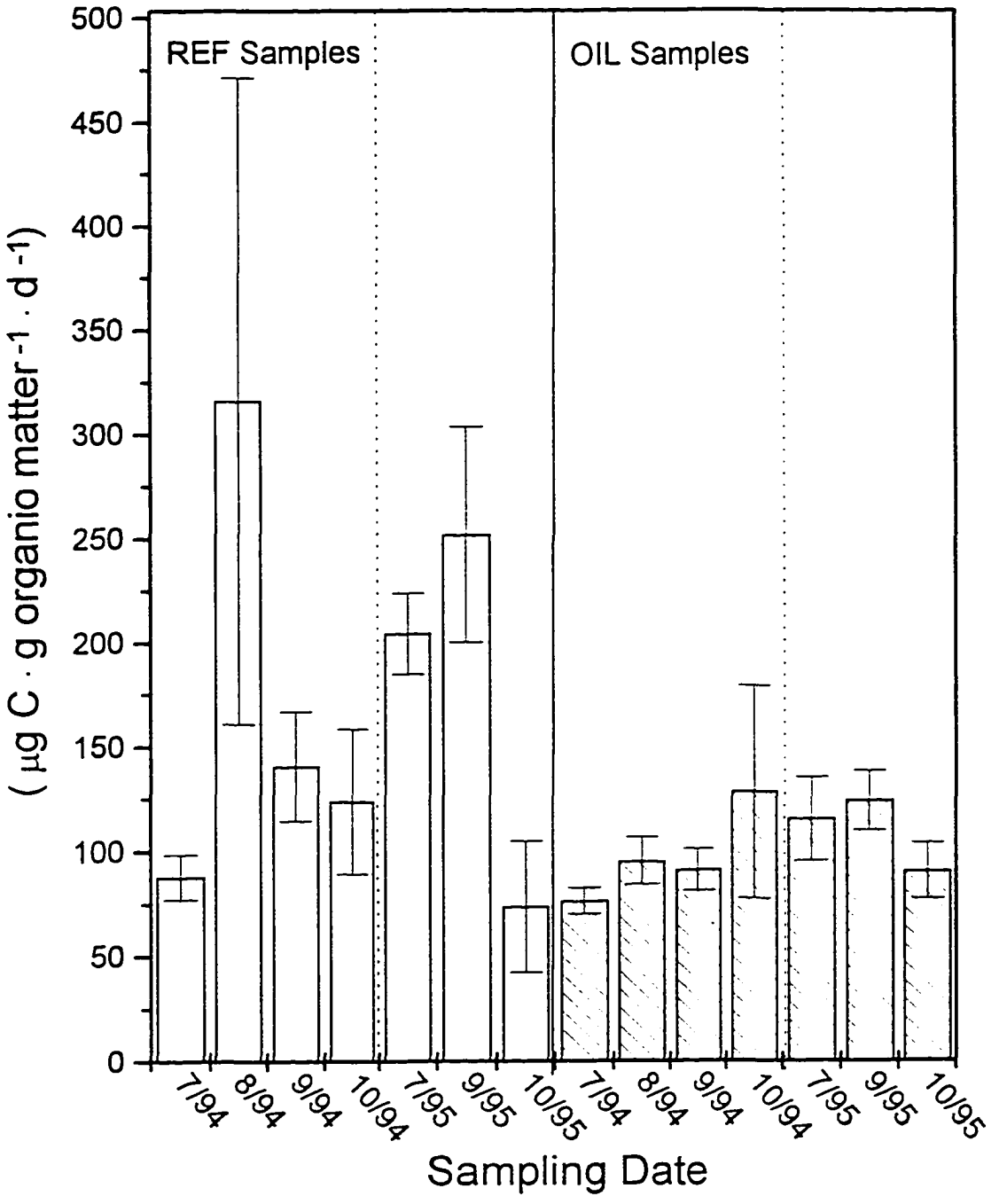


Fig. 3-11. Average daily soil C *in vitro* mineralization rates per g organic matter in REF and OIL samples for all sampling dates. Column heights represent mean values of C mineralization rates for all samples for that date; error bars are standard errors of the mean. Sample sizes are the same as given in Table 3-1 for each sampling date and sample type.

Organic soil respiration rate per g inorganic matter was strongly correlated in OIL samples with soil total C content and total N content, but much less so in REF samples (Table 3-6). Multiple regression analysis showed that total soil C ($p < 0.001$) and active bacterial biomass ($p = 0.001$) had the greatest influence in predicting soil respiration per g inorganic matter overall (multiple regression $r^2 = 0.733$). Multiple regression analysis was applied to OIL and REF samples independently. For OIL samples only soil total C ($p < 0.001$) had predictive value for soil respiration ($r^2 = 0.867$). In REF samples a fairly weak linear relationship ($r^2 = 0.36$) between soil respiration and total soil C was seen. Respiration per g inorganic matter in OIL samples was well-correlated with glutamate, hexadecane, and phenanthrene mineralization potentials, but these correlations were low in REF samples (Table 3-6). Soil respiration was not correlated with total or active bacterial or fungal biomass, or with protozoal numbers for either OIL or REF samples (Table 3-6). Biomass estimates for OIL samples were not correlated with soil C or N (with the possible exception of active bacterial biomass), while REF samples' total and active bacterial and fungal biomass estimates were fairly well correlated with soil C and N (Table 3-6). No correlation was observed between concentrations of hydrocarbons, or hydrocarbon fractions, and any biomass or respiration measurement, except for the clear differences in hydrocarbon degraders and active biomass estimates as a result of oiling (data not shown).

Expressed per g organic matter, soil respiration in both OIL and REF samples was positively correlated to total soil C and N (OIL: $r^2 = 0.66$ for soil C and $r^2 = 0.50$ for soil N; REF: $r^2 = 0.89$ for C and $r^2 = 0.85$ for N; $p < 0.001$ in all cases). The influence of soil C and N on soil respiration per g organic matter was examined in OIL and REF samples independently by multiple regression. For REF samples, a strong linear relationship ($r^2 = 0.847$) between respiration per g organic matter and total soil C ($p < 0.001$) and total soil N ($p = 0.031$) was seen. The same analysis for OIL samples showed a weaker relationship ($r^2 = 0.625$), and demonstrated that soil C was a good

Table 3-6. Correlations between various microbial and soil parameters. Soil respiration and specific substrate respiration data are mineralization potentials measured as described in Material and Methods. Bacterial and fungal data are biomass estimates, and protozoal data are numbers of flagellate protozoa. All data are compared on a g inorganic matter basis. Coefficients of determination ($r^2$) for parameter correlations are presented for each treatment (OIL or REF) along with the p-value indicating the statistical significance of the calculated correlation coefficient (r).

| Parameters compared | | OIL | | REF | |
|---|---|---|---|---|---|
| | | $r^2$ | p-value | $r^2$ | p-value |
| Soil respiration | Total soil C | 0.87 | <0.001 | 0.36 | 0.005 |
| Soil respiration | Total soil N | 0.69 | <0.001 | 0.30 | 0.022 |
| Soil respiration | Glutamate respiration | 0.57 | <0.001 | 0.24 | 0.020 |
| Soil respiration | Hexadecane respiration | 0.46 | <0.001 | 0.02 | 0.570 |
| Soil respiration | Phenanthrene respiration | 0.52 | <0.001 | 0.07 | 0.290 |
| Soil respiration | Total bacteria | 0.01 | 0.720 | 0.13 | 0.030 |
| Soil respiration | Active bacteria | 0.14 | 0.010 | 0.09 | 0.040 |
| Soil respiration | Total fungi | 0.20 | 0.002 | 0.12 | 0.030 |
| Soil respiration | Active fungi | 0.01 | 0.860 | 0.05 | 0.140 |
| Soil respiration | Protozoa | 0.01 | 1.000 | 0.07 | 1.000 |
| Total bacteria | Total soil C | 0.01 | 0.460 | 0.45 | <0.001 |
| Total bacteria | Total soil N | 0.01 | 0.560 | 0.50 | <0.001 |
| Active bacteria | Total soil C | 0.28 | 0.007 | 0.41 | 0.001 |

Table 3-6 (continued). Correlations between various microbial and soil parameters.

| Parameters compared | | OIL | | REF | |
|---|---|---|---|---|---|
| | | $r^2$ | p-value | $r^2$ | p-value |
| Active bacteria | Total soil N | 0.29 | 0.005 | 0.40 | 0.002 |
| Total fungi | Total soil C | 0.17 | 0.003 | 0.54 | <0.001 |
| Total fungi | Total soil N | 0.22 | 0.001 | 0.65 | <0.001 |
| Active fungi | Total soil C | 0.01 | 1.000 | 0.41 | 0.001 |
| Active fungi | Total soil N | 0.01 | 1.000 | 0.53 | <0.001 |

predictor of respiration ($p < 0.001$), while N had no predictive value for soil respiration ($p = 0.5$). No biomass estimates per g organic matter were correlated with soil C or N in OIL samples, while several of these parameters were positively correlated with soil C and N in REF samples. Total bacteria and total fungi per g organic matter were correlated with soil C ($r^2 = 0.53$ for bacteria and 0.85 for fungi) and soil N ($r^2 = 0.51$ for bacteria and 0.89 for fungi; $p < 0.001$ in all cases). Active fungi also were positively correlated with soil C ($r^2 = 0.59$) and soil N ($r^2 = 0.65$) in REF samples.

## DISCUSSION

Effects of the 1976 crude oil spill could still be seen in the site's physical, chemical and microbiological soil parameters almost 20 years after the event. The long-term presence of petroleum in the OIL plot caused alterations to the soil matrix that affected the physical and chemical milieux in which the microbial community resides. This selective pressure resulted in a shift in microbial community structure, as indicated in some of our microbiological measurements. Some of the traditionally measured microbial parameters clearly reflected this shift, while others appeared not to be affected by oil.

The choice of units for expressing the microbiological data carries with it implicit assumptions about the role of the added crude oil in the soil system. We wished to evaluate the data in a way that would detect treatment effects in microbial behavior unbiased by added oil mass. This implies oil is an inert soil "diluent" which would tend to depress values in the OIL plot as a result of the increase in mass content of the soil due to oiling. This assumption is only partly true, however, as the composition of crude oil includes both readily metabolizable and recalcitrant carbon substrates, as well as some compounds of known toxicity. By expressing the data per g inorganic matter, though, we may consider the microbial response to added oil substrates based on a conservative tracer that should not have changed as a result of oiling (i.e., little inorganic matter was added with the oil).

In order to examine the microbial response to oil in another light, we also expressed our microbial data per g organic matter (i.e., oil plus soil organic matter in the OIL plot). This assumes that oil is part of the total organic substrate pool for microbes, which also is only partly true due to its toxicity to some components of the microbial community, as well as the virtually undegradable nature of the asphaltene oil fraction. The value of this expression, however, is that it allows evaluation of the effect of substrate quality associated with the added oil. Thus, we have chosen to present microbial data mainly per g inorganic matter as a conservative tracer, but have also examined the data per g organic matter to assess oil C substrate quality effects.

Physical and Chemical Parameters

The soil temperatures measured at each sampling time always were higher in the OIL plot compared to the REF plot. The OIL plot's elevated temperatures are likely due to several soil physical changes caused by the spilled petroleum. The decreased albedo of the OIL plot's surface, and the death of the insulative moss layer, increased soil warming from insolation, resulting in an increased thaw depth and active layer thickness of the OIL plot (Collins *et al.*, 1994).

Crude oil residue in the OIL plot also affected the moisture regime of the soil matrix. The sorption of petroleum in the soil matrix decreased the soil's capacity to absorb water, resulting in relatively lower water holding capacities for OIL samples. Soil moisture on a g dry soil basis also was generally lower in OIL samples than in REF samples (Table 3-2), a phenomenon observed in other studies at this site (Johnson *et al.*, 1980; Sparrow and Sparrow, 1988). Changes in soil water potentials associated with the added petroleum probably also reflect a change in the soil microbial habitat.

The pH of the soil samples in our study was somewhat lower than reported for the site in 1980 (pH 4.8 to 5.6; Johnson *et al.*, 1980), but was in accordance with the mean reported by Sparrow and Sparrow (1988; pH 4.5), indicating no change in pH

over the last seven years. There was no difference in pH between OIL and REF plots, an observation consistent with other oil spill studies at this site (Johnson *et al.*, 1980; Sparrow and Sparrow, 1988) and others in Alaska (e.g., Sexstone *et al.*, 1978a).

Organic matter and total C on a dry soil mass basis overall were significantly higher in OIL than in REF samples (Table 3-3), as expected from OIL samples' added petroleum, while their total soil N was not different. The oil residue from the site had a C to N ratio of ca. 280 (85 % C and 0.3 % N by weight) so it would not be expected to contribute much to the soil N. Despite the fairly wide range in organic matter content of both sample types, their respective C to N ratios were quite constant (C/N of 23.0 for REF and 52.7 for OIL), though somewhat lower than values reported earlier (C/N of 30.9 for REF and 71.7 for OIL; Sparrow and Sparrow, 1988). Sparrow and Sparrow (1988) described similar results for organic C in their study, but found significantly higher total N in OIL compared to REF samples. Unlike studies undertaken earlier (e.g., Johnson *et al.*, 1980; Sparrow and Sparrow, 1988), we were not able to readily discriminate soil C from that introduced by the petroleum. Gravimetric recoveries of DCM-extractable material ("oil") in our study were similar to those reported by Sparrow and Sparrow (1988; 13.63 kg "oil" $\cdot$ $m^{-2}$ at a soil bulk density of 48.5 kg $\cdot$ $m^{-2}$, yielding a concentration of ca. 0.28 g $\cdot$ g dry $soil^{-1}$), but substantially higher than estimated by Collins *et al.* (1994; 0.004 $m^3$ $\cdot$ $m^{-2}$ at an assumed oil specific gravity of 0.89 and soil bulk density of 48.5 kg $\cdot$ $m^{-2}$, yielding ca. 0.07 g $\cdot$ g dry $soil^{-1}$). Extract residue analysis indicated C content (0.85 g C $\cdot$ g $residue^{-1}$) similar to that reported by Sparrow and Sparrow (1988), as well.

Differences in our soil C and N results from those of Sparrow and Sparrow (1988) may partially be explained through differences in sampling and analytical protocols between the two studies. Sample collection and treatments in this and Sparrow and Sparrow's study were similar for OIL organic horizon samples, yielding comparable values for "petroleum", and C and N data. However, REF samples in Sparrow and Sparrow (1988) included the live moss layer for C and N analysis, while

this layer was not included in our study. Thus, our mean (± s.e.) total C value for REF samples (0.169 ± 0.011 g C · g dry soil$^{-1}$) was somewhat lower than that reported in their study (7.08 kg · m$^{-2}$ at a soil bulk density of 36 kg · m$^{-2}$, yielding organic C content of ca. 0.2 g organic C · g dry soil$^{-1}$), and our calculated REF C to N ratio of 23 was lower than their reported value of 30.9. They also reported that total soil N on an areal basis was substantially higher in OIL than in REF samples in their study (REF total N of 0.23 kg · m$^{-2}$ vs. OIL total N of 0.29 kg · m$^{-2}$), but when the differing bulk densities of their OIL and REF samples are considered (REF soil bulk density of 36 kg · m$^{-2}$ vs. OIL soil bulk density of 48.5 kg · m$^{-2}$), total soil N concentrations in their OIL and REF study plots agreed well with our values (ca. 0.006 g N · g dry soil$^{-1}$).

Our inability to achieve similar soil organic C values between REF and OIL samples after subtracting DCM-extract residue C from samples is more difficult to explain, but may be a result of our hydrocarbon extraction and organic matter determination protocols. Sparrow *et al.* (1978) corrected their soil data by subtracting oil residue mass from soil mass, and Sparrow and Sparrow (1988), using the same protocol, found no difference in soil ("non-oil" ) organic C between REF and OIL samples using benzene-extracted residue mass to correct for petroleum C. Our soil organic matter and C data were determined using a CNS analyzer on oven-dried (105°C) soil samples and extractable hydrocarbons were measured using boiling DCM (80°C) to extract sodium sulfate-dried field moist samples, while Sparrow and Sparrow (1988) determined soil C using a wet chemical analytical procedure and determined extractable hydrocarbons in soils dried at 65°C. Thus, in our samples, hydrocarbon mass fractions exhibiting appreciable volatility between 65°C and 105°C were not measured as components of OIL sample organic matter or total C, yet some of this fraction was included in the value for gravimetrically determined "oil" residue. As a result, it appears "non-oil" soil organic matter and C data for our OIL samples are lower than would have been the case had our samples been treated at lower temperatures. Contrary to our expectations, this suggests that a significant proportion

of total hydrocarbon mass in our OIL samples resides in low boiling components (at least between 80° and 105°C) almost 20 years after the spill event. It also explains the lower C/N value for our OIL samples than reported previously.

Petroleum Hydrocarbons

Gravimetric recoveries of petroleum from OIL plot soil samples agreed well with values reported by Sparrow and Sparrow (1988) for their study of this site ten years after the initial spill and study. This suggests that, in the second decade after the spill, little mass loss of residual oil, either due to biotic or abiotic processes, occurred. Values for "spill intensity" of surface oiling in the OIL plot were reported by Collins *et al.* (1994) in units of $m^3 \cdot m^{-2}$, and yielded an estimate of ca. 0.07 g oil · g dry $soil^{-1}$. However, as the authors failed to report soil bulk density data, we relied on Sparrow and Sparrow's (1988) soil bulk density data for our calculation. It is possible that the low value from Collins *et al.* (1994) resulted as a calculation artifact from overestimating soil bulk density. Alternatively, their single surface sample for the winter oil spill plot may not have been representative of average degree of oiling at this site. Sparrow and Sparrow (1988) collected at least 20 soil cores in their study, and we determined extractable oil mass in 41 samples with a range of 0.069 to 0.502 g oil · g dry $soil^{-1}$. Thus, while Collins *et al.* (1994) reported a concentration in the range of values we found, it is likely our data and those of Sparrow and Sparrow (1988) more closely reflect the mean and range of oil concentrations at the site.

While our protocol did not provide for quantitative discrimination of all major hydrocarbon fractions in extracted petroleum residue, some information regarding gross residue composition may be drawn from the data we collected. The mean (± s.e.) asphaltene concentration of ca. 18% (± 3%) g · g $residue^{-1}$ we measured agrees closely with the value reported for the fresh crude oil spilled in 1976 (22% asphaltene; Jenkins *et al.*, 1978), suggesting little or no relative change for this petroleum fraction. Asphaltenes occur in crude oil as higher molecular weight (1,000 to 10,000)

hydrocarbon and nitrogen-, sulfur- and oxygen-containing compounds, and typically consist of 10 to 20 fused rings with aliphatic and naphthenic side chains (National Research Council, 1985). Their complexity, high molecular weight, and low solubility render them resistant to microbial degradation (Atlas, 1981), though evidence exists that they may be degraded co-metabolically under optimum conditions (Leahy and Colwell, 1990). Our data suggest that no change in this fraction has occurred relative to other fractions in our extracted residue. This suggests that either minimal degradation of this fraction occurred coupled with little mass loss in the remaining fractions (biotransformation with little mineralization), or that mineralization equivalent to that of the remaining petroleum mass occurred, yielding an unchanged proportion.

Based on the gas chromatographic data from our pentane-soluble SPE eluates, it appears that the extracted oil has become relatively enriched in higher MW hydrocarbons. The mass fractions in our samples exhibit relative abundances that increase with molecular weight, while the reverse is the case for similar fractions in fresh PBC oil (estimated from data contained in National Research Council, 1985; see Table 3-5). As this range of hydrocarbons boils above 205°C and is relatively insoluble and non-volatile, this selective enrichment in favor of heavier fractions suggests preferential removal of the lower MW components by some mechanism other than evaporation or dissolution in water and loss due to leaching.

Evidence that hydrocarbon oxidation has occurred in our OIL samples is seen in the difference between pentane-soluble extracts before and after SPE treatment. While ca. 82% of the DCM extract was soluble in pentane, only ca. 14% of the extracted mass was eluted through the polar matrix of the SPE tube, implying increased polarity of the pentane fraction compared to fresh crude oil. It has been observed that, in general, the relative amount of polar material in petroleum increases as it becomes more weathered (National Research Council, 1985). As we found that both alkane (hexadecane) and aromatic (phenanthrene) hydrocarbons were effectively recovered

through the SPE procedure, this suggests that the material present in the extract has undergone substantial chemical or biological oxidation since the oil was spilled in 1976. Collins *et al.* (1994) found that oil extracts of soil samples from this site exhibited compositional changes from the original spilled oil ranging from very weathered to almost unchanged after 15 years. Their data indicated that surface samples were more degraded than subsurface samples and that degraded samples showed evidence of biodegradation. Analysis of relative abundance of alkanes and aromatics indicated that, to the extent that any biodegradation of the oil occurred, this degradation proceeded at similar rates in both alkane and aromatic fractions.

Sexstone *et al.* (1978b) also found a lack of change in hydrocarbon class composition seven years after crude oil was spilled in tundra soils in Barrow. They suggested that the ratio of resolved to total integrated area of gas chromatograms would be a more sensitive index of biodegradation. As petroleum becomes more weathered, more of the sample is detected chromatographically in an "unresolved complex mixture" (UCM) and less is detected as discrete sample peaks in the chromatogram (National Research Council, 1985). By comparing relative areas of resolved and UCM components in residual oil to those in oil subjected to physical weathering, Sexstone *et al.* (1978b) concluded that bio-oxidation of their oil samples had occurred. Fresh oil for their 1976 study contained 31.2% resolvable compounds, artificially weathered oil contained 20.6 % resolvable material, and oil recovered seven years later from a wet meadow contained only 1.9% resolvable material. They noted that the decrease in this ratio could not be due to evaporative weathering since it was very much lower than that of the artificially weathered oil. Their evidence led them to their conclusion that "in the cold, nutrient limited soils" that they studied, "biodegradation occurs slowly with no major preferential utilization" of hydrocarbon classes (Sexstone *et al.*, 1978b).

In addition to our evidence of hydrocarbon oxidation based on increases in polar compounds, our data indicate that some bio-oxidation of the oil may have occurred.

The percent of total integrated area that showed resolvable component peaks in our gas chromatograms ranged from 6.1% to 22.1% with a mean value of 11.7%, indicating that most of our samples were substantially weathered. Compared to the value reported by Sexstone *et al.* (1978a) for artificially weathered 1976 PBC oil (20.6%), our samples' resolvable components appear on average to be depleted by ca. 43%. Without other mechanisms for oxidation, this suggests some biodegradation of petroleum has occurred.

It is possible that other, non-biological, oxidation reactions may have increased the relative amount of UCM in our samples. Photooxidation of aromatic compounds can be a major contributor to hydrocarbon oxidation, but this process is not thought to contribute significantly to alkane oxidation and is typically limited to low molecular weight aromatics (National Research Council, 1985). As our extraction procedure was performed at 80°C, it is expected that relatively little low molecular weight aromatic material was included in our GC analysis. Humification reactions in the soil matrix may contribute to oxidation of hydrocarbons, causing transformation of the parent material to intermediates that are subsequently incorporated into soil organic components (Shannon and Unterman, 1993). This process may be involved with decreasing the desorption potential and bioavailability of soil contaminants (Bollag *et al.*, 1988).

Microbiological Parameters

Biomass estimates of the microbial community in both OIL and REF samples indicate that, compared to fungi, bacteria may play a relatively minor role in soil activity at this site. Flanagan (1978) found that bacteria constituted less than 10% of total microbial biomass in his subarctic tundra and black spruce forest taiga sites and that fungi were the predominant decomposers in these systems. Our data show similar results, with total bacterial biomass never exceeding ca. 5% of total microbial biomass in either OIL or REF samples. The predominant ground cover at our field site (mosses, lichens and *E. vaginatum*), coupled with the saturated and permafrost-

underlain soils, suggest our site has characteristics similar to both black spruce forest and tundra (Van Cleve *et al.*, 1983). Fungi may dominate the soil microbial biomass at our site due to the selective pressures of low pH and abundance of recalcitrant carbon substrates in the form of peaty residues in the organic horizons. Fungi are better equipped for causing decay of and metabolizing insoluble plant remains than are bacteria, due to their physical form, mode of growth and enzymatic capabilities (Carlile and Watkinson, 1994).

Total fungal biomass estimates generally exhibited no differences attributable to oiling in our study (Fig. 3-1). Different results were seen in fungal biomass determinations based on direct microscopic observation in another oil spill study using northern soils (Miller *et al.*, 1978). They found that oil depressed fungal hyphae for three seasons following experimental crude oil spills on tundra near Barrow, Alaska. Our OIL plot data showing depressed active fungal biomass are consistent with filamentous fungal inhibition due to oiling. Total fungal biomasses were estimated in our study by phase-contrast microscopic observation of fungal hyphae. Thus our total fungal estimates simply reflect observable hyphae irrespective of whether actively respiring cytoplasm is present and do not necessarily represent metabolically active fungi.

Estimates of active fungal biomass based on FDA staining were very much lower than total fungal biomass in both OIL and REF samples (see Fig. 3-3A), but active fungal biomass was always lower in OIL than REF samples at each sampling (Fig. 3-2). We found active fungi were depressed in OIL samples by ca. 10- to 100-fold compared to REF samples (Fig. 3-2) and, as a proportion of total observed fungal hyphae, FDA-active hyphae were always more abundant in REF than OIL samples (Fig. 3-3A). These data also are consistent with the data of Sparrow and Sparrow (1988) showing ATP levels in OIL samples that were significantly lower than those found in samples from their control plot ten years after the original spill. They interpreted the diminished ATP levels in the oiled plot as suggestive of fungal growth

inhibition, as fungi represent the predominant microbial biomass in subarctic forest soils.

Total bacterial biomass estimates showed no obvious trend with respect to oiling (Fig. 3-4), but FDA-active bacterial cells always were lower in OIL than in REF samples (Fig. 3-5). As a fraction of total bacterial biomass, as well, REF soils often had higher proportions of active cells (Fig. 3-3B). Ten years after the spill Sparrow and Sparrow (1988) found evidence of microbial biomass and activity inhibition in oiled soils. As this was based on lower ATP levels in the oiled soil, they could not distinguish fungal from bacterial biomass or activity. However, their data are consistent with the relatively lower FDA-active fungal and bacterial biomasses we found in our OIL samples. The lower active microbial biomasses in OIL samples, both in absolute terms and as a fraction of total biomass measured, indicate that the quality of available substrate in REF soils may be superior to that in OIL soils. Alternatively, it may reflect a generally more toxic environment in the OIL compared to REF soils.

Evidence of microbial population shifts due to oiling can be seen in hydrocarbon degrader enumerations (Fig. 3-6) and hydrocarbon mineralization potentials (Fig. 3-9C, D, E and F). Enrichment of crude oil-emulsifying bacteria by at least two orders of magnitude was seen in OIL samples compared to REF soils. This observation is consistent with those made in other terrestrial hydrocarbon spills (e.g., Sexstone *et al.*, 1978b; Atlas *et al.*, 1991; Long *et al.*, 1995). The increase in numbers of hydrocarbon degraders in OIL compared to REF samples appears to coincide with elevated mineralization potentials for hexadecane (Fig. 3-9C and D) and phenanthrene (Fig. 3-9E and F). Samples with relatively higher mineralization potentials in this assay contain relatively more abundant and/or metabolically active microbial populations capable of using the substrate (Brown *et al.*, 1991). The much higher hexadecane mineralization potentials (Fig. 3-9C and D) in the OIL samples imply that populations in these samples have become acclimated to the n-alkane hexadecane, a common component of PBC oil (National Research Council, 1985). Interestingly, REF samples exhibited

significant mineralization potentials for hexadecane in this assay, as well (Fig. 3-9C). Time course data collected to determine optimum incubation periods for this assay (Fig. 3-8B) suggest that REF populations may have acclimated to the labeled-hexadecane *in vitro* during the first 48 h of the assay. OIL samples appear to contain populations of sufficient abundance and activity to mineralize a significant amount of added hexadecane within 24 h of inoculation, implying *in situ* acclimation of OIL populations. The hexadecane mineralization potentials of REF samples suggest that the genetic potential for catabolizing this substrate, while much lower than OIL samples, is still present in these microbial populations. This finding is not surprising when considering the likely high abundance of biowaxes from plants in this terrestrial system. Long-chain alkanes derived from plant lipid components (i.e., "biogenic hydrocarbons") have been demonstrated to acclimate microbial populations to catabolize oil hydrocarbons (Leahy and Colwell, 1990; Sugai *et al.*, 1997). By contrast, REF samples showed no appreciable potential for phenanthrene mineralization (Fig. 3-9E), while OIL samples demonstrated relatively high potential rates (Fig. 3-9F). Time course data for phenanthrene mineralization (Fig. 3-8C) suggest that REF samples possess little potential to mineralize phenanthrene, even after several days' exposure to the substrate in a nutrient-rich environment, while OIL samples rapidly metabolized the substrate following inoculation. This suggests that REF sample microbial communities rarely encounter phenanthrene-like substrates in the soil matrix and thus have no need to express or amplify the genetic potential for phenanthrene catabolism. It is evident that nearly two decades' exposure to crude oil has provided microbial populations in the OIL samples ample opportunity to acclimate to this common crude oil component.

Protozoal abundances showed no consistent effect due to oiling (Fig. 3-7). This general resistance to oil stress has been observed previously by other researchers (Rogerson and Berger, 1981; Foissner, 1994). The numbers of protozoa we found in different morphological classes were not evenly distributed, however. Ciliates were

rarely seen in any sample and were never seen in an OIL sample. Flagellates were numerically dominant, with naked amoebae 10- to 100-fold lower in abundance. It is generally thought that trophic interactions between bacteria and protozoa in soils result in inverse relationships in their population densities due to protozoal preferences for bacterial prey (Pussard *et al.*, 1994). Additionally, there is evidence that they may alter bacterial prey population distributions in favor of faster growing species (Sinclair and Alexander, 1989). It is not clear, however, what role protozoa play in affecting microbial community structure and activity in our soils. Our data show no correlations between protozoal numbers and bacterial biomass, fungal biomass, inorganic N or N mineralization, though the complexity of trophic and physical interactions involving protozoa likely precludes detailed resolution of protozoal effects from our data in these soils. It is apparent, however, that as indicator organisms in these soils, protozoal populations are poor predictors of oil stress or soil processes.

Total soil C mineralization rates expressed per g inorganic matter showed no consistent differences with respect to oiling (Fig. 3-10), a result also reflected in the glutamate mineralization data (Fig. 3-9A and B). Sparrow and Sparrow (1988) found similar results for *in vitro* soil respiration in their study of this site ten years after the spill, and suggested this indicated that oiled and unoiled soils contained similar amounts of available substrate. In studies of the Athabasca oil sands in Canada, Wyndham and Costerton (1981) showed that, despite enrichment in numbers of hydrocarbon degraders and elevated petroleum degradation rates, there was no difference in rates of glutamate uptake and soil respiration between oil sands and unoiled controls. This suggests that pre-exposure of the soil community is an important factor affecting these kinds of activity measurements (Pfaender and Buckley, 1984). Immediately after the spill at our site in 1976, *in vitro* respiration rates were significantly depressed in oiled soils compared to reference soils, while after one year they were significantly higher (Johnson *et al.*, 1980). These data suggested that the initial inhibition response to the spilled oil reflected toxicity due to abundant low MW

volatile hydrocarbons in the fresh oil. The elevated respiration seen one year later was attributed to a burst of microbial growth and activity due to release of readily utilizable carbon substrates from plants killed by oil. These authors noted that a lag time may occur before oil is utilized after a spill because of the time needed for development of abundant microbial populations able to degrade the oil. Our data indicate that, after nearly twenty years exposure to the crude oil at our site, the OIL soils' microbial populations have adapted to the oil to the extent that common indices of heterotrophic activity no longer discriminate between treatments.

When the soil respiration data are considered on both an inorganic and organic matter basis, there is evidence of both superior and seasonally varying substrate quality in REF soils and well-adapted hydrocarbon degrader communities in OIL soils. On an inorganic matter basis, the strong dependence ($r^2 = 0.867$) of OIL soil respiration on soil C ($p = 0<0.001$) but not on N ($p = 0.479$) reflects the relatively consistent quality of the substrate most abundant in these soils (petroleum hydrocarbons with very little organic N) and the hydrocarbon-adapted microbial populations using it. Soil respiration per inorganic matter was relatively constant irrespective of season in OIL samples, with the exception of July 1994 and October 1995 samplings (Fig. 3-10). Further evidence of the role the hydrocarbon-acclimated population plays in the OIL plot is seen in the relatively strong correlations of total soil C respiration with hydrocarbon mineralization potentials ($r^2 = 0.46$ and 0.52 for hexadecane and phenanthrene, respectively). Additionally, glutamate mineralization potential was well-correlated with hexadecane potentials in OIL samples ($r^2 = 0.49$), a finding in concert with the observed increase in amino acid degraders associated with alkane-specific degraders (Long *et al.*, 1995) and with crude oil contamination (Atlas *et al.*, 1991). In contrast, REF samples' respiration rates showed no correlation with hydrocarbon mineralization potentials ($r^2 < 0.1$ for both substrates). The relatively weaker relationship ($r^2 = 0.36$) of REF soil respiration with soil C ($p = 0.031$) and N ($p = 0.160$) on an inorganic matter basis implies greater variation in available labile

substrate from sample to sample, as might be expected in the heterogeneous environment of undisturbed taiga soils (Smith, 1992) with seasonally varying soil C inputs from litterfall and root exudates (van Elsas and Smalla, 1997). REF sample respiration (expressed either per g organic or per g inorganic matter) exhibited substantial seasonal variation (Figs. 3-10 and 3-11). OIL sample respiration data per g organic matter showed no variation with respect to season ($p = 0.47$; see Fig. 3-11). Multiple regression of the data per g organic matter revealed that REF soil respiration depended strongly ($r^2 = 0.847$) on both total soil C ($p < 0.001$) and soil N ($p = 0.031$), while OIL soils' relation to soil C and N was weaker ($r^2 = 0.625$) and did not depend on soil N ($p = 0.5$). This suggests that C mineralization in the OIL plot is not coupled tightly with N mineralization while the opposite is true in REF soils.

The evidence for disrupted N mineralization capabilities in the OIL soils from soil respiration data is supported by our observations of net N mineralization. Net nitrogen mineralization in the samples we assayed was not similar in OIL and REF samples ($p < 0.1$). Net nitrification was different between treatments ($p = 0.09$), and no net nitrification was observed in any OIL sample assayed. Mineral N ($NH_4^+$-N) was also more abundant in REF than in OIL field samples. Higher ammonium uptake or lower N mineralization rates in the OIL field plot may account for this observation.

In addition to our net N mineralization data, the Biolog multiple substrate use assay provides evidence of greater ability for REF samples to mineralize organic N. At all dilution factors, significantly more N-containing substrates were used faster by REF samples than would be predicted if there were no difference between treatments. No N-containing substrates were ever used faster by OIL samples at any dilution. While this assay measures dye reduction due to oxidation of the C source in the microplate wells and does not directly measure N mineralization, these data indicate that the REF soil communities are better able to metabolize N containing compounds. These data are consistent with the higher net N mineralization we saw in REF samples.

As there is evidence of little difference in soil C respiration potentials between OIL and REF samples, indicating no difference in available substrates, the questions arise as to what effect the oil has had on soil microbial populations and why the pollutant persists. Some of the answers lie in what our assays measure, as well as in the nature of the contaminant itself. The *in vitro* respiration assay we used measures the integrated effects of soil C substrate quality and the activity of the extant microbial community. Thus, what constitutes "available" substrates depends in part on the metabolic capabilities of the microbes present. We have found orders of magnitude higher populations of hydrocarbon degrading microbes (crude oil emulsifiers) in OIL than in REF samples (Fig. 3-6), and the genomic potentials for alkane and polynuclear aromatic hydrocarbon (PAH) degradation are present (Fig. 3-9D and F). Further, there is evidence of oil weathering, some of which may be biologically mediated (Collins, 1991 and our data). This evidence points to microbial communities in OIL soils that have been altered with respect to population structure and metabolic potential for hydrocarbon degradation. Therefore, it is likely that much of the respired C we measured in OIL plot samples was derived from crude oil components in these soils.

The lowered overall net N mineralization in OIL samples indicates a soil environment of diminished functional ability due to pollution. Analysis of community-level substrate metabolism in soils from this site suggests a lower diversity of populations exists in OIL soil communities, and these populations may be less specialized physiologically (see Chapter 2). Absence of nitrification in OIL samples may be due to inhibition or loss of the nitrifying populations from oiling; only a small group of related bacteria is involved and potential physiological redundancy for this process across different populations is limited (De Boer *et al.*, 1996). In most soils availability of $NH_4^+$ is a principal factor controlling autotrophic nitrification, though acidity and low $O_2$ availability may also control rates (Firestone and Davidson, 1989). As pH was similar between the two soils, and we measured N mineralization in aerobic jar assays, these factors are not likely to account for the differences observed.

Ammonium limitation is a possibility, as we measured less ammonium in OIL than in REF field samples, though measurable ammonification occurred in both OIL and REF jar assays. Nitrifiers are considered to be poor competitors for ammonium compared to heterotrophic microbes (Firestone and Davidson, 1989), populations similarly active in OIL and REF jar assays. The much higher C/N ratio in OIL compared to REF soils (ca. 52 and 23, respectively) suggests the likelihood of N immobilization associated with heterotrophy would be higher in OIL than in REF soils, given their similar amounts of available carbon (based on soil respiration). Additionally, the key enzyme responsible for autotrophic nitrification, ammonium mono-oxygenase, has broad specificity and has been shown to oxidize a variety of low MW hydrocarbons (Hyman *et al.*, 1988; Hyman *et al.*, 1985). It is possible that nitrifying microbial populations have been "starved" to extinction in the OIL plot, either through inability to compete with heterotrophs for $NH_4^+$-N, through wasting energy on incidental oxidation of oil hydrocarbons, or a combination of these factors.

Despite evidence that OIL soils' community structure has shifted towards an enhanced potential for hydrocarbon degradation, crude oil remains abundant at the site. Enrichment of this residue in higher MW components (see Table 3-5) suggests that mineralization of crude oil is occurring at the expense of the lower MW hydrocarbons present. Minimal change in asphaltene content of the residue from values reported when the spill occurred (see Table 3-4) indicates that little mass loss in residual oil is associated with metabolism of these compounds. Initial degrees of oiling reported when the spill occurred are quite variable and range from ca. 7% to 190% oil in the organic soil horizons (O2/A1; Johnson et al, 1980), so a precise determination of oil mass loss from 1976 is not possible. However, little change in residual crude oil mass has occurred in the ten years since Sparrow and Sparrow (1988) visited the site (see Table 3-4). *In situ* respiration data were not collected, so we do not have information regarding rates of petroleum mineralization in the field, but as hydrocarbon degraders are abundant and the genomic potential for hydrocarbon

mineralization is present, this suggests other factors may be responsible for the oil's persistence.

There is a growing body of evidence (e.g., Leahy and Colwell, 1990; Weißenfels *et al.*, 1992; Providenti *et al.*, 1993) that sorption of hydrocarbons to organic matter in soil may limit their uptake and degradation by soil microbes. Weißenfels *et al.* (1992) found that sorption of PAHs to soil organic matter decreased the amount biodegraded in soils from contaminated sites. Mihelcic and Luthy (1988) showed that the desorption rate of the PAHs acenaphthene and naphthalene was a factor influencing their biodegradation. Boethling and Alexander (1979) found that low solution concentration of synthetic organic chemical substrates may be an important factor limiting their biodegradation by natural microbial communities. Sorption to soil organic matter can remove these compounds from the dissolved state and, in general, sorption of neutral hydrophobic compounds depends on soil organic C content (Providenti *et al.*, 1993). It has been shown that the duration of contact with soil matrix components ("aging") affects the desorption, bioavailability and biodegradability of hydrocarbons, even when organic matter is not abundant (Hatzinger and Alexander, 1995). Humification reactions in the soil matrix, mentioned above, may cause transformations of residual hydrocarbons to compounds incorporated into recalcitrant soil organic matter pools (Shannon and Unterman, 1993). It is possible that slow degradation of lower and moderate MW oil fractions not protected by soil organic matter is proceeding in OIL soils at rates comparable to that for labile substrates in REF soils, within the limits imposed by temperature and inorganic nutrient availability. Enrichment of the oil in higher MW hydrocarbons, coupled with soil aging and sorption effects, implies that this residue will become less degradable with time, as higher MW components are, in general, considered to be more recalcitrant to degradation (Atlas, 1991).

We can use our *in vitro* gross soil C mineralization data coupled with crude oil residue concentration data to estimate the minimal time the crude oil is expected to

remain at the site. The mean oil concentration for our OIL samples was ca. 0.541 g oil · g inorganic matter$^{-1}$, with a C content of 85%, and mean *in vitro* soil C mineralization potentials were 72 μg C · g inorganic matter$^{-1}$ · d$^{-1}$. Assuming that soil in the OIL plot is warm enough for significant microbial activity for 100 days per year, we calculate a residence time of ca. 64 years for the crude oil at the site. This calculation is based on potential rates determined under optimized laboratory conditions, and assumes that all C respired in our assay came from the oil and that all crude oil carbon is equally available and labile. Therefore, rates of oil disappearance based on these assumptions are overestimates and *in situ* rates will be much slower.

The effects of this oil spill on the microbial community may have broader ecological consequences in the OIL plot. While total C mineralization potentials are similar between OIL and REF soils, net N mineralization and data collected regarding community-level multiple substrate use show that OIL soils' microbial communities are relatively constrained in their ability for organic N transformations. Mineralization of crude oil C substrates releases little or no associated N, so any available inorganic N would tend to be immobilized by oil degraders. Oiling also appears to inhibit nitrifiers. As soil organic matter in tundra and taiga ecosystems constitutes potentially the most readily available reserve of nutrients for plant growth (Van Cleve, 1977), diminished N mineralization capabilities by soil microbial communities may have significant implications for plant recolonization and recovery at the site.

Vegetation recovery in the OIL plot may provide evidence that this microscopic perturbation has visible effects. Most mosses, lichens and shrubs were killed shortly after the oil spill (Jenkins *et al.*, 1978) and generally remain absent in surface oiled areas, and no sign of black spruce recovery (new seedling establishment or new growth on damages trees) was seen through 1994 (Collins *et al.*, 1994). While much of this mortality was probably due to direct toxicity of petroleum on plant roots and shoots (solvent effect on lipid membrane structures of the cells; Bossert and Bartha, 1984), plant community recovery following oil spills has been shown to be limited by

nutrient availability (Odu, 1972). Almost twenty years after the spill, essentially the only plants surviving in the oiled area are *E. vaginatum* tussocks which appear to have flourished since the original experimental spill (Collins *et al.*, 1994). Other observations of ecological disturbance in Alaska (e.g., tundra fires) have shown these plants to benefit from removal of other species, probably due to competitive release (Racine *et al.*, 1987). It has been suggested that tussocks are resistant to spilled oil due to their elevated growth form and annual root growth penetrating to unoiled mineral soil (Johnson, 1980), thus minimizing exposure to oil. In a recent study at this site (Collins *et al.*, 1994), however, excavations of *Eriophorum* tussocks demonstrated that oil was present within the root mass, suggesting that root toxicity alone does not explain slow plant recovery at the site. Studies of this plant species have indicated that organically bound soil nutrients, including N, cycle 3-10 times more rapidly within the tussock than beneath it due to increased tussock soil temperatures and the ability of individual tussocks to retain their own organic nutrient capital within tussock soil (Chapin *et al.*, 1979). It may be that *E. vaginatum* litter within the tussocks provides higher nutrient quality (higher organic N) substrate than the surrounding oiled soils, allowing higher N mineralization and nutrient cycling rates that benefit these plants. Removal of shading shrubs and insulative moss layers probably account for recovery of *E. vaginatum* in the OIL plot, but nutrient cycling within the tussocks must be occurring for these plants to flourish.

We have seen that adding crude oil to our taiga field site has resulted in substantial changes to soil microbial communities. Long-term exposure to oil has led to an adapted community able to respire oil carbon in the OIL plot at rates similar to soil carbon in the REF plot. Multiple regression analysis suggests that N mineralization is occurring as organic matter is degraded in REF soils, but the high C/N ratios and shift to a predominantly oil-degrading community in the OIL plot has resulted in lower N mineralization in OIL soils. Absence of nitrification in OIL soils suggests that an important functional component of the microbial community has been disrupted due to

oiling, probably either due to toxicity or competitive exclusion by active heterotrophs. As the oil becomes ever more enriched in higher MW compounds due to hydrocarbon biodegradation and spends more time in contact with organic matter in the soil, it will likely become less degradable. Therefore, despite the presence of a well-acclimated microbial community of hydrocarbon degraders at this site, we expect that residual crude oil will persist for decades. Further, as long as natural biodegradation of the oil residue continues, disruption of normal mineral nutrient cycling activity in the OIL plot is probable.

# CONCLUSIONS

The experimental oil spill plot at Caribou-Poker Creeks Research Watershed provided us a unique opportunity to examine the effects of long-term crude oil contamination in a subarctic environment. In the context of ongoing efforts to exploit the remaining oil reserves in the Arctic, research regarding the factors affecting recovery of spill-affected northern terrestrial systems assumes ever greater importance. Nearly twenty years after the spill occurred at this taiga site, a substantial amount of crude oil remained in the soil. The continued presence of this environmental contaminant resulted in changes to the soil's physical, chemical and biological properties. We used this site to study the factors contributing to the long-term persistence of the oil and its influence on the composition and function of the native microbial community.

Among the variables assessed at the site, several parameters characterizing some functional aspects of the microbial community were measured. Broad-based microbiological data exhibited similar responses between oiled and oil-free soils at the site, while more narrowly-focused assays suggested more profound changes in the microbial community structure as a result of oiling. Soil respiration, total bacterial and fungal biomass, and soil protozoan assays showed no consistent differences attributable to oiling between oil and oil-free soils. However, active bacterial biomass, active fungal biomass, net N mineralization and nitrification, substrate-specific mineralization and microbial (bacterial) community diversity assays showed differences in soils likely attributable to oiling.

We measured whole soil C mineralization potentials and found little difference overall in respiration rates between oiled and oil-free soils. This suggests that, for the microbial communities present in the two soil types, similar amounts of labile carbon were available, or that other environmental factors (e.g., nutrient limitation, temperature, etc.) common to both soils have resulted in similar soil community

responses. However, regression analysis of these data suggest that C mineralization in unoiled soils, but not oiled soils, is tightly coupled to N mineralization. This information, combined with our microbial enumeration and substrate-specific mineralization data, indicates that the selective pressure generated by the contaminant has shifted microbial community metabolism toward petroleum substrate use, but this does not appear to be sufficient for rapid destruction of the residual crude oil.

Net nitrogen mineralization and nitrification for the samples we assayed were different between oiled and oil-free soils, and no net nitrification was observed in oiled soil samples. Our inability to detect net nitrification in oiled soils indicates that the two soil treatments have differing microbial populations. It is possible that hydrocarbon toxicity has eliminated nitrifiers from the soil system. Alternatively, competition from heterotrophs for ammonium may have resulted in loss of their metabolic energy source. In either case the N dynamics of the oiled soils are different from oil-free soils, another indication of altered soil ecology due to crude oil presence. As N mineralization from soil organic matter represents the primary source of inorganic N for plant growth, reduced N mineralization due to oiling may have implications for revegetation and long-term recovery of the site.

The use of multiple substrate analysis to assess community diversity indicates oiled soil community diversity was relatively lower than that in our oil-free soils. These data are consistent with the information we collected regarding oil-induced population enrichment, as total biomass estimates were similar between the two treatments. Reductions in population diversity appear to correspond to selection for metabolic "generalists" in oiled soils (see chapter 2), but the communities so affected exhibited slower N substrate oxidation rates overall (see chapter 3). These data provide further evidence that oil pollution at the site has disrupted the soil system's normal nutrient cycling processes.

*In vitro* respiration measurements suggest that C mineralization potentials were similar between oiled and oil-free soils, but there was little apparent mass loss in crude

oil residue in the second decade after the spill. Calculations based on these *in vitro* measurements under optimized conditions and average oil concentrations imply the oil will persist for at least another six decades. This estimate assumes that all components of the residual oil are equally available labile C sources and that all respired C is from the oil. Given the molecular complexity and size of some of the heavier crude oil components remaining, the high natural organic matter content of the oiled soils, and the length of time since the spill (increasing the probability of "protecting" oil substrates from microbial attack), however, 60 years is an optimistic estimate.

Long-term presence of crude oil at this site has disrupted some of the organic soil's gross functional characteristics. We found that some traditional microbiological assays were insufficient for discriminating soil treatments at our site, suggesting these techniques alone would be inappropriate for monitoring the site's recovery. Assays focusing on specific functions, on the other hand, were able to readily differentiate soils based on treatment. In particular, the multiple-substrate assay not only discriminated samples by treatment but also by season sampled, indicating detectable temporal bacterial heterogeneity. Thus, microbiological assessment of soils appears to be of potential utility in evaluating the effects of contaminant stress in soils, but the assays chosen need to be of the proper metabolic resolution.

The broad-scale physiologies assessed through total C mineralization and total biomass estimates showed little difference between oiled and oil-free soils due to treatment (oiling), though clearly the two treatments yielded different ecosystem effects in the two decades following the spill (e.g., increased active layer thickness, widespread plant death and little plant recolonization in the oiled plot). Viewed from the perspective of broadly-defined physiology, the oiled and oil-free areas' microbiological components behaved similarly. The apparent loss of microbial populations due to oiling, however, has caused a metabolic shift in the surviving communities affecting processes important for normal functioning of the taiga

ecosystem. Without human intervention, it is anticipated this site will remain contaminated and devoid of a healthy plant community for the foreseeable future.

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