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Survival and Phospholipid Fatty Acid Profiles of Surface and Subsurface Bacteria in Natural Sediment Microcosms

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Although starvation survival has been characterized for many bacteria, few subsurface bacteria have been tested, and few if any have been tested in natural subsurface porous media. We hypothesized that subsurface bacteria may be uniquely adapted for long-term survival in situ. We further hypothesized that subsurface conditions (sediment type and moisture content) would influence microbial survival. We compared starvation survival capabilities of surface and subsurface strains of *Pseudomonas fluorescens* and a novel *Arthrobacter* sp. in microcosms composed of natural sediments. Bacteria were incubated for up to 64 weeks under saturated and unsaturated conditions in sterilized microcosms containing either a silty sand paleosol (buried soil) or a sandy silt nonpaleosol sediment. Direct counts, plate counts, and cell sizes were measured. Membrane phospholipid fatty acid (PLFA) profiles were quantified to determine temporal patterns of PLFA stress signatures and differences in PLFAs among strains and treatments. The *Arthrobacter* strains survived better than the *P. fluorescens* strains; however, differences in survival between surface and subsurface strains of each genus were not significant. Bacteria survived better in the paleosol than in the nonpaleosol and survived better under saturated conditions than under unsaturated conditions. Cell volumes of all strains decreased; however, sediment type and moisture did not influence rates of miniaturization. Both *P. fluorescens* strains showed PLFA stress signatures typical for gram-negative bacteria: increased ratios of saturated to unsaturated fatty acids, increased ratios of *trans*- to *cis*-monoenoic fatty acids, and increased ratios of cyclopropyl to monoenoic precursor fatty acids. The *Arthrobacter* strains showed few changes in PLFAs. Environmental conditions strongly influenced PLFA profiles.

Microbial activity has been documented in, and microbes have been recovered from, numerous subsurface terrestrial environments. These include deep water-saturated environments (4, 24, 26) and unsaturated (vadose-zone) environments (2, 18, 23, 26). In some cases, viable microorganisms have been recovered from deep, geohydrologically isolated sediments and rocks, which implies that microorganisms have persisted in situ for millennia (8, 14, 18, 23). Pristine, uncontaminated subsurface environments generally have very low nutrient concentrations (10, 29, 30). Microorganisms isolated from these subsurface environments have been shown to survive similarly nutrient-poor conditions (1) and may be uniquely adapted to extreme nutrient deprivation in the subsurface. The persistence of subsurface heterotrophic communities through geologic time (thousands to millions of years) is possible and may be attributed to rates of endogenous metabolism that are orders of magnitude lower than those of surface communities (11, 33). Microbial survival over similar time scales has been documented for other environments, as well (9, 20).

Physical and chemical conditions in the subsurface strongly influence microbial activities (10, 29). Water availability is an especially important parameter, as evidenced by generally higher abundance and activities of microorganisms in saturated zones than in unsaturated subsurface environments (23, 24, 26). Residual organic matter can also influence subsurface microorganisms (24, 30). Paleosols have been found to have higher numbers and activities of microorganisms than contiguous sediments that did not undergo soil development, possibly due to larger initial populations and/or the presence of residual organic matter as an energy source (8, 23).

The study presented here determined the survival patterns of strains of two common subsurface bacterial genera (*Arthrobacter* and *Pseudomonas*), incubated for over 1 year as pure cultures in microcosms containing natural sediments. Closely related strains of each genus, one isolated from the subsurface and one from a surface environment, were compared. A factorial design was used to test the effects of sediment type (a paleosol versus a similar sediment that did not undergo soil development) and moisture conditions (saturated versus unsaturated) on survival. It was hypothesized that subsurface isolates would survive better than closely related strains isolated from surface environments. It was also hypothesized, based on observations of microbial abundance in various subsurface environments, that the paleosol would be more conducive to survival than the nonpaleosol and that bacteria would persist better under saturated conditions than in unsaturated microcosms.

Membrane phospholipid fatty acid (PLFA) profiles of the bacteria were tested throughout the experiment. Bacteria are known to alter their membrane lipids in response to stress (39, 40), including subsurface bacteria in porous media (25). Stressed gram-negative bacteria typically show increases in the following ratios: saturated to unsaturated fatty acids, *trans*- to *cis*-monoenoic fatty acids, and cyclopropyl fatty acids to their monoenoic precursors (17, 25, 36). Stressed gram-positive *Arthrobacter* spp. have been found to alter their membrane lipids relatively little, at least during experiments lasting only a few days (25, 27). PLFAs were characterized in the present study in

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order to determine PLFA profiles during long-term (>1 year) stress and to determine the influences of environmental parameters (sediment type and moisture content) on PLFA stress signatures.

MATERIALS AND METHODS

Bacterial strains. Three of the bacterial isolates chosen for this experiment were obtained from the Subsurface Microbiology Culture Collection, maintained by David Balkwill at Florida State University, Tallahassee. A surface soil *Arthrobacter* sp., strain SMCC B776, was identified to the genus level based on 16S rRNA sequencing (5) and application of a probe for *Arthrobacter* (31); however, the sequence does not cluster with any previously described species and so is thought to represent a novel, thus far unnamed, species of *Arthrobacter*. The subsurface *Arthrobacter* isolate (SMCC B672) was obtained from the P-24 borehole in the Middendorf aquifer at the U.S. Department of Energy (DOE) Savannah River Site in South Carolina, at a depth of 259 m. Identification of this isolate as an *Arthrobacter* sp. was based on the same testing as for the surface soil isolate; 16S rRNA comparison of the two strains indicates identity at approximately the species level (5). Strain SMCC B672 was not a dominant organism among those cultured from the particular sample from which it was collected; however, closely related strains have commonly been isolated from a variety of subsurface environments, including other coastal plain sediments at the Savannah River Plant, unsaturated sediments in south-central Washington State, and volcanic tuff at the Nevada Test Site (2, 5, 18). Overall, *Arthrobacter* was the most commonly cultured genus (approximately 20% of all isolates) in samples from four different boreholes at the Savannah River Site (5).

A subsurface *Pseudomonas* isolate (SMCC B623) was obtained from the P-24 borehole in the Middendorf aquifer, at a depth of 244 m. Strain SMCC B623 was dominant among the organisms cultured from the 244-m sample, accounting for approximately 25% of the colonies (5). Sequencing of 16S rRNA from this fluorescent strain showed it to be closely related to *Pseudomonas fluorescens* (98 to 99% similarity) (5); Nierzwicki-Bauer reported that this isolate reacted to her fluorescent pseudomonad group probe (31). The surface strain of *Pseudomonas* used in this study is the type strain of *P. fluorescens* (ATCC 13525), whose 16S rRNA has been sequenced and found to be similar at approximately the species level to that of SMCC B623 (5). *P. fluorescens* is commonly isolated from diverse subsurface environments, including the coastal plain sediments at the Savannah River Site (5) and volcanic tuff at the Nevada Test Site (1, 2, 18).

Description of sediments and preparation of microcosms. The two sediment types used in our microcosms, a sandy paleosol and a fluvial silt, were obtained from a section of the Hanford formation exposed at White Bluffs near the DOE's Hanford Site in south-central Washington State (8). These sediments were chosen because large quantities were available in outcrops and because previous findings showed that they harbored microorganisms (8, 23). The Hanford formation comprises a sequence of deposits from catastrophic floods that occurred throughout the Pleistocene along the ancestral Columbia River valley. Sediment was sieved (2-mm-diameter wire mesh) to remove large clasts, including some pedogenic carbonate fragments present in the paleosol. Sediments were homogenized by dividing and remixing in a large-volume (~10 liter) sediment sample splitter. Grain-size analysis of the <2-mm size fraction showed the paleosol to be a silty sand (56% sand, 39.5% silt, 4.5% clay). The other sediment was a glaciofluvial sandy silt (17% sand, 82% silt, 1% clay) and is referred to hereafter as a fluvial silt. Total organic carbon contents of both the paleosol and the fluvial silt were 0.05%. Inorganic carbon was 0.03% in the paleosol and 0.33% in the fluvial silt. Total carbon (organic and inorganic) was measured with a Leco CR-12 carbon analyzer (Leco, St. Joseph, Mich.). Inorganic carbon was measured by acidification with a Coulometric System 140 (U.I.C., Inc., Joliet, Ill.). Subtraction of inorganic carbon from total carbon yielded total organic carbon. Carbon analyses were performed by Huffman Laboratories (Golden, Colo.). Sediment pHs (measured in a 1:1 slurry of sediment and water) were 7.9 for the paleosol and 8.1 for the fluvial silt. For preparation of microcosms, sediments were air dried at room temperature and then dispensed in 400-g amounts into 1-quart home canning jars. In order to sterilize the sediment, the microcosms were autoclaved for 1 hour on 3 successive days. Sterile microcosms were stored at 5°C and warmed to room temperature prior to inoculation.

Inoculation of microcosms. To prepare cells for inoculation of microcosms, cultures were incubated in 1-liter batches at 16°C with intermittent shaking in 100% PTYG broth (for *Arthrobacter* cultures) or 10% PTYG broth (for *P. fluorescens* cultures). The 100% PTYG broth contained (per liter of H_2O) 10 g of glucose, 10 g of Difco yeast extract, 5 g of Difco Bacto Peptone, 5 g of Difco tryptone, 0.6 g of $MgSO_4 \cdot 7H_2O$, and 0.07 g of $CaCl_2 \cdot 2H_2O$; 10% PTYG consisted of 10% of the amount of each of the organic ingredients, with the same concentrations of the inorganic ingredients. After growth to late log phase, the cultures were concentrated by centrifugation in sterile bottles at 5,100 × *g* for 20 min. The pellets were resuspended in 50 to 75 ml of filter-sterilized, autoclaved 0.01 M Tris buffer (pH 7) and recentrifuged. This rinsing procedure was repeated, and the rinsed pellets were resuspended in 100 ml of Tris buffer and combined. The resuspended washed cells were then quantified with duplicate or triplicate direct cell counts with a Petroff-Hausser counting chamber and by phase-contrast microscopy. The culture was then diluted with autoclaved, filter-sterilized NANOpure water (Barnstead, Dubuque, Iowa) to achieve a concentration of 4.0×10^9 cells ml^{-1} for use as an inoculum.

Ten milliliters of inoculum was added to each sterile microcosm, and autoclaved, filter-sterilized NANOpure water was added to achieve approximate gravimetric water contents of 37 to 42% (saturated) and 15 to 20% (unsaturated; 33 to 39% field moisture content; water potential, approximately −0.06 MPa, measured by thermocouple psychrometry). Saturated microcosms actually contained a few air-filled voids after mixing and thus were not completely saturated throughout. However, the bulk of the sediment was at or near water saturation. The inoculum and added water were mixed into the sediment by stirring with stainless steel spoons that had been sterilized and rendered free of organic residue by heating for 5 h at 500°C. All aseptic operations were conducted in a sterile (HEPA-filtered air) hood. Canning jar lids were screwed on tightly and further sealed with Parafilm. Microcosms were incubated in the dark at 16°C. For each bacterial strain, 20 microcosms of each of the following treatments were set up: (i) fluvial silt, unsaturated; (ii) fluvial silt, saturated; (iii) paleosol, unsaturated; and (iv) paleosol, saturated. Several uninoculated experimental controls were prepared for each combination of treatment and bacterial strain by replacing the inoculum with 10 ml of sterile NANOpure water.

Sampling schedule and procedures. Microcosms of each of the four bacterial isolates were set up on separate days. Microcosms were sacrificed for sampling at 0, 1, 2, 4, 8, 16, 32, and 64 weeks of incubation. Each successive sampling interval was doubled, as we hypothesized that initial responses to microcosm conditions would change rapidly and be more variable. At each sampling time and for each treatment (bacterial strain, sediment type, and moisture condition), two microcosms were sacrificed. Each of these duplicate microcosms was opened; the contents were aseptically mixed; and subsamples were then collected for plate counts, direct counts, and PLFA analysis. Remaining microcosms were left unopened and undisturbed until their scheduled sampling dates. Week zero sampling was conducted as soon as possible after inoculation—within 18 h for all strains except the surface *Arthrobacter* strain, which was sampled 36 h after inoculation. Single experimental control (uninoculated) microcosms for each treatment combination were sacrificed for sampling at 0 and 16 weeks (*Arthrobacter* microcosms) and at 0, 8, and 16 weeks (*P. fluorescens* microcosms).

Direct microscopic counts. Direct microscopic counts were performed with the acridine orange method of Ghiorse and Balkwill (15) as previously described (23, 24). This involves elution of cells from sediment in a sodium pyrophosphate solution, fixing in glutaraldehyde, making a slurry in molten agar, smearing onto a defined area of a microscope slide, staining the smear with acridine orange, and counting cells with epifluorescence microscopy. All solutions were filter sterilized and autoclaved. Procedural blanks were made from each of the containers of autoclaved, filter-sterilized 0.1% sodium pyrophosphate used in sampling. Four replicate slides were made from each of the duplicate microcosms that were sacrificed at each time point. To count cells, slides were observed under ×630 magnification with a Zeiss Axioskop epifluorescence microscope. Twenty fields were counted on each of four duplicate slides per sample; the minimum detection limit of this protocol was approximately 3.0×10^4 cells per g (wet weight) of the original sample. In some cases, samples were stored at 5°C after the fixation step for a month or more; storage of fixed samples is routinely done and appears not to affect final counts (15, 21). In a few cases, slides were stored in a sterile container prior to staining and viewing for periods ranging from overnight to a few weeks; experiments in our laboratory have shown that storage at this stage does not affect final counts. In all cases, microscopic counting was performed within a few hours after staining.

Plate counts. Microcosm sediment (1.0 g [wet weight]) was dispensed aseptically into a test tube containing 10 ml of sterile 0.1% sodium pyrophosphate (pH 7.0) and vortex mixed for 1 min to elute cells from the sediment. These samples were then stored on ice for up to 6 h until serial dilutions were prepared and plated. Serial 10-fold dilutions were prepared with 9-ml blanks of sterile phosphate-buffered saline (2.22 g of $Na_2HPO_4 \cdot 7H_2O$, 0.223 g of $NaH_2PO_4 \cdot H_2O$, 8.5 g of NaCl per liter of H_2O). Triplicate 1% PTYG agar plates (0.1 g of glucose, 0.1 g of Difco yeast extract, 0.05 g of Difco Bacto Peptone, 0.05 g of Difco tryptone, 0.6 g of $MgSO_4 \cdot 7H_2O$, 0.07 g of $CaCl_2 \cdot 2H_2O$, 15.0 g of Difco Bacto agar per liter of H_2O) (4) were inoculated and spread with 0.1 ml of suspension from each of the dilution tubes. The plates were incubated at 22°C. Colonies were counted 5 to 7 days and also 2 months after plating. No significant growth of new colonies was observed on any plates between the initial and 2-month counts.

Cell volume analysis. Acridine orange-stained cells observed under epifluorescence microscopy were photographed with slide film. The resultant images were projected onto a screen, and all cells were measured for length and width. Measurements were calibrated by comparison to the size of a projected image of a photographed stage micrometer scale. Fields to be photographed were randomly selected; 2 to 10 cells were measured per sample. Cell volumes were calculated by the following relationship: volume = $[(\pi)\ (w/2)^2\ (l - w)] + [(4/3)\ \pi\ (w/2)^3]$, where l is length and w is width. This formula calculates the volume of rod-shaped and spherical cells. Volume may be underestimated for those cells oriented obliquely or end-on; however, if the assumption is made that cells on all slides orient randomly, then data generated by this method accurately reflect relative sizes. Cell volumes of week-64 *P. fluorescens* samples were not measured.

PLFA analyses. Samples for PLFA analyses were removed and frozen at −70°C immediately after opening of the microcosms. Week-64 samples of *P.*

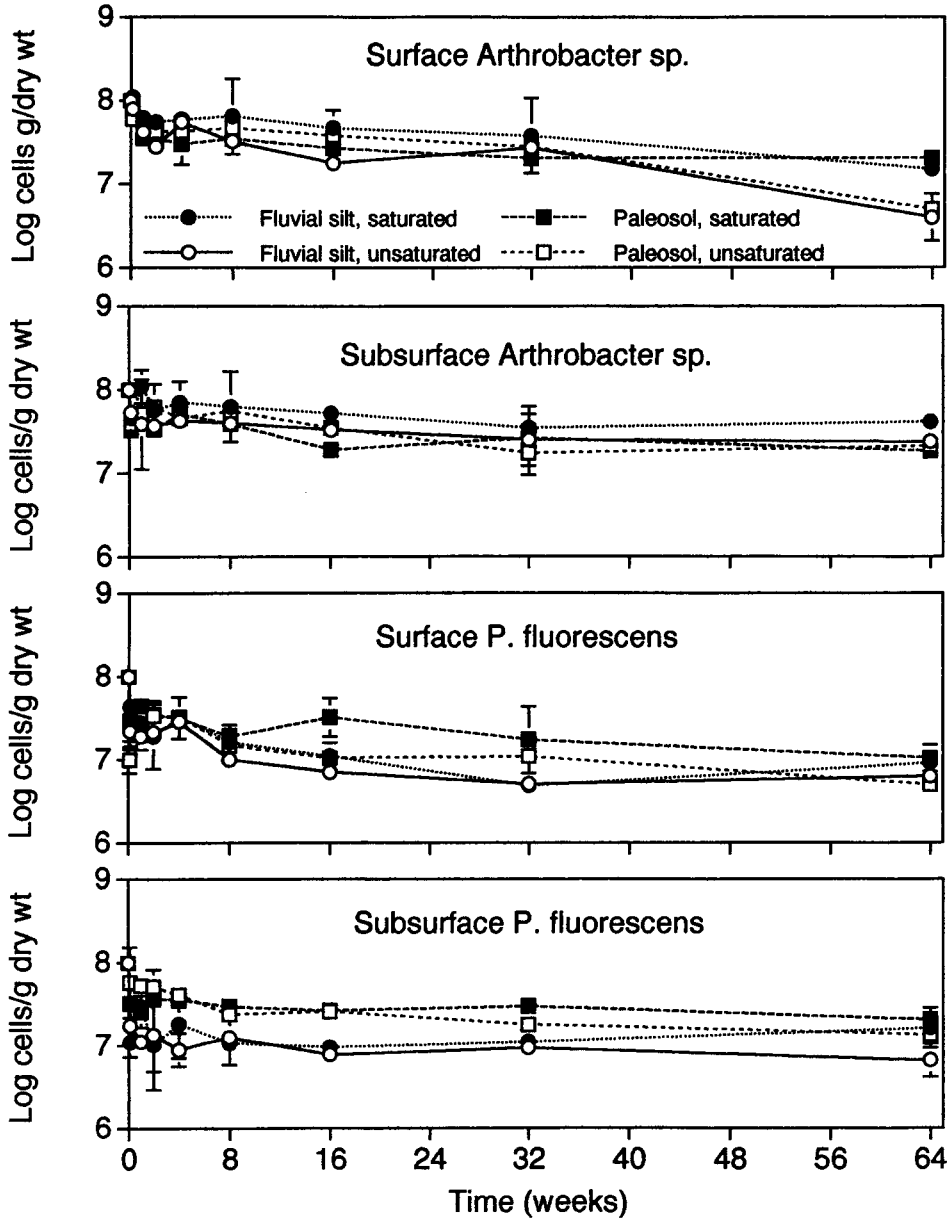

FIG. 1. Direct microscopic counts of surface and subsurface strains of an *Arthrobacter* sp. and surface and subsurface strains of *P. fluorescens* in sediment microcosms during 64 weeks of incubation. Error bars show 1 standard deviation ($n = 2$).

fluorescens microcosms were not analyzed for PLFAs. Samples were sent frozen on dry ice to the University of Tennessee for analysis. Extraction and quantification of PLFAs were performed essentially as described by Kieft et al. (25). Fatty acid designations are as described by Ringelberg et al. (37).

Statistical analyses. Factorial analyses of variance (ANOVA) were performed with the general linear method (SYSTAT, version 5.2, for the Macintosh; SYSTAT, Inc., Evanston, Ill.) on plate count, direct count, cell volume, and PLFA data. For all statistical analyses, the sample size (n) for each date and treatment was 2, reflecting the number of microcosms sacrificed. Although replicate microbiological analyses were performed on each of these microcosm samples as described above, the results of these replicate analyses were averaged to avoid pseudoreplication. We tested the main effects of genus, strain, sediment type, saturation condition, and time, along with two-way interactions of time with each of the other factors. Testing two-way interactions with time elucidated differences between bacterial types and between microcosm conditions in the responses of the bacteria over time without the need to fit curves to the data. Direct count and plate count data were $\log_{10}$ transformed for ANOVA to ensure that variances were independent of the means.

RESULTS

Direct counts. Declines in total bacterial counts ranged from less than 1 to nearly 2 orders of magnitude during the 64-week incubation, depending on the type of organism and treatment (Fig. 1). For nearly all microcosms, numbers of cells observed on all sampling dates were well below the number of inoculated cells (10^8 cells g of sediment [dry weight]$^{-1}$). The two exceptions were the saturated fluvial silt microcosms of both the surface and subsurface *Arthrobacter* strains, in which the cell numbers initially increased. ANOVA showed significant main effects of time ($P < 0.001$), genus ($P < 0.001$), and sediment type ($P = 0.006$); differences between surface and subsurface strains and between moisture conditions were not significant. ANOVA of interactions with time showed that

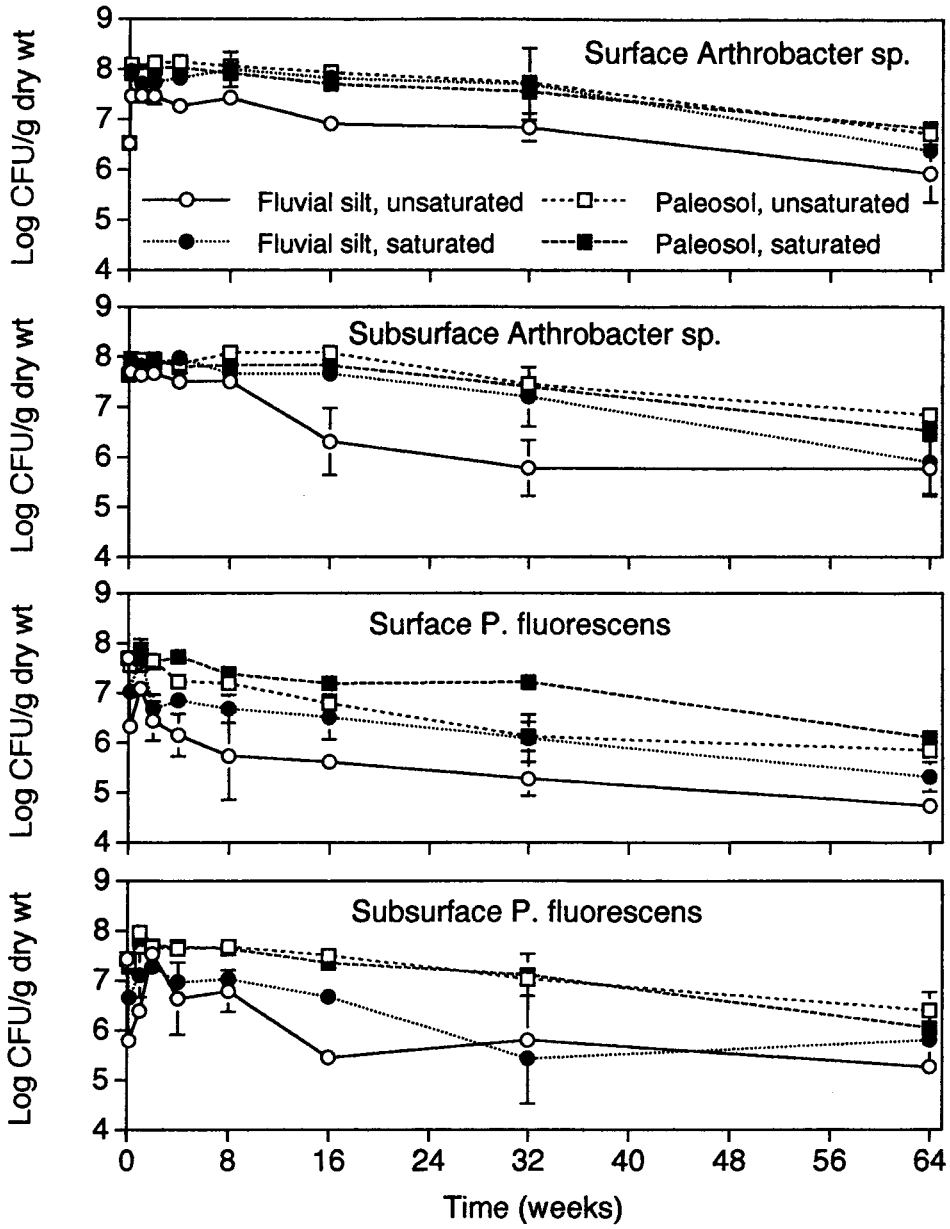

FIG. 2. Plate counts of surface and subsurface strains of an *Arthrobacter* sp. and surface and subsurface strains of *P. fluorescens* in sediment microcosms during 64 weeks of incubation. Error bars show 1 standard deviation ($n = 2$).

declines in direct counts varied significantly between bacteria of the two genera ($P < 0.001$) and between moisture conditions ($P = 0.006$); differences between surface and subsurface strains and between sediment types were not significant. *Arthrobacter* cell counts were generally higher than *P. fluorescens* cell counts in the first few weeks of the experiment. Total cell counts of *P. fluorescens* were higher in paleosol microcosms than in fluvial silt microcosms.

Plate counts. Culture counts of the inocula (calculated based on plate counts of cell suspensions at the time of inoculation and dry weight of microcosm sediments and assuming uniform distribution of cells) were 3.39×10^6 CFU g (dry weight) of microcosm sediment^{-1} for the surface *Arthrobacter* strain; 4.38×10^7 CFU g (dry weight) of sediment^{-1} for the subsurface *Arthrobacter* strain; 5.08×10^7 CFU g (dry weight) of sediment^{-1} for the surface *P. fluorescens* strain; and 2.68×10^7 CFU g (dry weight) of sediment^{-1} for the subsurface *P. fluorescens* strain. Culture counts declined in all microcosms over the course of the experiments (Fig. 2); however, differences occurred among bacterial types and between treatments. The main effects of time, genus, sediment type, and moisture were significant (ANOVA, $P < 0.0001$ for each); differences between surface and subsurface strains were not significant ($P = 0.568$). Differences in culturability, tested by ANOVA as interactions with time, were significant between sediment types ($P < 0.039$); differences in culturability with time between genera, between surface and subsurface strains, and between moisture conditions were not significant. Survival was generally greater in the *Arthrobacter* microcosms than in the *P. fluorescens* microcosms, greater in the paleosol than in the fluvial

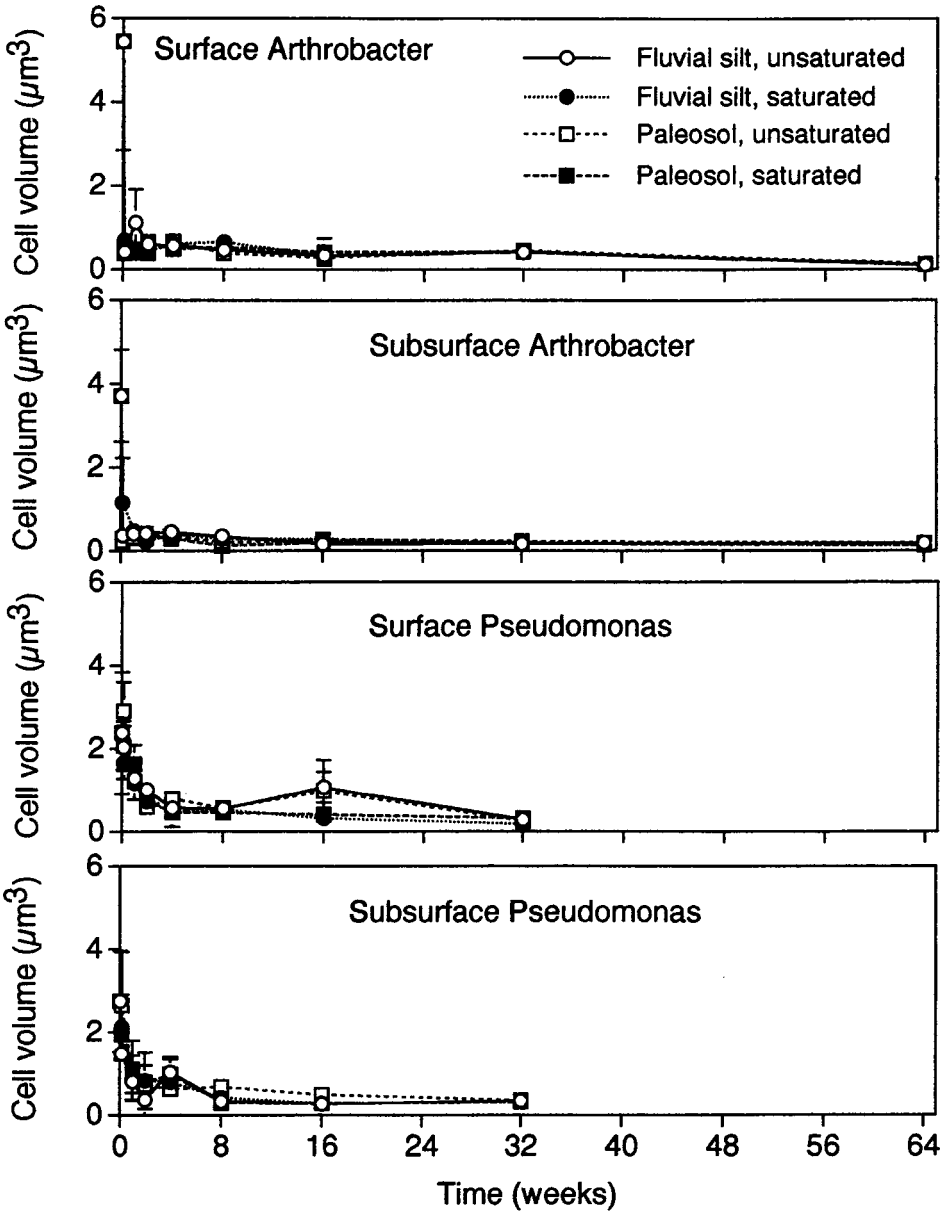

FIG. 3. Cell volumes of surface and subsurface isolates during 64 weeks of incubation in sediment microcosms. Error bars show 1 standard deviation ($n = 2$).

silt, and greater under saturated than under unsaturated conditions.

All *Arthrobacter* plate counts showed increases in culture counts over those of the inoculum within 18 to 36 h (i.e., at week zero sampling). Culture counts decreased in all *P. fluorescens* microcosms between inoculation and the first sampling event. All *P. fluorescens* microcosms demonstrated a recovery of culturability within the first week of incubation, to levels close to or exceeding those of the inocula.

Throughout the experiment, culturability of both strains of *P. fluorescens* was significantly lower than that of both strains of *Arthrobacter*. This was particularly evident in fluvial silt microcosms and less pronounced in the saturated paleosol microcosms. Culture counts of all strains were generally lowest in the unsaturated fluvial silt microcosms. Culturability of the surface *P. fluorescens* strain was generally lower in unsaturated microcosms than in saturated microcosms.

Cell volume. All cells underwent miniaturization, beginning immediately upon inoculation into microcosms (Fig. 3). The main effects of time and genus were highly significant (ANOVA, $P < 0.001$ for each); differences between strains were also significant ($P = 0.048$). Differences in cell sizes, tested as interactions with time, were significant between the two genera ($P < 0.001$); the effects of strain type, sediment type, and degree of saturation on cell sizes were not significant. Both *Arthrobacter* strains were initially larger than either *P. fluorescens* strain, but all four strains showed considerable variation in inoculum cell volume. Both the aquifer and surface strains of *Arthrobacter* responded similarly across all treatment combinations. Reduction in *Arthrobacter* cell volume was im-

mediate and profound. Cell volume was reduced about 10-fold between inoculation and the first sampling event, a period of 36 h for the surface *Arthrobacter* strains and 18 h for the aquifer *Arthrobacter* strain. *P. fluorescens* cell volumes declined more gradually over the first few weeks, but volumes at 32 weeks were generally comparable to *Arthrobacter* volumes on that date. Mean volumes continued to decline gradually over the remainder of the experiment.

PLFAs. Total PLFAs averaged 21 pmol g^{-1} for the surface *Arthrobacter* strain, 40 pmol g^{-1} for the subsurface *Arthrobacter* strain, 182 pmol g^{-1} for the surface *P. fluorescens* strain, and 129 pmol g^{-1} for the subsurface *P. fluorescens* strain over the course of the experiment. The experimental controls from uninoculated microcosms averaged 4 pmol g^{-1}; these were predominantly palmitic acid (16:0).

The *Arthrobacter* isolates showed minimal changes in PLFA profiles during the course of the experiment (Fig. 4 and 5). Ratios of saturated to unsaturated fatty acids rose transiently in the surface *Arthrobacter* strain but returned to preincubation levels within 8 weeks. The subsurface *Arthrobacter* strain showed only minor changes in the saturated-to-unsaturated ratio; these ratios were slightly higher in the unsaturated microcosms than in the saturated microcosms. The ratio of saturated to unsaturated fatty acids did not differ significantly with time or between genera, strains, sediment types, or moisture conditions (ANOVA). Chain lengths, indicated by the ratios a15:0/a17:0 and 16:0/18:0, increased transiently in both strains to differing degrees depending on microcosm conditions. These ratios were generally higher in the unsaturated than in the saturated samples (Fig. 4 and 5). Ratios of anteiso- to iso-branched fatty acids did not vary significantly.

The two *P. fluorescens* strains showed typical stress responses in their PLFA profiles: increased ratios of saturated to unsaturated fatty acids, increased ratios of *trans*- to *cis*-monoenoic fatty acids, and increased ratios of cyclopropyl fatty acids to their monoenoic precursor fatty acids (Fig. 6 and 7). Differences were observed between surface and subsurface strains and especially between different treatments. Ratios increased to a greater extent in the surface *P. fluorescens* strain than in the subsurface isolate. Ratios returned to near preincubation levels in some treatments in the surface *P. fluorescens* strain. The *trans*-to-*cis* and cyclopropyl-to-monoenoic ratios were generally higher in the paleosol microcosms than in the fluvial silt microcosms; the cyclopropyl-to-monoenoic ratios remained significantly higher after 32 weeks of incubation in the paleosol microcosms than in the fluvial silt. The ratios of *trans*- to *cis*-monoenoic fatty acids rose to significantly higher levels in the surface *P. fluorescens* strain than in the subsurface strain (Fig. 6 and 7).

DISCUSSION

All microcosms continued to support viable populations after incubations exceeding 1 year. This is not unusual, in that *Pseudomonas* and *Arthrobacter* are common genera in soil and the subsurface (2, 10, 18), both of which are nutrient-poor environments considered to select for starvation survivors. Species of both *Arthrobacter* and *Pseudomonas* have been shown to survive starvation in liquid (1, 6, 28). *Arthrobacter* spp. are considered to be oligotrophic bacteria adapted to carbon-limited terrestrial environments (35). The subsurface isolates studied here were recovered from borehole samples collected from great depths (>200 m) in the Middendorf aquifer at the Savannah River Site, in a portion of the aquifer where groundwater age has been estimated by ^{14}C dating to be 8,000 years (30). The groundwater age and low organic carbon content of the porewaters (<0.1 to 1.0 mg of C $liter^{-1}$) indicate low nutrient flux and, thus, starvation conditions. It should be noted that in the context of this study, survival refers to the net survival of microbial populations; cell division may have occurred concomitant with cell death. Poor survival of a *Pseudomonas aureofaciens* strain and an *Arthrobacter protophormiae* strain under nutrient-limited but moist conditions in quartz sand was reported by Kieft et al. (25); however, quartz sand may be a less hospitable medium for survival than the natural sediments of the present study.

The subsurface strains and surface strains of the same species survived equally well in this experiment, thus refuting our first hypothesis. This may be explained if one assumes that subsurface microorganisms are derived from surface populations. The groundwater age of the sediments from which these subsurface strains were isolated (8,000 years) suggests that these microbes could have resided in the subsurface for at least that long. However, this may have been insufficient time for selection of greater starvation survival characteristics, especially given the low growth rates in the subsurface (33). It may also be that starvation survival traits are selected for as strongly in surface environments as they are in the subsurface. These bacteria may also be at energetically constrained limits of adaptation to starvation survival. Yet another possibility is that differences in survival between surface and subsurface strains might have become evident if the experiment had been continued longer.

The hypotheses that sediment type and moisture conditions would influence survival were supported by the data. Better survival in the paleosol than in the fluvial silt may be explained by qualitative differences in organic carbon. Although both sediment types had the same quantity of organic carbon, organic substrates in the paleosol may have been more readily available and/or more easily metabolized. Differences in texture and mineralogy may also be important. Clay minerals have been shown to protect bacteria from various stresses, including starvation (13); the slightly higher clay content of the paleosol may have enhanced survival. Differences due to moisture content are to be expected. An important effect of unsaturated conditions is to decrease nutrient availability, as discussed by Kieft et al. (23). The thin water films of the unsaturated sediments decrease nutrient diffusion and bacterial mobility, thus limiting microbial access to nutrients. The water potentials were high enough in both treatments to prevent cell desiccation (near zero for the saturated sediments and >-0.1 MPa in the unsaturated sediments).

Differences in patterns of culturability between genera were most pronounced immediately following inoculation. All *P. fluorescens* microcosms lost culturability between inoculation and the first sampling event, whereas all *Arthrobacter* microcosms gained in culturability within the same interval. The initial gain in *Arthrobacter* culture counts was probably related to expression of a myceloid growth form in the inoculum. Myceloid cells occur in *Arthrobacter* as clusters and chains of branching, incompletely divided cells (12). Myceloid cells were predominant in our *Arthrobacter* inocula. In counting cells in our inocula, we designated each portion of a myceloid that appeared to be a single cell as one individual. Deutch and Perera (12) reported that myceloid cell aggregates could not be disrupted by mechanical agitation. When our inocula were plated, we would expect each myceloid cluster of cells to form a single colony, yielding a total culturability well below the actual number of cells (by a factor corresponding to the average number of cells per myceloid). Subsequent recovery of culturability in the microcosms may reflect disaggregation of myceloids. While making direct cell counts, we observed a

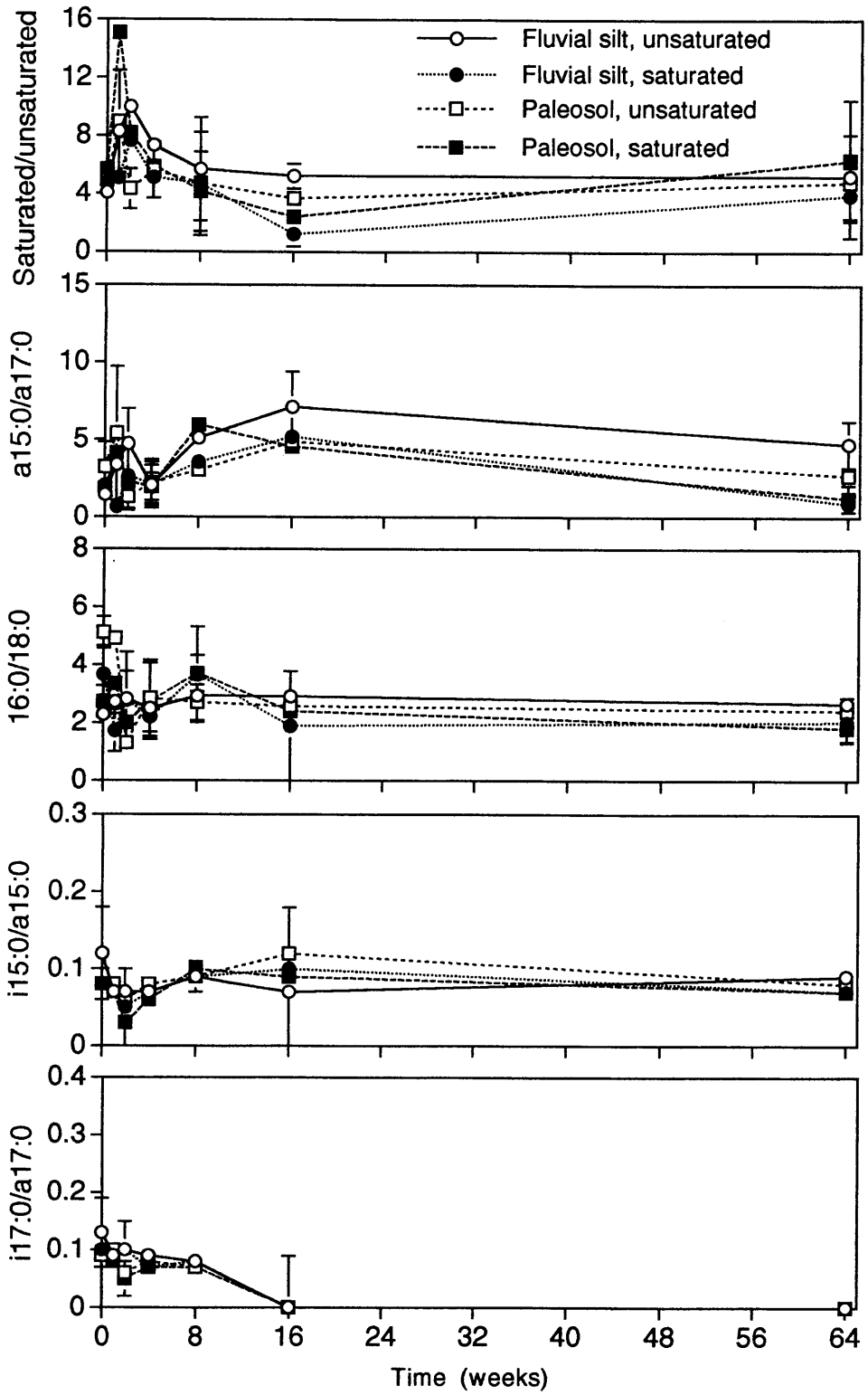

FIG. 4. PLFA ratios reflecting membrane fluidity of a surface *Arthrobacter* strain in sediment microcosms during 64 weeks of incubation. Error bars show 1 standard deviation ($n = 2$).

small but significant proportion of pairs, clusters, and chains of *Arthrobacter* cells in samples collected early in incubation. These disappeared after a few weeks, suggesting that myceloid clusters slowly disaggregated following inoculation.

The initial drop in total cell numbers observed in all microcosms from levels of the inocula may have been caused by the sudden stress imposed by cell washing and inoculation into the microcosm sediments. The decline may also be explained, at

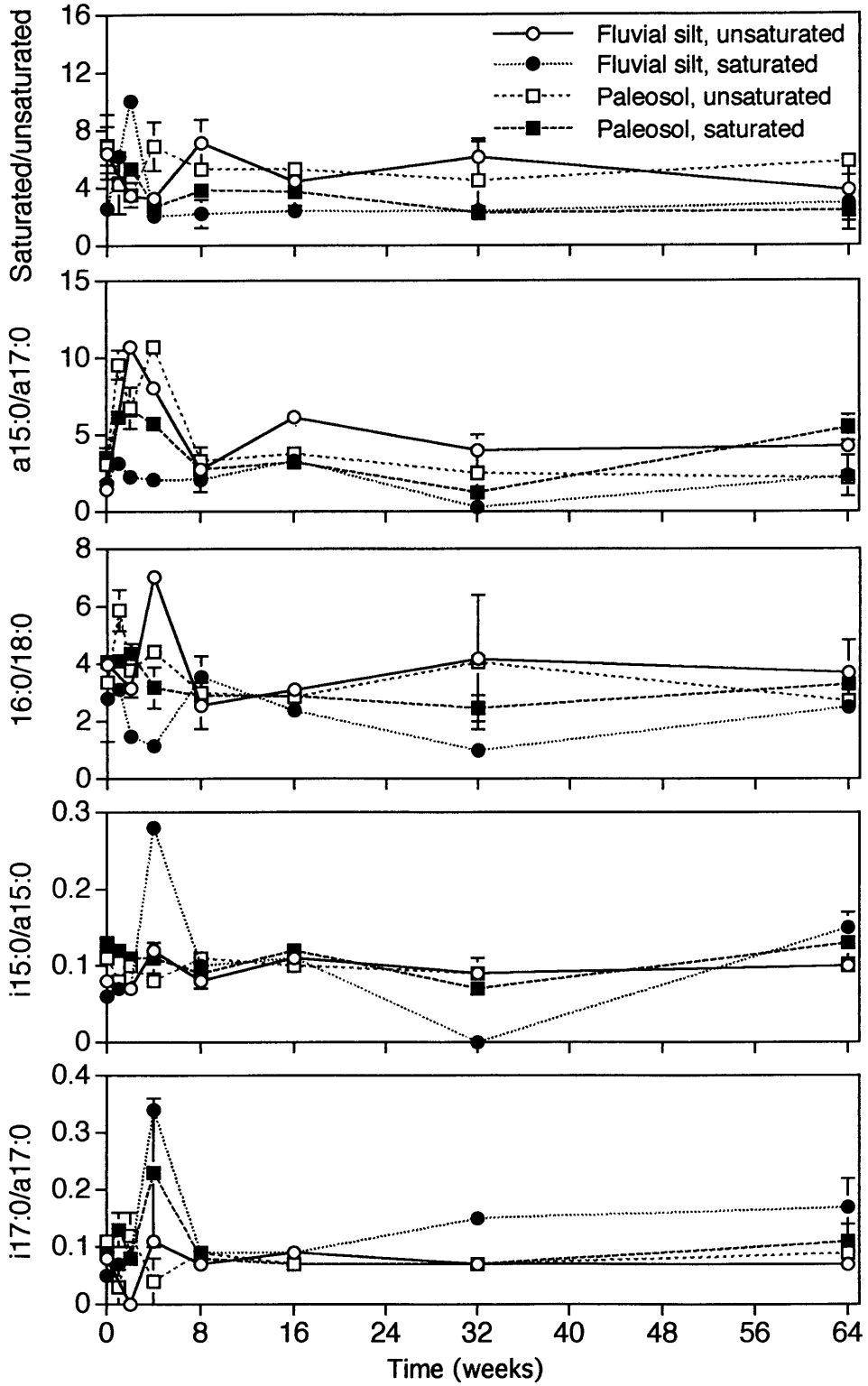

FIG. 5. PLFA ratios reflecting membrane fluidity of a subsurface *Arthrobacter* strain in sediment microcosms during 64 weeks of incubation. Error bars show 1 standard deviation ($n = 2$).

least in part, by attachment effects. Irreversible cellular attachment to sediment particles may have prevented elution of cells during preparation for direct counting, and so direct counts may have underestimated total cell counts. Attachment may also have increased during the course of the experiment and could therefore have contributed to the gradual declines in total cell counts. Attachment effects cannot be definitively invoked, as the experiment was not designed to distinguish be-

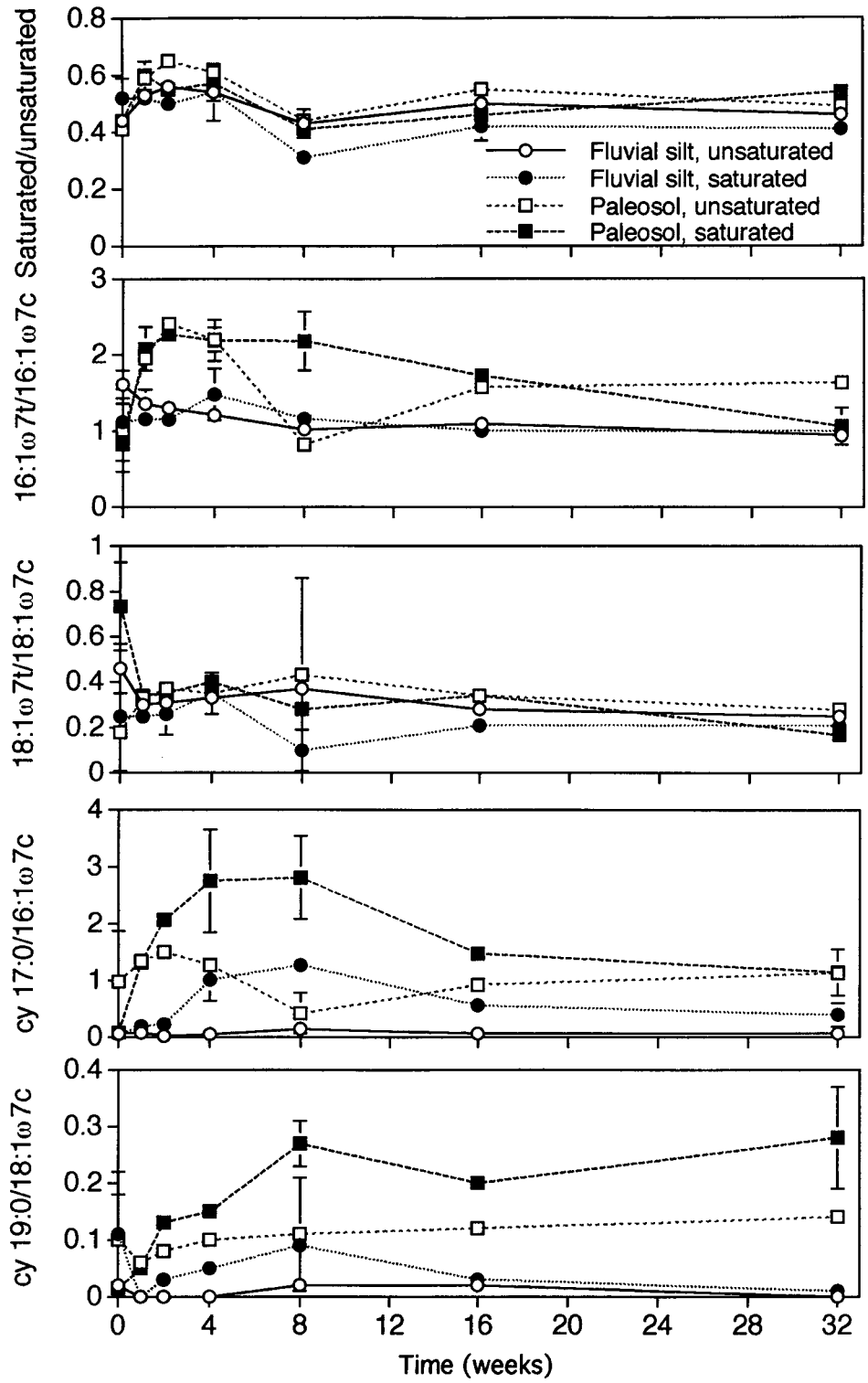

FIG. 6. PLFA stress-associated ratios of a surface *P. fluorescens* strain in sediment microcosms during 64 weeks of incubation. Error bars show 1 standard deviation ($n = 2$).

tween attachment and cell death followed by disintegration. However, numbers of culturable cells exceeded total cell numbers in several microcosms of both *Arthrobacter* and *P. fluorescens*, particularly during the early stages of incubation, a finding that could be explained by attachment.

Cell size measurements and cell volume calculations demonstrated a dramatic miniaturization of all strains. Reduction in cell volume during starvation has been documented for a variety of bacterial genera (16, 22). The miniaturization of *Arthrobacter* in this experiment contrasts with the absence of

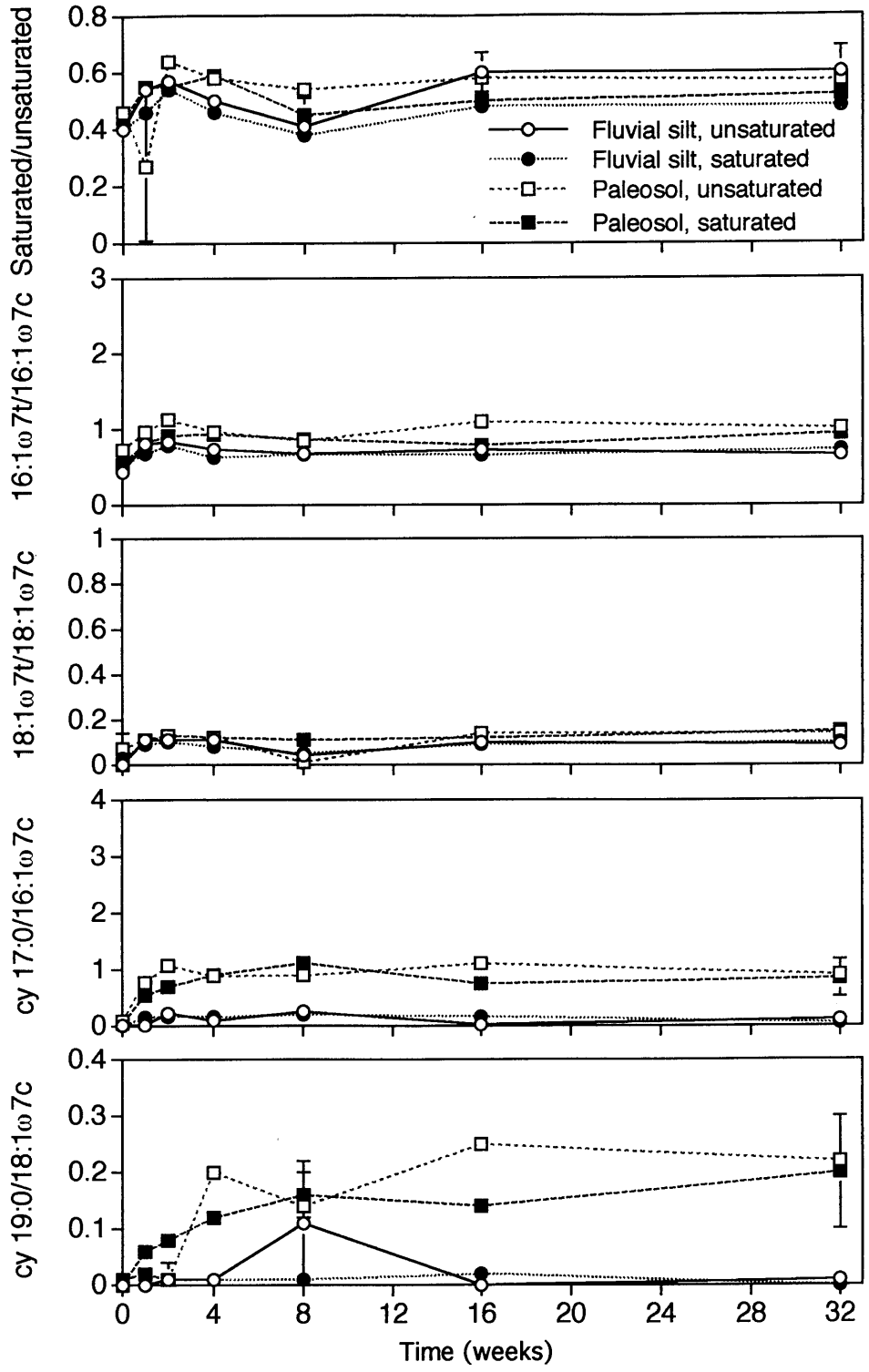

FIG. 7. PLFA stress-associated ratios of a subsurface *P. fluorescens* strain in sediment microcosms during 64 weeks of incubation. Error bars show 1 standard deviation ($n = 2$).

miniaturization in *Arthrobacter crystallopoietes* noted by Boylen and Pate (7). Differences in morphological responses may be due to differences between species and/or differences in starvation conditions. Miniaturization of cells can occur concomitant with fragmentation (cell division without growth) (3, 28, 32); however, fragmentation was not evident in our experiment. Although cell volumes were greatly diminished after 64 weeks from those of the inocula, they were still larger than the ultramicrocells often observed in soils and the subsurface (22). This suggests that slow endogenous metabolism could continue

to fuel starvation survival for even longer periods of time than this experiment without exhaustion of cellular reserves.

The lack of significant membrane fatty acid changes in the *Arthrobacter* strains is consistent with the findings of a previous relatively short-term starvation experiment (25). The particular PLFA ratios examined in this study are those that would be expected to affect membrane fluidity in *Arthrobacter*. The membrane lipids of these bacteria are dominated by branched-chain fatty acids. Membrane fluidity can be modified by changes in the ratio of saturated to unsaturated fatty acids, changes in fatty acid chain length, and changes in the ratios of iso- to anteiso-branched fatty acids (19). Apparently the adaptations to starvation that *Arthrobacter* spp. undergo, e.g., cell miniaturization (this experiment) and sharp decreases in endogenous respiration (6), do not require changes in membrane fluidity and are not accompanied by changes in PLFA profiles, even after over 1 year of incubation. Lack of an apparent PLFA stress signature in *Arthrobacter* spp. may be an essential aspect of their extraordinary survival capabilities.

The changes in membrane PLFA profiles observed in both *P. fluorescens* strains are consistent with previous observations of stressed gram-negative bacteria (17, 25, 36). Typical stress-induced changes in PLFA ratios occurred in both strains. Microcosm conditions had a strong effect on the degree of this PLFA response: *trans*-to-*cis* ratios and cyclopropyl-to-monoenoic ratios were generally higher in the paleosol microcosms than in the fluvial silt. Increases in cyclopropyl-to-monoenoic ratios have been found to be associated with anaerobic conditions, as well as nutrient stress (25); however, because organic carbon contents of the sediments were low and microbial metabolism was likely very slow, it is unlikely that oxygen was depleted in our microcosms. The return of the stress ratios to preincubation levels after 16 to 32 weeks in the surface *P. fluorescens* strain is of interest. This may signal a long-term adjustment or acclimation to the relatively nutrient-poor, static conditions of the sediments. It may also relate to observations of PLFA profiles in subsurface sediment communities collected directly from nature. The high PLFA ratios normally associated with stressed cells are often lacking in these profiles, even where the environments are severely nutrient limiting (34, 38). The membrane lipids of bacteria undergoing truly long-term survival, i.e., over geologic time periods, may not differ appreciably from those of apparently nonstressed cells.

Our studies demonstrated that surface and subsurface strains of *Arthrobacter* and *P. fluorescens* survived well in natural porous media for over 1 year and that environmental conditions strongly influenced survival and PLFA profiles. Unfortunately, it is difficult to extrapolate data from 1-year incubations to thousands or millions of years. However, a growing body of evidence suggests that survival over such time scales has occurred in nature (20). Our experiment was intended to simulate natural conditions with actual subsurface sediments; nevertheless, the experimental protocol, including rapid shift-down during cell washing, may have been harsher than that which occurs during sediment burial or during bacterial transport from the surface. Thus, survival of these strains under laboratory conditions could indicate an even greater potential for survival in nature. The trends in morphology and physiology of the bacteria in this experiment during extended incubation in laboratory microcosms are consistent with the apparent status of bacteria as they exist in the subsurface. We observed cell miniaturization and decreasing culturability relative to total numbers of cells in laboratory microcosms; this is consistent with previous findings that subsurface communities are generally characterized by a high proportion of miniaturized, nonculturable cells (22).

It is also difficult to generalize about subsurface bacteria based on a comparison of these two subsurface strains with closely related surface isolates. In many ways, these strains are representative of bacteria from pristine terrestrial environments. These and related strains of *Arthrobacter* and *Pseudomonas* are the most commonly isolated gram-positive and gram-negative bacteria, respectively, from a variety of saturated and unsaturated subsurface environments, as well as soils (1, 2, 5, 18). They are aerobic bacteria, typical of the microorganisms of organic-carbon-poor, electron-donor-limited, uncontaminated subsurface environments (30). Although coastal plain aquifers such as the ones from which these strains were isolated may be atypical in having relatively large numbers of indigenous microorganisms, the microbial community structure of these aquifers is qualitatively similar to those of other subsurface environments (5). However, even though the subsurface and surface bacteria of this study are representative of the communities from such environments, it will be necessary to compare many more strains, using molecular as well as physiological approaches, before solid paradigms can be established regarding the relationship of subsurface microbes to surface microorganisms.

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