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Post-Sampling Changes in Microbial Community Composition and Activity in a Subsurface Paleosol

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ABSTRACT

Laboratory storage of deep vadose zone sediments has previously resulted in an increase in the abundance of cultured microorganisms by as much as 10,000-fold, without concomitant increases in total microscopic counts. In the present study, factors contributing to the time-dependent stimulation of various microbiological parameters were examined during a 224 d post-sampling period, using a factorial-design experiment that partitioned the effects of storage time, sediment condition (intact blocks or homogenized) during storage, and O_2 concentration (0.5, 4.5, and 21%) during storage at 15°C. Stored samples were analyzed at selected intervals, to determine direct microscopic counts, viable biomass, lipid biomarker profiles, cultured aerobic heterotrophic microorganisms, and microbial activity. Time of storage prior to analysis of the samples was the most important factor affecting the microbiological response. Sediment condition influenced the stimulation response: microbial activity and the population of cultured microorganisms increased faster, and reached slightly higher values, in the homogenized samples, although maximum values were reached at similar times in the homogenized and intact samples. O_2 concentration also influenced the response, but was the least important of the factors evaluated. Total cells and viable biomass, measured as total phospholipid fatty acids, changed little during storage. Maximum cultured populations and activity were attained at 63 to 112 d, with culture counts approximating the total numbers of microscopically counted cells. At approximately the same time, unbalanced growth (evidenced by high ratios of polyhydroxybutyrate to phospholipid fatty acid) indicated that inorganic nutrients became limiting. Lipid biomarkers indicative of Gram-positive bacteria, including actinomycetes, became dominant components of the community profiles in samples maintained at 0.5% and 4.5% O_2. The shift in the microbial community from relatively inactive, predominantly uncultured microorganisms to metabolically active populations that were nearly all cultured highlights the need for rapid initiation of analyses after sample acquisition, if measurement of in situ

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microbiological properties is desired. The fact that these processes also occur in intact sediment blocks suggests that minor perturbations in the chemical or physical properties of subsurface sediments can result in major changes in the activity and composition of the microbial community.

Introduction

Interest in the microbiology of deep terrestrial environments has expanded rapidly in the past 10 years, because of the potential for using indigenous microorganisms for in situ bioremediation and because of the role of microorganisms in catalyzing biogeochemical processes. Research has been largely focused on deep, saturated sediments because of the interest in protecting uncontaminated groundwaters and remediating contaminated aquifers. In contrast, relatively little information is available on the microbiology of deep vadose zone sediments. Recharge rates to deep (>10 m), unsaturated sediments are commonly quite low (0.03 to 50 mm y^{-1}) in arid and semiarid regions of the western United States [9] and severely limit the influx, diffusion, and availability of nutrients. Although chemical contamination of deep vadose zones has occurred on many federal and private lands in the western U.S. [26, 34], little is known about the ability of microorganisms to survive and grow under these harsh conditions.

Recently, viable microorganisms have been reported in deep, unsaturated sediments of the western United States [4, 6, 14, 21]. We have been investigating the microbiology of deep vadose zone sediments impacted by artificial (i.e., anthropogenic) moisture recharge. Artificial recharge is an important process, because, in most cases, contaminants enter the vadose zone by infiltration or injection of processing waters; for example, more than 8.6×10^{11} l of water have been discharged to the upper vadose zone at DOE's Hanford Site in south-central Washington State [5]. The impact of artificial recharge on subsurface microbial communities may be an important factor in assessing microbiological influences on contaminant fate and transport, and the potential for using indigenous microflora for in situ bioremediation.

In a previous study of deep vadose zone paleosols (buried soils) impacted by artificial recharge, we found that cultured populations increased up to 10,000-fold in unsaturated subsurface paleosols during 75–83 d of sample storage at 4°C [4]. Post-sampling alterations in microbiological properties appear to be a common phenomenon. They have been noted in other Hanford Site deep vadose zone and saturated sediments, including vadose zone sediments impacted by contaminants [4, 13], and in unsaturated and saturated ashfall tuff rock from the Nevada Test Site [14, 18, 19].

The objectives of this study were to determine the factors controling the post-sampling changes in microbiological properties and to assess changes in community structure following sample acquisition. The roles of sample homogenization and O_2 concentration were examined, during a timecourse extending to 224 d post-sampling, by measuring total biomass, culturable populations, and metabolic activity. In addition, viable biomass, community composition, and physiological status were measured over the timecourse by lipid biomarker analysis.

Materials and Methods

Site Description and Sample Acquisition

The White Bluffs are located adjacent to the Hanford Site on the east side of the Columbia River in south-central Washington State. An actively eroding, vertical landslide scarp has exposed Ringold Formation sediments, including a weakly developed paleosol, described previously [4]. Three large blocks of sediment (approx. 0.5 m × 0.5 m × 0.25 m) were excavated from behind a fresh face, which was exposed by excavating approximately 0.5 m into the scarp. The blocks were handled with sterile gloves, wrapped and sealed in sterile plastic bags, transported to the laboratory in coolers, and processed within 24 h.

Sample Preparation and Experimental Design

A small, battery-operated, circular saw with a disinfected blade was used to remove the outer several cm and to cut the large blocks into small rectangular blocks 3–5 cm on a side. Four to six small blocks (total of ~300 g) each were distributed to 108 sterile, acid-washed, one-pint, wide-mouth cannings jars. Blocks in 54 of these jars were homogenized by aseptically crushing them with a mortar and pestle, and sieving through a 2-mm screen. Blocks in the remaining 54 jars were left intact. Small blocks from contiguous locations in a large block were distributed to different jars to minimize bias due to spatial variability at a scale approximating that of the small blocks. All sample processing was performed with sterile gloves. The jars were sealed with sterile, butyl rubber stoppers.

Both the intact and homogenized samples were held at 15°C (the in situ temperature) in atmospheres that contained 21, 4.5, or 0.5% O_2. Custom gas mixtures (Norco, Boise, ID) were used for the 4.5 and 0.5% O_2 concentrations. They contained 0.03% CO_2 with the remaining volume as N_2. The gas mixtures were passed through

activated charcoal to remove organic compounds, a 0.2-µ filter, and, finally, sterile water, to saturate the air before the mixture entered a portable glove bag. Jars were placed in the portable glove bags. The atmosphere was replaced with the prescribed gas mixture by flushing the glove bag for 15 min. Atmospheric air was also displaced from the jars by slipping a cannula between the rubber stopper and the side of the jar, and flushing the jars with the prescribed gas mixture for 2 min. Jars containing homogenized samples were slowly rolled during flushing to displace atmospheric air between sediment particles. The rubber stoppers were sealed in the jar mouth using duct tape.

At t = 0 and after 21, 35, 63, 112, and 224 d of storage, three jars (i.e., triplicate samples) from each of the 6 treatments were sacrificed for microbiological measurements. For each timepoint, microscopic direct counts, lipid biomarker analysis (viable biomass, community composition, and physiological status), culturable microorganism enumeration (aerobic heterotrophs, fungi, heat-resistant bacterial spores, and heat-resistant fungal spores), ^{14}C-glucose mineralization assays, and glucose-induced respiration assays were performed on the triplicate samples. Culturable microorganism and microbial activity assays were performed at 21% O_2 due to practical considerations.

Physical and Chemical Analyses

Moisture content was determined for all treatments at all timepoints by gravimetric analysis. Duplicate aliquots of ~20 g of sediment were dried at 105°C for 24 h. Bulk density, particle density, porosity, and air-filled porosity were determined using standard methods [20].

At sampling, 4 sets of 3 intact sediment samples were taken for geochemical analysis. Each sample consisted of 6 small blocks of sediment, as described. One set was composed of samples immediately adjacent to one another, with each sample composed of contiguous blocks. A second set was sampled in the same manner, but from a different large block. Each sample in the third and fourth sets was composed of noncontiguous blocks. The 12 samples were separately homogenized by crushing and seiving, as described previously.

For bulk sediment analyses, a 1-g aliquot of homogenized sediment was taken from each sample and analyzed for total carbon by combustion using a model CR-12 Carbon Analyzer (Leco Corp., St. Joseph, MI), for inorganic carbon by titration after acid digestion (Method ASTM D513, section G), and organic carbon was determined as the difference between total carbon and inorganic carbon. Detection limits for these measurements were 50 µg g^{-1} sediment. Kjeldahl nitrogen (Method ASTM D3179; detection limit 100 µg g^{-1} sediment, with sensitivity in increments of 100 µg g^{-1} sediment) and total phosphorus (Method ASTM D3682, modified for dissolution; detection limit 50 µg g^{-1} sediment) were determined on 1-g aliquots by standard acid-digestion methods. Analyses were performed by Huffman Laboratories (Golden, CO). Bulk sediment pH was determined on a 1:1 (wt:wt) slurry of homogenized sediment in 10 ml of 10 mM $CaCl_2$ [25].

Extractable ions were measured on 25-g aliquots of homogenized sediment by placing sediment and 50 ml of deionized water in a glass centrifuge tube, shaking the tube for 24 h on a wrist action shaker, centrifuging for 30 min at 4000 rpm, and filtering the supernatant through a 0.22-µm syringe filter. Extractable sulfate and ortho-phosphate were analyzed by ion chromatography (model 2020i, Dionex, Sunnyvale, CA). Detection limits were 0.05 µg ml^{-1} and 1.0 µg ml^{-1}, respectively. Extractable nitrite and nitrate were analyzed by a colorimetric, segmented, rapid flow analyzer (Alpkem, Clackamas, OR). Detection limits were 0.01 µg ml^{-1} and 0.02 µg ml^{-1}, respectively. Extractable organic carbon, inorganic carbon, and total carbon were analyzed directly, using an aqueous-phase carbon analyzer (model DC-80; Dohrmann, Santa Clara, CA; detection limit 0.02 µg ml^{-1} for all three analytes).

Microbiological Analyses

Culturing. Aerobic heterotrophic bacteria and fungi were enumerated by adding 10 g of sediment to 95 ml of 0.1% sterile sodium pyrophosphate (pH 7.0) in a sterile blender container and subjecting the sediment to two 30-sec bursts separated by a 30-sec interval. Ten-fold serial dilutions of the suspension were immediately prepared by placing 10 ml of suspension into 90 ml of sterile sodium pyrophosphate. Spread-plates were performed, in duplicate, on 1% peptone-tryptone-yeast extract-glucsoe (PTYG) medium [1] for heterotrophic bacteria and on Rose-Bengal medium supplemented with 30 µg streptomycin ml^{-1} for fungi [8]. Heat-resistant bacterial and fungal spores were enumerated by holding the dilutions at 80°C for 10 min prior to plating. Plates were incubated at 25°C. Colonies were enumerated after 21 d.

Radiorespirometry. Intact samples were homogenized to equalize substrate availability and byproduct capture rates between samples that were stored intact and those that were stored homogenized. Subsamples (10 g) were placed in sterile, 175-ml bottles, and 1.0 ml (0.5 µCi) of a [^{14}C(U)]-glucose solution (New England Nuclear/DuPont Research Products, Boston, MA) was distributed evenly over the sediment, to obtain a final concentration of 3.0 µg glucose g^{-1} sediment. A 7-ml, sterile, scintillation vial containing 1 ml of 0.3 M KOH was suspended in the bottle, with Teflon tape, to trap $^{14}CO_2$. The bottles were sealed with a sterile rubber stopper wrapped with Teflon tape, and incubated at 25°C in the dark. Vials were removed at 1, 2, 3, 4, and 7 d, for scintillation counting (model LC7000; Beckman Instruments, Fullerton, CA), and replaced with fresh vials. Regression of the linear portion of the $^{14}CO_2$ evolution curves (generally 4 data points) was used to calculate a mineralization rate. The lag time for glucose mineralization was determined by nonlinear regression of the curves, using a statistical analysis program (Systat, Evanston, IL). Controls without sediment and with sterile sediment (autoclaved on 3 consecutive days) also received [^{14}C(U)]-glucose.

Glucose-induced respiration. Intact samples were maintained as such during the assay, to compare with the intact samples that were homogenized immediately prior to the radiorespirometry assay. Subsamples (100 g) were placed into sterile, 250-ml, wide-mouth flasks. Ten ml of glucose solution was distributed evenly over the

sediment, to obtain a final concentration of 300 μg glucose g^{-1} sediment. The flasks were sealed with a sterile rubber stopper, connected to a 20-channel, computer-automated respirometer (Micro-Oxymax system, Columbus Instruments, Columbus, OH), and incubated at 25°C. The headspace was automatically purged every 12 h for 7 d, and analyzed for O_2. Regression of the linear portion of the O_2 utilization curves (generally ⩾ 4 data points) was used to calculate a respiration rate. Controls were as described above for radiorespirometry. This assay was performed in addition to radiorespirometry, because assimilated glucose can be incompletely metabolized at concentrations ⩽ 36 μg g^{-1} sediment. This could lead to underestimation of metabolic activity as measured by radiorespirometry [3]. Because carbonate dissolution could complicate the interpretation of CO_2 production, O_2 utilization was used as a measure of respiration.

Direct microscopic counts. Intact samples were homogenized. Subsamples (20 g) were prepared by placing sediment in 50-ml tubes and replacing atmospheric air with the appropriate gas mixture, as described previously. Tubes were capped and shipped on ice, by overnight express mail, to the laboratory of T. Kieft. Direct counts of total microorganisms were performed by a modified [33] acridine-orange method [15], and were initiated within 24 h of receipt. Four slides were prepared from each subsample. Twenty fields on each of the four slides were examined at 630× magnification, yielding a minimum detection limit of ~1.5×10^5 cells g^{-1} dry sediment.

Phospholipid fatty acids (PLFA). Intact samples were homogenized. Subsamples (100 g) were frozen at −70°C and shipped on dry ice, by overnight express mail, to the laboratory of D.C. White. Samples (75 g) were placed into 250-ml glass centrifuge bottles, and 142.5 ml of a Bligh and Dyer extraction solvent system [2], modified to include a phosphate buffer [40], was added. Following 2 min of sonication at room temperature, the single-phase extraction was allowed to progress for 3 h. Samples were centrifuged at 650× g for 30 min, after which the liquid phase was decanted into a separatory funnel. Chloroform and water (37.5 ml each) were added, resulting in two phases that were allowed to separate overnight. The organic phase was collected and concentrated using rotary evaporation. The total lipid extract was then further separated into neutral, glyco-, and polar lipids on a silicic acid column (100-200 mesh Unisil, Clarkson Chemical Co., Williamsport, PA) [17]. The polar lipid fraction was subjected to a mild alkaline methanolysis, from which the transesterified fatty acid methyl esters were recovered [40]. Fatty acid methyl esters were further separated and quantified by capillary column gas chromatography (GC)/mass spectrometry, as described by Ringelberg et al. [28]. Poly-hydroxy butyric acid (PHB) was recovered from the glycolipid fraction by strong acid ethanolysis prior to quantification by GC [11]. The following modified GC parameters were used: Restek Rtx-5, 30-m, 0.35-mm capillary column with a 0.5-μm film thickness, held at 60°C for 10 min, then programmed to 280°C at 10°C min^{-1}. Methyl nonadecanoic acid was used as the internal standard. Fatty acidy methyl esters are designated, as previously described [27]. To determine how a specific biomarker (e.g., biomarker A) changed within and between different treatments, the levels of biomarker A were normalized to changes in total viable biomass by applying the equation: (mass, biomarker $A_{\text{sample of interest}}$/mass, biomarker $A_{t=0\ \text{sample}}$) × (mass, total $PLFA_{t=0\ \text{sample}}$/mass, total $PLFA_{\text{sample of interest}}$).

Statistical Analyses

The factorial experimental design allowed the effects of time, sediment condition, and O_2 concentration to be determined. Three-factor analysis of variance (ANOVA) was performed with the NCSS statistical package, version 4.21 (J. Hintze). Because caution is required in interpreting effects of factors when many interactions are present, one-factor ANOVA was used to compare (a) 0.5% vs. 21% O_2 concentrations at each timepoint, for each sediment condition, (b) 0.5% vs. 21% O_2 concentrations at each timepoint, regardless of sediment condition, (c) homogenized vs. intact samples at each timepoint, for each O_2 concentration, and (d) homogenized vs. intact samples at each timepoint, regardless of O_2 concentration. One-factor ANOVA was performed with StatView II (Abacus Concepts, Berkeley, CA) using the Fisher protected least-significant-difference statistic. ANOVA for the PHB/PLFA ratio was performed with a more conservative estimate of statistical significance, the Tukey statistic for difference between means, because the ratio does not consider variability in the individual terms. Statistical significance was considered to be $P \leqslant 0.05$ for all ANOVA tests.

For community composition analyses, the cyclopropyl fatty acids, cy 17:0 and cy 19:0, were grouped with the monoenoic PLFA. In addition, 18:1ω9c was removed from the monoenoic PLFA group, because it is also a precursor of polyenoic formation in some eukaryotes. Principal component analyses were performed using the multivariant statistical package Ein*sight (Infometrix, Seattle, WA). The normal saturates palmitic (16:0) and stearic (18:0) acid, which are common to all biological membranes, were removed from the data set to improve the test's diagnostic power.

Results

Physical and Geochemical Properties

The particle size distribution averaged 80 ± 5% clay, 19 ± 4% silt, and 1 ± 1% sand. Although the paleosol was obtained from the unsaturated zone, the calculated air-filled porosity was ⩽ 5%. Water content after 224 d of incubation was similar in the homogenized and intact samples; it ranged between 25.1 and 29.0%, corresponding to an air-filled porosity of 25 and 16%, respectively. In situ measurements of O_2 partial pressure were not possible in the clay-rich, high bulk density sediment. However, the high clay content and low air-filled porosity suggested that the sediment may have contained anaerobic regions.

Sediment geochemical properties and their variabilities are summarized in Table 1. Extractable ions varied more than bulk sediment properties. Extractable organic carbon, inorganic carbon, and sulfate showed the highest variability.

Table 1. Sediment geochemical properties in 12 replicate samples

Property	Mean	SD	Range Low	Range High
Bulk sediment[a]				
pH	6.81	0.06	6.72	6.90
Total phosphate	101	11	78	120
Kjeldahl nitrogen	133	50	100	200
Organic carbon	722	123	510	900
Extractable ions				
Ortho-phosphate	1.12[b]	0.96[b]	<1	2.50
Nitrate	13.5	6.79	5.20	26.0
Organic carbon	29.5	18.8	9.60	64.9
Inorganic carbon	28.3	30.2	5.80	80.5
Sulfate	606	372	173	1150

[a] Ammonium and inorganic carbon in the bulk sediment were below detection in all 12 samples.
[b] Calculated by an assigned value of 50% of the detection limit to samples below the detection limit.

Means for geochemical properties in contiguous and noncontiguous samples were statistically similar, except for bulk sediment organic carbon, and variability in properties was not consistently higher in either type of sample (data not shown). The two contiguous sample sets were statistically different from each other in extractable organic carbon, inorganic carbon, and sulfate, whereas statistically different geochemical properties were not detected in the two noncontiguous sample sets (data not shown). The data suggest that significant spatial variability existed in some geochemical properties at a scale approximating that of the small blocks. Although the spatial variability of microbiological properties was not measured, the distribution of small blocks from contiguous locations to different jars may have been a critical strategy for dealing with microbiological spatial variability in the factorial experimental design.

Cultured Populations

As storage time increased, aerobic heterotrophic bacteria able to grow on 1% PTYG medium increased 100- to 1000-fold in all 6 treatments (Fig. 1A and B). Cultured populations generally increased more rapidly over the first 63 d, and tended to be higher in the homogenized samples than in the intact samples. Of 10 pairwise comparisons between the 0.5% and the 21% O_2 concentrations, 4 showed a greater population at the 0.5% O_2 concentration. Only one comparison had a greater population at the 21% O_2 concentration. Based on the ANOVA analysis, all three factors (post-sampling time, sediment condition, and O_2 concentration) contributed to the response (Table 2). Heat-resistant bacterial spores constituted about 3% of the cultured population at t = 0. They stayed at approximately the same population size during the timecourse in both the homogenized and intact samples (Fig. 1C and D), while the total number of cultured bacteria increased greatly. Post-sampling time was the only significant factor that contributed to the response in heat-resistant bacterial spores (Table 2).

Culturable aerobic fungi were not detected at t = 0; however, fungal propagules typically increased to 100 to 10,000 g^{-1}, as post-sampling time increased (Fig. 1E and F). Propagule numbers increased more rapidly, and tended to be higher, in the homogenized samples than in the intact samples. Of 10 pairwise comparisons between the 0.5% and the 21% O_2 concentrations, 3 showed a greater fungal population at the 0.5% O_2 concentration. None of the comparisons had a greater population at the 21% O_2 concentration. As in the case of aerobic heterotrophic bacteria, all three factors contributed to the response (Table 2). Heat-resistant fungal spores were also not detected at t = 0; however, low numbers of propagules (generally < log 1.0 cfu g^{-1}) were present in 17 of 30 (56%) post-sampling time/treatment combinations (data not shown). There were no statistically significant factors affecting the number of heat-resistant fungal spores (data not shown).

Activity Measurements

Rates of ^{14}C-glucose mineralization increased, and lag times decreased, during storage, in all treatments (Fig. 2). The ^{14}C-glucose mineralization rate was linear 2–7 d after ^{14}C-glucose addition for samples assayed prior to 63 d storage, and from 0.5–3 d for samples assayed at $\geqslant$ 63 d of storage. Evolution of $^{14}CO_2$ in the controls was negligible. Mineralization rates increased at 21 and 35 d in the homogenized samples, but did not change in the intact samples (Fig. 2A and B). Rates in the homogenized samples were generally higher than in the intact samples at 63 and 112 d. All three factors made statistically significant contributions to the observed response (Table 2). Responses for ^{14}C-glucose mineralization rate (Fig. 2B) and O_2 utilization rate (Fig. 2F) were remarkably similar, although the intact samples were homogenized just prior to the ^{14}C-glucose mineralization assay and were left intact during the O_2 utilization assay. This indicates that homogenization of the intact samples just prior to the ^{14}C-glucose mineralization assay did not result in significant artifacts during the linear portion of the response. Lag times for glucose mineralization, as determined

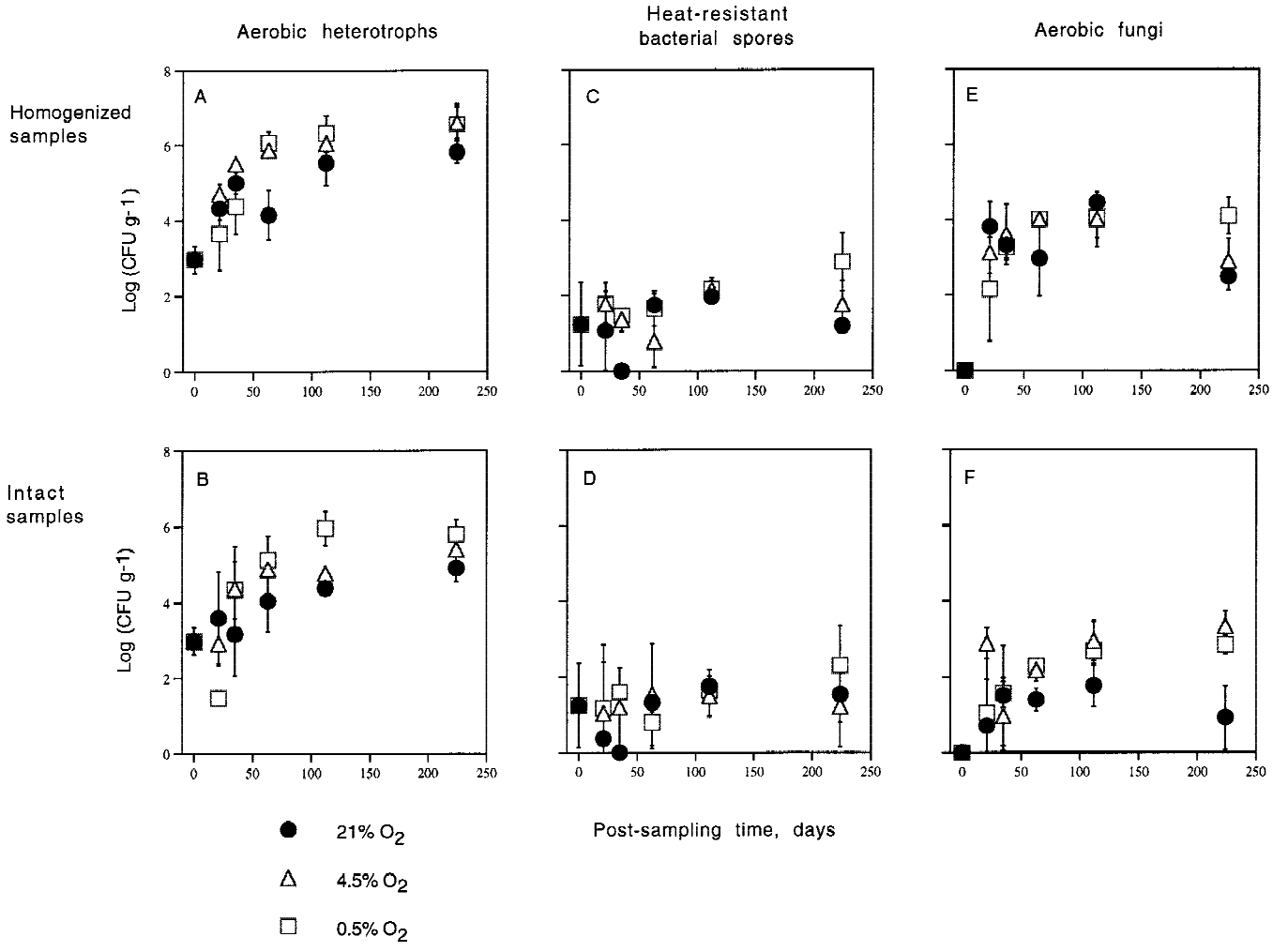

Fig. 1. **(A–F)** The relationship between populations of culturable aerobic microorganisms in sediment samples held at three different O_2 concentrations and increasing lengths of time between sample acquisition and sample analysis. Each value is the mean of triplicate samples. Error bars represent one standard deviation.

by nonlinear regression, decreased sharply at 21 d in the homogenized samples and slowly in the intact samples. The results were shorter lag times in the homogenized samples at 21 through 63 d (Fig. 2C and D). Post-sampling time and sediment condition significantly contributed to lag time (Table 2).

Oxygen utilization in the glucose-induced respiration assay (300 µg g^{-1} sediment) was highest at 63 and 112 d and declined at 224 d (Fig. 2E and F), similar to the response observed for the ^{14}C-glucose mineralization rate (3 µg g^{-1} sediment). The elevated O_2 utilization values in several of the homogenized, reduced O_2 samples at 63 and 112 d (the timepoints with maximum activity and number of cultured heterotrophs) suggest O_2 limitation may have existed in some samples at these timepoints, in the presence of the 100-fold higher carbon input. Utilization rates were linear over the same time periods, after glucose addition, as described above for the ^{14}C-glucose mineralization assay. Oxygen utilization response was a function of all three factors (Table 2).

Biomass

Microscopic direct cell counts were low throughout storage (Fig. 3A and B). Of the 368 cases where 20 fields were counted, cells were not observed in 53% of the cases. The maximum number of cells per 20 fields was 31. As a standard method of applying statistical analyses to data that are below detection, subsamples containing no cells in 20 fields were assigned a value of 50% of the detection level [16], or 8.3×10^4 cells g^{-1}. With this transformation, the cell numbers in all treatments were within log 1.0 cells g^{-1} of the

Table 2. *P*-values for microbiological properties by 3-factor ANOVA

Factor	Heterotrophs	Heat-resistant bacterial spores	Fungi	Mineralization[a]		Oxygen utilization	AODC	Total PLFA
				Lag	Rate			
Post-sampling time (A)	<0.001*	0.046*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.018*
Sediment condition (B)	<0.001*	0.092	<0.001*	<0.001*	0.044*	<0.001*	<0.001*	0.079
O_2 concentration (C)	0.001*	0.212	0.002*	0.331	<0.001*	<0.001*	0.157	0.337
A × B interaction	0.004*	0.741	<0.001*	<0.001*	0.827	<0.001*	<0.001*	0.045*
A × C interaction	<0.001*	0.523	0.001*	0.009*	<0.001*	<0.001*	0.007*	0.318
B × C interaction	0.531	0.699	0.015*	0.535	<0.001*	0.007*	0.519	0.376
A × B × C interaction	0.111	0.925	0.030*	0.383	<0.001*	<0.001*	0.301	0.520

[a] Statistically significant values ($P \leq 0.05$) are followed by an asterisk.

initial (t = 0) cell number. Storage time and sediment condition contributed to the response (Table 2). The rapid dephosphorylation of cellular phospholipids upon cell death makes total PLFA a useful measure of the biomass of living cells [40]. Total PLFA was lower and relatively constant after t = 0, except for elevated values in intact samples at 112 and 224 d (Fig. 3C and D). Compared to t = 0, individual treatment means for viable biomass (excluding elevated values referred to above) were 2- to 3-fold lower. Viable biomass was a function of storage time only (Table 2).

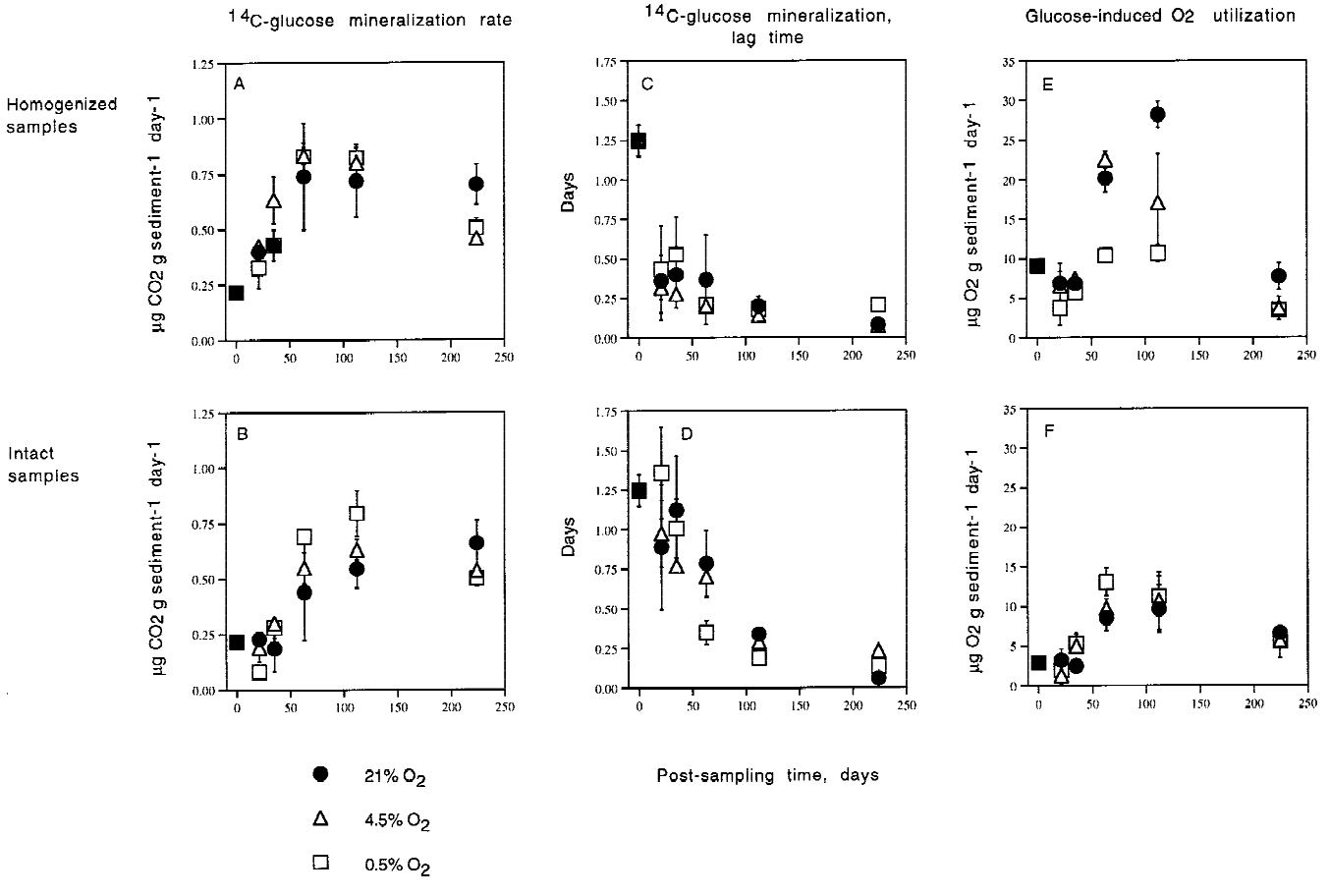

Fig. 2. **(A–F)** Relationship between substrate-induced metabolic rates and lag time in sediment sample held at three different O_2 concentrations and increasing lengths of time between sample acquisition and sample analysis. Each value is the mean of triplicate samples. Error bars represent one standard deviation.

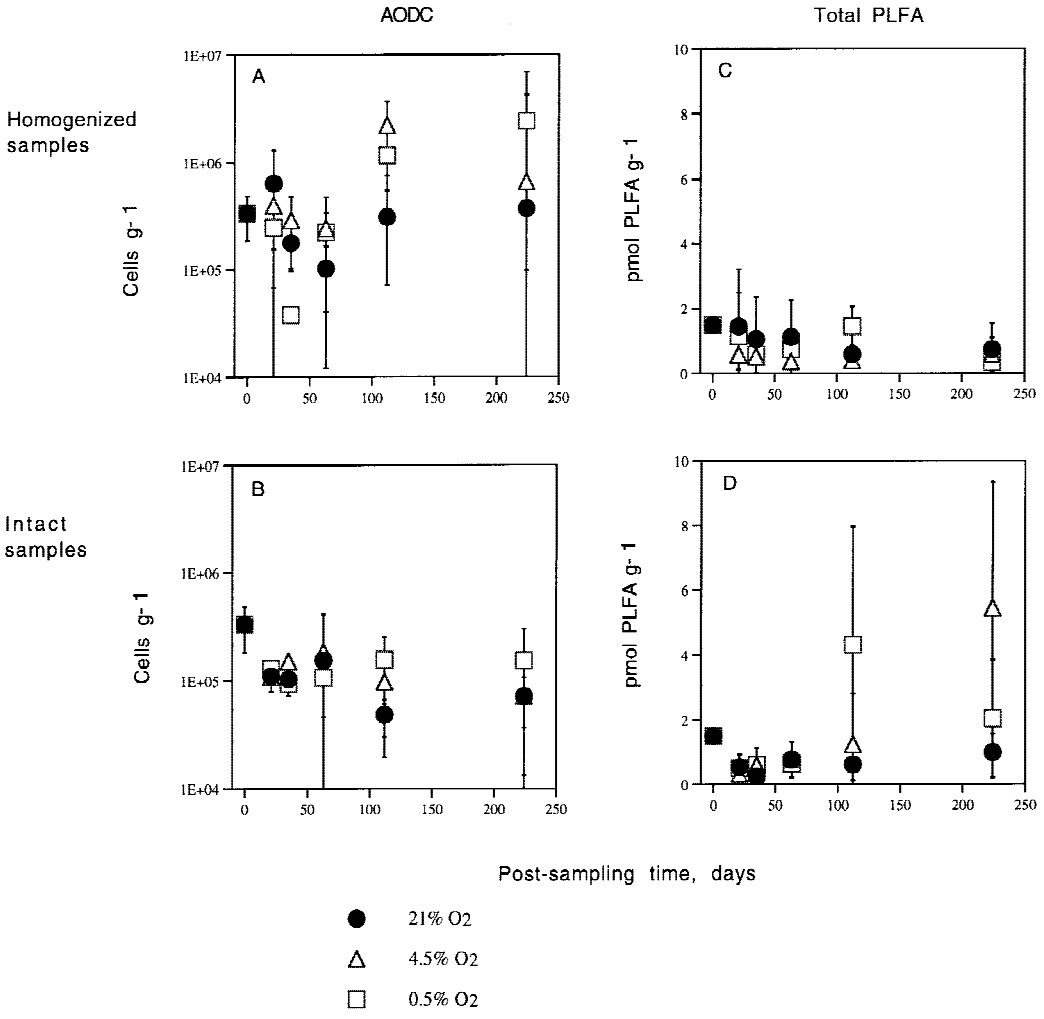

Fig. 3. **(A–D)** Relationship between total biomass and viable biomass in sediment samples held at three different O_2 concentrations and increasing lengths of time between sample acquisition and sample analysis. Each value is the mean of triplicate samples, except the t = 0 total PLFA represents a single measurement performed on pooled triplicate samples. Error bars represent one standard deviation.

Community Composition and Physiological Status

Mole percentages of terminally branched saturated and mid-chain branched saturated PLFA, biomarkers indicative of Gram-positive microorganisms [24], increased during storage. They became the dominant biomarkers in intact sediments stored at 0.5%, and intact and homogenized sediments stored at 4.5% O_2 (Fig. 4A, B, C, and D). When normalized for changes in viable biomass, these PLFA increased >10-fold (range = 11 to 27) in 9 treatments (Fig. 4B, C, and D). The intact samples stored at 0.5% O_2 for 112 and 224 d, and at 4.5% O_2 for 224 d, possessed the highest viable biomass (Fig. 3D); the largest (biomass-normalized) increases in terminally branched saturated and mid-chain branched saturated PLFA (19 to 27-fold; data not shown); and the largest (biomass-normalized) increases in the terminally branched saturate i16:0 (81 to 114-fold; data not shown). Monoenoic PLFA, indicative of Gram-negative microorganisms [35], increased in percentage to 63 d in the intact samples, and remained relatively constant from 21 to 112 d at each O_2 concentration in the homogenized samples. The microeukaryotic biomarker 18:2ω6 [10] generally decreased in percentage after 21 d in all treatments, except the homogenized samples at 21% O_2 (Fig. 4E).

Principal component analysis showed that the biomarker explaining the largest portion of the variability in community structure was the Gram-positive biomarker i16:0 (Table 3). This biomarker was the most positively weighted variable of principal component 1 (PC1) for all sample types, and of PC1 for the i ntact samples. il6:0 was also the most positively

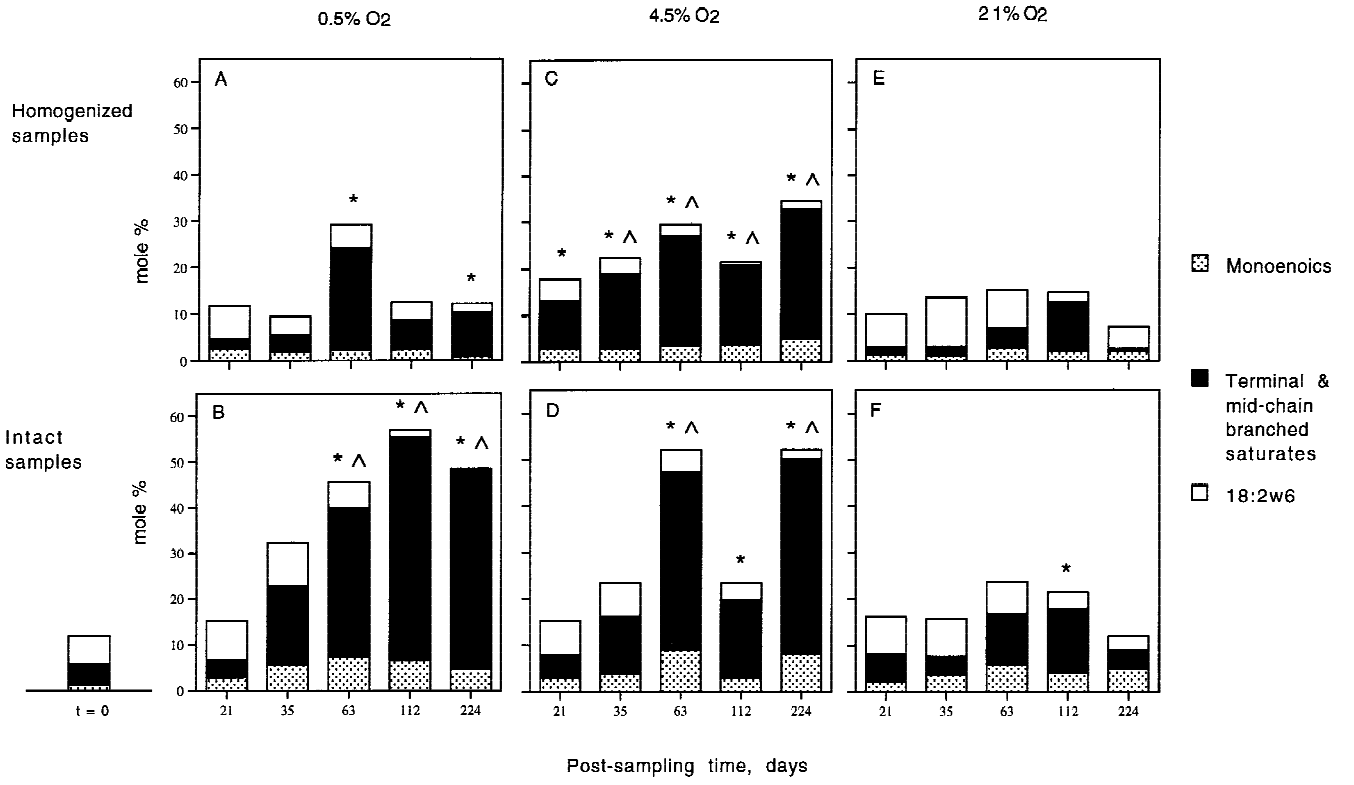

Fig. 4. **(A–F)** Mole percent of phospholipid signature biomarkers in sediment samples with increasing lengths of time between sample acquisition and sample analysis. Samples were held at three different O_2 concentrations. Each value is the mean of triplicate samples, except the t = 0 sample represents a single measurement performed on pooled triplicate samples. *, >10-fold increase in iso 16:0 compared to t = 0, after normalized for changes in viable biomass; ^, >10-fold increase in terminally branched and mid-chain branched saturated PLFA compared to t = 0, after normalized for changes in viable biomass.

weighted variable of PC2, and an important variable of PC1 in the homogenized samples. When normalized for changes in viable biomass, iso16:0 increased >10-fold (range = 17 to 114) in 14 treatments (Fig. 4A, B, C, D, and F).

The ratio of the endogenous storage lipid PHB to total PLFA was very low, or PHB was not detected, from t = 0 to 63 d (Table 4). In contrast, the ratios were much higher at 112 and 224 d. Variability within triplicate samples was similar to or greater than between treatments at the 112 and 224 d timepoints; however, analysis of the data showed that samples $\leqslant$ 63 d and $\geqslant$ 112 d were statistically different.

Discussion

In a previous report, we described dramatic post-sampling changes in the microbiological properties of subsurface paleosols over 75–210 d [4]. In that study, sediment from the center of the core was removed, resulting in fragmentation and some mixing of the sediment. We hypothesized that disturbance could increase the availability of limiting nutrients by redistributing cells and solid-phase nutrients, by increasing gaseous exchange, and by facilitating movement of moisture via evaporation or condensation to generate new microscale chemical gradients at surfaces [4]. In this study, we incubated intact sediment blocks and homogenized sediment at different levels of O_2 and used a factorial experimental design to separate out the effects of storage time, sediment condition, and O_2 concentration. To minimize the effects of spatial heterogeneity, samples were composed of sediment from many noncontiguous locations. Analyses were performed on triplicate samples for each treatment at each timepoint.

Time of storage prior to analysis of samples was the predominant factor in the microbiological response; the effect of time was significant for all 8 microbiological parameters. Six and 4 of the parameters were significantly affected by sediment condition and O_2 concentration, respectively (Table 2). In addition, large shifts in signature biomarkers indicated that the composition of the community was influ-

Table 3. Principal components for the combined, intact, and homogenized sample sets

	Combined		Intact		Homogenized	
Fatty acid	PC1[a]	PC2	PC1	PC2	PC1	PC2
14:0				0.20		
i16:0	0.62		0.75		0.32	0.73
16:1ω7c			0.16			0.10
a17:0			0.18			0.18
17:0	0.17	0.12		0.17	0.25	0.21
18:2ω6	0.28	0.38	0.22	0.48	0.35	
18:1ω9c	0.57	0.30	0.50	0.46	0.61	
10me18:0						0.14
20:0	0.28	0.47		0.28	0.53	
21:0		0.10				
% variance explained:	77	20	70	22	72	16

[a] Coefficients for the five most positively weighted variables are shown for each principal component (PC).

enced by O_2 concentration and sediment homogenization. Statistically significant interactions between the 3 factors were common for most of the parameters. Because they were so common, the roles of the main factors were further investigated using one-factor ANOVA. With one exception, the one-factor ANOVA comparisons confirmed the statistical significance and relative importance of the main factor.

Table 4. Ratio of PHB/PLFA in homogenized and intact samples at different O_2 levels

		Sample type	
Days	O_2 concentration	Homogenized	Intact
0			0.02 a
21	0.5%[a]	0.02 a	0.05 a
	4.5%	2.8 ab	0.02 a
	21.0%	0.19 a	0.01 a
35	0.5%	bd[b]	bd
	4.5%	bd	bd
	21.0%	bd	bd
63	0.5%	bd	0.01 a
	4.5%	bd	0.08 a
	21.0%	bd	bd
112	0.5%	5.4 ab	11 b
	4.5%	126 b	21 b
	21.0%	40 b	97 b
224	0.5%	175 b	67 b
	4.5%	3.3 b	3.5 ab
	21.0%	170 b	3.5 b

[a] Values are means of triplicate samples, except the t = 0 sample represents a single measurement performed on pooled triplicate samples. Numbers in the same column that are followed by the same letter are not significantly different ($P \leq 0.05$).

[b] bd, PHB was below the detection limit.

The exception was the one-factor ANOVA for ^{14}C-glucose mineralization rate, which concluded that sediment condition was statistically significant and O_2 concentration was not.

The pattern over the timecourse for both direct counts and viable biomass was an initial small decrease to values that then remained relatively stable. Similar results have been observed with storage in other subsurface materials [4, 19]. However, the size of the aerobic cultured population increased 2 to 3 orders of magnitude, to approximate the microscopic direct counts at 63 d and thereafter. This shift of the community from a predominantly uncultured state to nearly total culturability may be due to (i) succession or biomass turnover, i.e., growth of specific microorganisms that are able to rapidly respond to the new environmental conditions by utilizing sediment-associated nutrients, including nutrients in dead and moribund cells; (ii) metabolic stimulation of microorganisms that were dormant or otherwise unable to grow immediately following sample acquisition; or (iii) a combination of these two processes. Decreases in total PLFA after t = 0 were common, suggesting that some of the population may have been O_2-sensitive. Although the sampled stratum was in the unsaturated zone, O_2 levels may have been low due to the dominance of clay-sized particles and nearly saturated conditons. O_2-sensitive (aerobic or anaerobic) microorganisms could have died under the more oxygenated conditions of the first 21 d, and contributed to the available nutrients for other microorganisms.

Changes in the mole percents of biomarkers can result from the growth (defined as an increase in cell populations, in cell size, or both) or the death and loss of specific microbial types (which typically results in the growth of other types). Several changes in the biomarkers indicated an increase in the biomass of Gram-positive microorganisms. Principal component analysis showed the Gram-positive biomarker iso16:0 explained the largest portion of the variability in community structure between treatments. i16:0 is indicative of a number of Gram-positive bacteria [24] and accounts for up to 30% of the total fatty acids in a *Streptomyces* species (D. Ringelberg, unpublished data). Other highly weighted variables indicative of Gram-positive microorganisms: 1) The terminally branched saturate, a17:0, which accounts for up to 12% of the total fatty acids in *Bacillus subtilis* [23] and 7% of the total fatty acids in a subsurface *Arthrobacter* [22], and; 2) the mid-chain branched saturate, tuberculostearic acid (10me18:0), found in a number of the actinomycetes [24] (Table 3). Because minor changes in viable biomass complicate the analysis of

community structure, levels of i16:0 and terminally branched and mid-chain branched saturated PLFA were normalized to the t = 0 levels of the biomarkers. This normalization showed that i16:0 increased >10-fold in 14 treatment-time combinations. Thirteen of the 14 cases were associated with the 0.5% and 4.5% O_2 treatments, and 12 were associated with timepoints $\geqslant$ 63 d (Fig. 4A, B, C, D, and F). Following normalization, terminally branched and mid-chain branched saturated PLFA increased >10-fold in 9 treatment-time combinations. All 9 cases were associated with the 0.5% and 4.5% O_2 treatments (although the intact/0.5% O_2 treatment was not represented), and 8 of the cases were associated with timepoints $\geqslant$ 63 d (Fig. 4B, C, and D). Thus, there was a clear association of greatly elevated levels of PLFA biomarkers (both specific and collective) indicative of Gram-positive microorganisms with the later timepoints in the reduced O_2 treatments. Moreover, the greatest increases in both specific and collective Gram-positive biomarkers occurred in the 3 samples with the highest levels of viable biomass. Together, these data clearly reflect substantial growth of Gram-positive microorganisms at the lower O_2 levels. The data cannot address whether the nutrient sources allowing growth were primarily from biomass turnover or from other sediment-associated nutrients.

A number of Gram-positive bacteria, including *Streptomyces* and *Bacillus*, which were indicated to be present in the sediment by lipid analysis, produce heat-resistant spores. Heat-resistant spores comprised about 3% of the cultured population at t = 0. In another study at the Hanford Site, the majority of the bacteria cultured from deep vadose zone sediments were identified microscopically as spore-formers (H. Bolton, personal communication). In addition, fungi were not detected at t = 0, but the signature biomarker 18:2ω6 showed that microeukaryotic biomass may have been present. Protozoa can be excluded as a major contributor, based upon the low biomass of the community; significant numbers of protozoa are typically present only when bacterial biomass is $\geqslant 10^8$ cells g^{-1} sediment (J. Sinclair, personal communication). Thus, fungi may have been initially present as uncultured spores. These results suggest that germination of spores and subsequent growth could play an important role in the post-sampling response of low nutrient flux environments, such as deep vadose zones in arid regions.

The accumulation of PHB reflects excess carbon availability, relative to inorganic nutrients, or unbalanced growth [11]. The ratio of PHB to PLFA normalizes PHB content to the level of viable biomass, and provides an index for community physiological status. Varying PLFA levels could confound the interpretation of PHB/PLFA ratios. However, the PLFA levels vary no more than 20-fold (and $\leqslant$ 4-fold in 23 of the 30 samples), although PHB varies by 4.5 orders of magnitude. Thus, PHB/PLFA ratios in this study are very insensitive to PLFA levels, and high ratios in both the homogenized samples and intact samples at 112 and 224 d indicate unbalanced growth conditions. Maximum cultured populations, maximum activity, and minimum mineralization lag time were attained in both homogenized samples and intact samples at this time. These results indicate that sediment-associated nutrients were not limiting to microorganisms in either the homogenized samples or intact samples until after 63 d storage, and that inorganic nutrient limitation was correlated to a halt in increasing cultured populations and activity. The trend of decreasing ^{14}C-glucose mineralization and O_2 utilization rates between 112 and 224 d may be related to the gradual shutdown of metabolic processes in response to prolonged, unbalanced growth.

Although the microbial community in the homogenized samples and intact samples experienced inorganic nutrient limitation at approximately the same time, the homogenized samples showed a more rapid increase in cultured aerobic heterotrophs and fungi, and glucose mineralization rates; and a more rapid decrease in glucose mineralization lag time. It is likely that homogenization increased the availability rate of limiting nutrients by redistributing cells, and solid-phase and soluble nutrients, enabling a more rapid microbial response. It is also possible that O_2 was less limiting in the homogenized samples. Given the high clay content and high water-filled porosity, a higher fraction of the total sediment volume may have been exposed to diffusing O_2 in the homogenized state (< 2-mm crumbs) than in the intact state (blocks 3 to 5 cm on a side). However, the lack of consistently higher activity in the 21% O_2, as compared to the 0.5% O_2 treatments, in either the homogenized samples or intact samples, suggests that O_2-limitation had little or no effect on activity (except in some homogenized samples at 300 µg glucose g^{-1} sediment, when microbial activity and cultured populations were highest). Thus, at the indigenous levels of carbon and biomass, O_2 availability during storage appears to have been adequate even at 0.5% O_2.

Nutrient flux and the controls it exerts on microbial communities probably explains the differences between microbial response to mixing and storage-induced changes in the studied subsurface sediments [4, 14, 18, 19] versus in other environments. Surface soils and estuarine sediments have

relatively high nutrient fluxes and biomass. Mixing material in these systems results in a very rapid (hours to few days) flush of microbial activity [7, 12, 32]. Respiration decreases in soil that has been mixed and stored for up to several months [29, 30, 31, 36, 37, 38, 39]. This response reflects the diminishing supply of the nutrients that remain after the initial flush of activity. In constrast, subsurface environments can have extremely low moisture recharge rates through the vadose zone, and very low saturated hydraulic conductivities. These conditions can severely limit the supply of soluble nutrients that a microorganism receives. In these environments, perturbation results in a more dynamic microenvironment for microorganisms, by inducing a variety of processes that increase the supply of nutrients to indigenous microorganisms. In deep vadose sediments, viable biomass measured by PLFA is several orders of magnitude lower than in surface soils, microorganisms exist primarily in inactive or dormant forms, and large volumes of sediment may not contain viable or metabolically active microorganisms (Brockman et al., submitted). As a result, resuscitation and growth responses may require significant periods of time for expression. Timescales for equivalent processes are typically weeks to months for subsurface sediments, in contrast to hours to days for soils and estuarine sediments.

The results demonstrate that assays and experiments should be initiated as soon as possible after sample acquisition, if measurement of in situ microbiological properties is desired. Rapid sample processing is especially critical when sediment is homogenized in the field to reduce spatial heterogeneity and yield ''replicate'' subsamples for multi-investigator research teams. In such situations, assays should be initiated on-site. In addition, the fact that these processes also occur in intact samples suggests that minor perturbations in the chemical or physical properties of subsurface sediments can result in major changes in the activity and composition of the microbial community.

Inducing small perturbations in the subsurface may have benefits such as enhancing microbial activity and obtaining a community structure more favorable for degrading contaminants *in situ*. In addition, when very few microorganisms are initially cultured from sediments, post-sampling incubation may be a useful approach for maximizing recovery in culture. Likewise, when biomass is very low and much of the biomass is dormant, clone libraries from extracted rDNA or the corresponding cDNA often contain very few sequences. In these situations, growth or increased activity of previously dormant populations could be used to increase the recovery of microbial diversity.

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References

1. Balkwill DL, Ghiorse WC (1985) Characterization of subsurface bacteria associated with two shallow aquifers in Oklahoma. Appl Environ Microbiol 50:580–588
2. Bligh EG, Dyer WM (1959) A rapid method of lipid extraction and purification. Can J Biochem Physiol 35:911–917
3. Bremer E, Kuikman P (1994) Microbial utilization of ^{14}C[U] glucose in soil is affected by the amount and timing of glucose additions. Soil Biol Biochem 26:511–517
4. Brockman FJ, Kieft TL, Fredrickson JK, Bjornstad BN, Li SW, Spangenburg W, Long PE (1992) Microbiology of vadose zone paleosols in south-central Washington State. Microb Ecol 23: 279–301
5. Brown MJ, P'Pool PK, Thomas SP (1990) Westinghouse Hanford Company effluent discharges and solid waste management report for calendar year 1989: 200/60 areas, WHC-ER-0141-2. Westinghouse Hanford Company, Richland, WA
6. Colwell FS (1989) Microbiological comparison of surface soil and unsaturated subsurface soil from a semiarid high desert. Appl Environ Microbiol 55:2420–2423
7. Craswell ET, Waring SA (1972) Effect of grinding on the decomposition of soil organic matter: oxygen uptake and nitrogen mineralization in virgin and cultivated cracking clay soils. Soil Biol Biochem 4:435–442
8. Difco (1984) Difco Manual, 10th edition. Difco Laboratories, Detroit, MI
9. Driscoll FG (1985) Groundwater and Wells, 2nd edition, Johnson Division, St. Paul, MN
10. Federle TW (1986) Microbial distribution in soil-new techniques. In: Megusar F, Gantar M (eds), Perspectives in Microbial Ecology. Slovene Society for Microbiology, Ljubljana, p 493–498
11. Findlay RH, White DC (1983) Polymeric beta-hydroxyalkanoates from environmental samples and *Bacillus megaterium*. Appl Environ Microbiol 45:71–78
12. Findlay RH, Trexler BT, Guckert JB, White DC (1990) Labo-

ratory study of disturbance in marine sediments: response of a microbial community. Mar Ecol Prog Ser 62:121–133
13. Fredrickson JK, Brockman FJ, Bjornstad BN, Long PE, Li SW, McKinley JP, Wright JV, Conca JL, Kieft TL, Balkwill DL (1994) Microbiological comparison of pristine and contaminated deep vadose sediments from an arid region. Geomicrobiol J 11:95–107
14. Fredrickson JK, Li SW, Brockman FJ, Haldeman DL, Amy PS, Balkwill DL (1995) Time-dependent changes in viable numbers and activities of aerobic heterotrophic bacteria in subsurface sediments. J Microbiol Meth 21:253–265
15. Ghiorse WC, Balkwill DL (1983) Enumeration and morphological characterization of bacteria indigenous to subsurface environments. Develop Ind Microbiol 24:213–224
16. Gilbert RO (1987) Statistical methods for environmental pollution monitoring. Van Nostrand Reinhold, New York
17. Guckert JB, Antworth CP, Nichols PD, White DC (1985) Phospholipid, ester-limited fatty acid profiles as reproducible assays for changes in prokaryotic community structure of estuarine sediments. FEMS Microbiol Ecol 31:147–158
18. Haldeman DL, Amy PS, White DC, Ringelberg DB (1994) Changes in bacteria recoverable from subsurface volcanic rock samples during storage at 4°C. Appl Environ Microbiol 60: 2697–2703
19. Haldeman DL, Amy PS, Ringelberg D, White DC, Garen RE, Ghiorse WC (1995) Microbial growth and resuscitation alter community structure after perturbation. FEMS Microbiol Ecol 17:27–38
20. Hillel D (1980) Fundamentals of Soil Physics. Academic Press, New York
21. Kieft TL, Amy PS, Brockman FJ, Fredrickson JK, Bjornstad BN, Rosacker LL (1993) Microbial abundance and activities in relation to water potential in the vadose zones of arid and semiarid sites. Microb Ecol 26:59–78
22. Kieft TL, Ringelberg DL, White DC (1994) Changes in ester-linked phospholipid fatty acid profiles of subsurface bacteria during starvation and desiccation in porous media. Appl Environ Microbiol 63:1531–1542
23. Microbial Identification Inc (1994) Tryptic soy broth agar library, version 3.8, Newark DE
24. O'Leary WM, Wilkinson SG (1988) Gram positive bacteria. In: Ratledge C, Wilkinson SG (eds), Microbial Lipids, vol. 1:117–201. Academic Press, London
25. Peech M (1965) Hydrogen ion activity. In: Black CA (ed), Methods of Soil Analysis, Part 2, Chemical and Microbiological Properties. American Society of Agronomy, Madison, WI, p 914–926
26. Riley RG, Zachara JM (1992) Chemical contaminants on DOE lands and selection of contaminant mixtures for subsurface science research. U.S. Department of Energy, Washington DC
27. Ringelberg DB, Davis JD, Smith GA, Pfiffner SM, Nichols PD, Nickels JB, Hensen JM, Wilson JT, Yates M, Kampbell DH, Reed HW, Stocksdale TT, White DC (1989) Validation of signature polar lipid fatty acid biomarkers for alkane-utilizing bacteria in soils and subsurface aquifer materials. FEMS Microbiol Rev 62:39–50
28. Ringelberg DB, Townsend GT, Deweerd KA, Suflita JM, White DC (1993) Detection of the anaerobic dechlorinating microorganism *Desulfomonile tiedjei* in environmental matrices by intact samples signature lipopolysaccharide branded-long-chain hydroxy fatty acids. FEMS Microbiol Ecol 14:9–
29. Ross DJ (1991) Microbial biomass in a stored soil: A comparison of different estimation procedures. Soil Biol Biochem 23:1005–1007
30. Ross DJ, Tate KR (1984) Microbial biomass in soil: Effects of some experimental variables on biochemical estimations. Soil Biol Biochem 16:161–168
31. Ross DJ, Tate KR, Cairns A, Meyrick KF (1980) Influence of storage on soil microbial biomass estimated by three biochemical procedures. Soil Biol Biochem 12:369–374
32. Rovira AD, Greacen EL (1957) The effect of aggregate disruption on the activity of microorganisms in the soil. Aust J Agric Res 8:659–673
33. Sinclair JL, Ghiorse WC (1989) Distribution of aerobic and anaerobic bacteria, protozoa, algae, and fungi in deep subsurface environments. Geomicrobiol J 7:15–31
34. U.S. Environmental Protection Agency (1985) Substances found at proposed and final NPL sites through update number three. Document NPL-U3-6-3, U.S. Environmental Protection Agency, Washington DC
35. Vestal JR, White DC (1989) Lipid analysis in microbial ecology. Bioscience 9:535–541
36. Visser S, Fujikawa J, Griffiths CL, Parkinson D (1984) Effect of topsoil storage on microbial activity, primary production and decomposition potential. Plant Soil 82:41–50
37. Wardle DA, Parkinson D (1990) Comparison of physiological techniques for estimating the response of the soil microbial biomass to soil moisture. Soil Biol Biochem 22:817–823
38. West AW, Ross DJ, Cowling JC (1986) Changes in microbial C, N, P and ATP contents, numbers and respiration on storage of soil. Soil Biol Biochem 18:141–148
39. West AW, Sparling GP, Grant WD (1987) Relationships between mycelial and bacterial populations in stored, air-dried, and glucose-amended arable and grassland soils. Soil Biol Biochem 19:599–605
40. White DC, Davis WM, Nickels JS, King JD, Bobbie RJ (1979) Determination of sedimentary microbial biomass by extractable lipid phosphate. Oceologia 40:51–62