MICROBIAL ECOLOGY

Microb Ecol (1998) 35:156–171

Microbial Communities in High and Low Recharge Environments: Implications for Microbial Transport in the Vadose Zone

D.L. Balkwill,[1] E.M. Murphy,[2] D.M. Fair,[1] D.B. Ringelberg,[3] D.C. White[3,4]

[1] Department of Biological Science, Florida State University, Tallahassee, FL 32306-3043, USA
[2] Environmental and Energy Sciences Division, Pacific Northwest National Laboratory, Richland, WA 99352, USA
[3] Center for Environmental Biotechnology, University of Tennessee, Knoxville, TN 37932-2567, USA
[4] Environmental Sciences Division, Oak Ridge National Laboratory, Oak Ridge, TN 37831, USA

Received: 6 November 1996; Accepted: 9 May 1997

ABSTRACT

Microbial communities along vertical transects in the unsaturated zone were evaluated at five sites in the Pasco Basin, in southeastern Washington State. Sites with contrasting recharge rates were chosen to maximize or minimize the potential for microbial transport. Pore water ages along the vertical transects were established using natural chloride tracers, and ranged from modern to either ~15,000 yBP (years before present) or ~30,000 yBP at the two low-recharge sites. Unsaturated flow processes were short-circuited by preferential flow at two of the three high-recharge sites, resulting in rapid movement of water through the vertical transects. Microbial numbers and biomass, based on plate counts, and phospholipid fatty acid (PLFA) concentrations decreased with depth at all sites. The majority (55–90%) of the culturable chemoheterotrophs recovered from most samples were streptomycete bacteria. 16S rRNA gene sequence and MIDI analyses indicated that 75% of the remaining isolates were Gram-positive bacteria (most likely species of *Arthrobacter* and *Bacillus*) 25% were Gram-negative bacteria (probably members of several genera in the alpha- and gamma-*Proteobacteria*). Comparison of microbial communities at low-recharge sites vs. high-recharge sites, where preferential flow occurs, revealed several differences that might be attributed to vertical transport of microbial cells at the high-recharge sites. Plate counts and PLFA analyses indicated that the proportion of streptomycetes, which were abundant at the surface but present in the subsurface as spores, decreased, or remained constant, with depth at the low-recharge sites, but increased with depth at the high-recharge sites. PLFA analyses also indicated that Gram-negative bacteria displayed increased nutrient stress with depth at the high-recharge sites characterized by preferential flow, but not at the low recharge site. This may be a result of advective transport of microbes to depths where

Correspondence to: D.L. Balkwill; Fax: (904) 644-4865; E-mail: balkwill@bio.fsu.edu

it was difficult for them to compete effectively with the established community. Moreover, PLFA community structure profiles fluctuated considerably with depth at the low-recharge sites, but not at the high-recharge sites. This might be expected if transport were distributing the microbial community along the vertical profile at the high-recharge sites. In contrast to the high-recharge sites at which preferential flow occurs, filtration likely prevented vertical transport of microorganisms at the high-recharge site that was characterized by unsaturated flow.

Introduction

The ability of microorganisms to survive and adapt to oligotrophic environments, such as deep vadose zones and groundwater, has become an important issue as the use of *in situ* bioremediation as a clean-up technology increases [10, 33, 49, 50]. In the arid and semi-arid areas of the western United States, microorganisms can be transported to groundwater through unsaturated zones that may range in depth from a few meters to hundreds of meters. Past disposal practices at many defense facilities took advantage of these deep vadose zones by disposing of waste directly into soil cribs (*e.g.,* drainage fields), trenches or settling ponds, and underground storage tanks that have occasionally developed leaks [13, 31, 44]. An understanding of the processes by which microorganisms move and survive under unsaturated flow conditions is important for designing bioremediation methods that involve seeding of non-native species [60] or introduction of nutrients to stimulate intrinsic biodegradation [25, 41].

Field-tracer experiments have effectively elucidated the transport of microbial analogs (microspheres) in saturated porous media over relatively short time period and distance scales [23, 24]. These experiments focused on the important ''passive'' transport processes, such as advection, dispersion, exclusion, filtration, and adsorption. Passive transport may be magnified under unsaturated flow, in which processes such as advection and dispersion may only occur during relatively short, seasonal rainfall events when soil water contents are sufficiently high to create continuous water films [15, 20, 47]. Under unsaturated flow, microorganisms are transported in water films that move by capillary forces [68]. As the water content decreases, capillary forces retain water in increasingly smaller pore spaces. Therefore, in arid and semi-arid environments, microorganisms may be transported through the smallest pore spaces. This invariably increases the likelihood of physical filtration [18, 45] and attachment to particle surfaces [26].

In natural systems, active processes such as growth and decay, adhesion, and taxis [43, 69] may either promote or retard transport, and are superimposed on the passive processes. Although some reports have highlighted growth as an important dispersal mechanism in porous media [27, 28, 43, 52], others have dismissed it as insignificant process [9]. When nutrients are limiting, active processes may only be manifested over long time scales, making short-term, direct observations of transport difficult. The time-frames of transport through arid vadose zones may range from days to thousands of years. These time-frames affect the survival response of microorganisms. Under rapid transport, microorganisms may need to respond with relatively short-term physiological or behavioral changes, while, under slow transport, microbial communities may respond by long-term adaptation or selection of phenotypes [6]. Microorganisms have developed specific survival mechanisms for oligotrophic environments, ranging from changes in cell structure (*e.g.,* spore-formation or alteration of cell membrane properties) to capacities for resisting stressful environmental factors (*e.g.,* heat, osmotic changes, or oxidative stress) [65]. Transported microorganisms may exhibit stress from competition with established microbial communities (*e.g.,* if they are transported into an environment favoring survival strategies that they have not evolved; see [2]). Little is known about the interaction of species within microbial communities during the transport process.

In this study, we investigate the potential transport of microorganisms in a semi-arid environment through indirect observation of microbial communities along a vertical, unsaturated flow path. Although transport cannot be unequivocally established by indirect observation, multiple lines of evidence are employed in this comparative field study to show variations between microbial communities at sites where the conditions for microbial transport are maximized, and those at sites where microbial transport through water films would be extremely slow. Transport times for vadose-zone pore waters are established using natural geochemical tracers [38], and provide time-frames over which microorganisms could be transported by water films.

Materials and Methods

Description of Study Site

Five sites in the Pasco Basin of southeastern Washington State were chosen for study with contrasting recharge rates (Fig. 1). Recharge in this semi-arid area is largely controlled by topography; run-on accumulates in topographic low areas, resulting in rapid recharge, whereas adjacent high areas may have extremely low rates of recharge. If microbial cells are carried by the water phase, then the likelihood of transport is maximized at the high-recharge sites. A low-recharge ("control") site, where microbial transport would be far less likely, was paired with two of the high-recharge field sites.

The Benson Ranch and Cold Creek Alluvium sites are located in low drainage. These sites are dry much of the time, but run-on can accumulate rapidly in the spring as a result of snowmelt or intense rainfall. This results in focused recharge to the unconfined aquifer, and, in some years, ponded water lasting a few days to a week. In contrast, and to provide a field experimental control to the high-recharge sites, the Benson Springs and Yakima Barricade sites are located in topographically high areas, where run-on does not occur and infiltration from precipitation is low. The Yakima Barricade site served as the low-recharge control for the Cold Creek Alluvium site. Benson Springs was the low-recharge control for the Benson Ranch site. At a fifth site, Wye Barricade, recharge was expected to be high because the site is characterized by coarse sands, no surface silt loam layer, low shrub cover, and, hence, low evapotranspiration. Unlike the Benson Ranch and Cold Creek Alluvium sites, however, the Wye Barricade site is not located in a drainage. Focused run-on does not accumulate at this site.

Apart from the occasional spring run-on (annual to decennial events) that can occur in low drainage, the Pasco Basin was inundated by catastrophic floods when glacial dams in western Montana and northern Idaho were breached during the late Pleistocene, spilling massive volumes of glacial melt water (primarily glacial Lake Missoula) across eastern and central Washington. The last series of floods occurred ~13,000 yBP (years Before Present by radiocarbon [37]; equivalent to ~15,000 calendar years) and reached a high water elevation of ~350 m [4,39]. Based on elevation, all of the study sites except Benson Springs were affected by these late Pleistocene floods. It has been estimated that dozens of catastrophic floods may have occurred during an ~2000 y period near the end of the Pleistocene, effectively flushing the vadose zone with glacial melt water over a large portion of eastern Washington state (see inset, Fig. 1).

Aseptic Drilling Techniques

Two common drilling methods were used: a truck-mounted, hollow stem auger (all sites except Yakima Barricade) or cable tool drilling (Yakima Barricade). The drilling components were steam cleaned prior to use. Regardless of the drilling method, a continuous sediment core was collected using a split-tube sampler. The sampler was advanced ~60 cm in front of the auger or cable flights. Autoclaved Lexan liners were inserted in 60 × ~6 cm stainless steel,

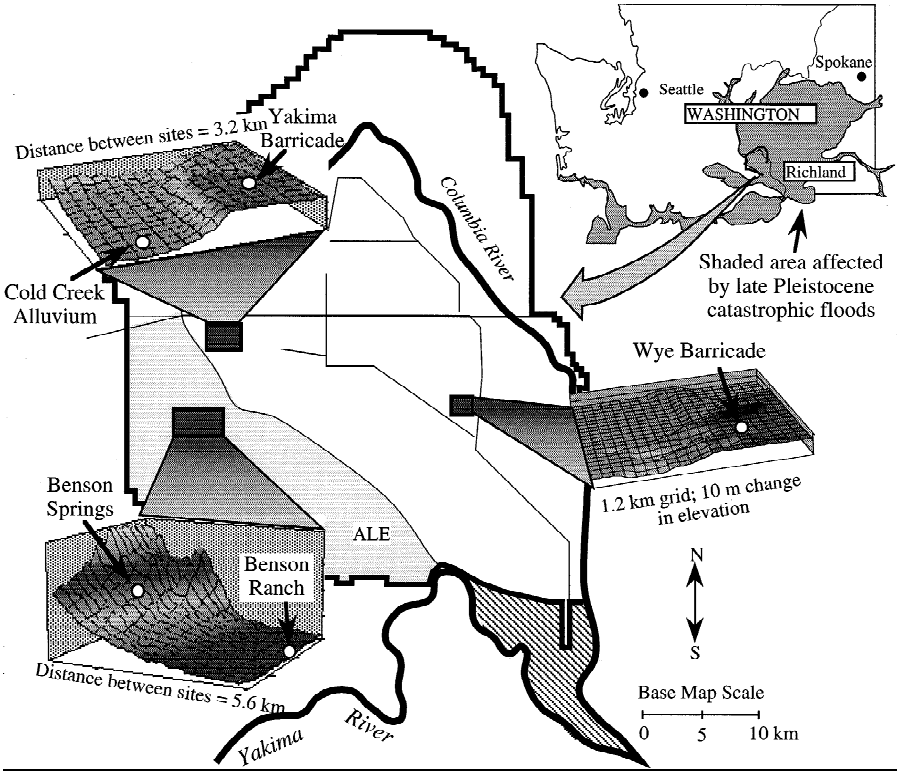

Fig. 1. Location and surface topography of study sites in the Pasco Basin of southeastern Washington State. The shaded area of the insert shows the area affected by late Pleistocene catastrophic floods.

split-tube samplers that were cleaned with ethanol and flame sterilized in the field. On recovery, the cores were capped immediately with sterile caps, labeled, and placed in an ice chest. The cores were then transported to a sterile laminar flow hood for laboratory processing. Split core samples for microbiological analyses were packaged in sterile containers, on ice, and sent by overnight express to Florida State University and the University of Tennessee, for further processing. Core samples for chemical and physical measurements were placed in air-tight containers and stored at 4°C. All microbiological samples were processed within 24 h of receipt.

Chloride Mass Balance (CMB) Method

The natural tracer method based on CMB is one of the simplest, least expensive, and most useful for determining recharge in arid climates [1], and has been used for over two decades. In this method, recharge is determined by applying a mass-balance argument on the chloride ion, in which the difference between the chloride concentration of the soil water and the atmospheric input concentration is due to evapotranspirative enrichment. Simply stated, the chloride concentration in the pore water is inversely proportional to the flux of water through the sediments. CMB estimates of recharge often represent averages over hundreds or thousands of years. Recharge is determined by the relationship

$$J_R = \left(\frac{Cl_O}{Cl_{SW}}\right) \times p \qquad (1)$$

where J_R is the net downward residual flux (e.g., recharge in cm y^{-1}), Cl_O is the average atmospheric chloride concentration in local precipitation and dry fallout (mg l^{-1} or equivalent units of g m^{-3}), Cl_{SW} is the average chloride concentration in the soil water (mg l^{-1}), and p is the average annual precipitation (cm y^{-1}). Cl_O can be expressed as the total chloride mass deposited at ground surface, q_{Cl} divided by precipitation, p.

The fundamental mass-balance relation (mass input = mass present) allows determination of the pore water age, t, at a given depth interval:

$$t \times q_{Cl} = \sum_i (Cl_i \, z_i \, \rho_b) \qquad (2)$$

where Cl_i is the chloride concentration in the interval i (g^{Cl}/g_{soil}), z_i is the thickness of the interval i (m), ρ_b is the bulk density (g m^{-3}). The term $\sum_i(Cl_i \, z_i \, \rho_b)$ represents the total chloride mass of the peak, in g m^{-2}, over depth interval z_i.

The chloride concentration profile was determined at approximately 0.3 m intervals over the depth of the borehole at each site. Bulk density and gravimetric water contents were first determined by removing ~20 cc of sediment from the intact core using a modified disposable syringe. After the volume was recorded, the samples were quickly weighed, dried in an oven at ~100°C for 24 h, and reweighed. Approximately 10 g of dried sample was mixed with 20 g of Milli-Q water in a 25-ml glass Corex tube, shaken overnight, and centrifuged at 4800 × g. The supernatant was filtered through a 0.22-μm Millipore filter prior to the chemical analyses. Chloride concentrations were determined by ion chromatography. The concentrations of chloride in the soil water were corrected using the gravimetric water content [56]. Very little natural chloride is present in the basalt and siliceous sediments found at these sites. Sediment size distributions were determined for the five different study sites using standard sieving and hydrometer methods [19].

Microbial Enumerations

The number of culturable aerobic (or facultatively anaerobic), chemoheterotrophic microorganisms in each sample was determined by colony counting on media plates with different concentrations and sources of organic carbon. The samples were prepared for plating by blending for 1 min in 0.1% $Na_2P_4O_7 \cdot 0H_2O$ at pH 7.0 [5]. Serial dilutions of the blended samples were prepared in a phosphate-buffered saline solution containing the following ingredients l^{-1} of distilled water: Na_2HPO_4, 1.18 g; $NaH_2PO_4 \cdot 1H_2O$, 0.22 g; NaCl, 8.5 g (pH 7).

For plate counting, serial dilutions were spread-plated in triplicate on four media: (i) PTYG (peptone-tryptone-yeast extract-glucose agar; [5]), (ii) 1% PTYG (a 1:00 dilution of PTYG, except that the concentrations of agar, $MgSO_4 \cdot H_2O$, and $CaCl_2 \cdot H_2O$ were the same as in PTYG), (iii) 5% TSA (BBL Trypticase Soy Agar; a 1:20 dilution of the BBL formula except for the agar), and (iv) 0.5% TSA (a 1:200 dilution of the BBL formula except for the agar). All plates were incubated aerobically at 25°C. PTYG plates were counted after 5 days and 2 weeks. 5% TSA and 1% PTYG plates were counted after 5 days and 4 weeks. 0.5% TSA plates were counted after 1 week and 6 weeks.

Analysis of Colony Morphologies on Plates

Plates counts were evaluated, first, by determination of the total number of colonies, and then by description of each visibly distinct type of colony present at countable dilutions, based on relative size, color, opacity, surface and edge characteristics, and elevation [54]. Colony characteristics typical of specific groups of microorganisms (*e.g.,* streptomycetes) were also noted. The number of distinct colony types and the morphological characteristics of each were recorded in a digitized database. The data were then sorted and analyzed to determine how the diversity of viable aerobic chemoheterotrophs (*i.e.,* the number of distinct colony types) varied between depths at each sample site.

Flotation Procedure for Examination of In Situ Microbial Cell Morphology

In situ microbial cell morphologies were examined with the modified flotation method described by Bone and Balkwill [8]. Flotation films were produced with 1% aqueous solutions of polyvinylpyrrolidone (PVP-360), retrieved on glass cover slips, and stained with acridine orange as described previously [8]. The edges of the cover slips were sealed with transparent fingernail polish instead of dental wax. Each film was examined extensively with epifluoresence optics (at 400 ×) to determine microbial cell shapes and sizes, the presence of dividing cells or mycelia (in the case of streptomycetes and

fungi), and the presence of microcolonies or other distinctive microbial features.

MIDI Analyses

Bacterial isolates were incubated on 5% TSA plates for 24 h to one week, depending on growth characteristics. Cells were harvested and processed for fatty acid composition after growth was observed in the third quadrant, as described in the Microbial Identification System operating manual, version 5 (MIDI, Inc., Newark, Del). Briefly, cells were subjected to saponification in methanolic NaOH, resulting in cell lysis and the liberation of fatty acids from cellular lipids. The fatty acids were then methylated in methanolic HCl and extracted for analysis by gas chromatography in hexane/methyl-*tert*-butyl. Modifications to the MIDI protocol involved the use of a 100°C heating block in place of a water bath and a vortex mixer instead of a test tube rotator. All solvents and reagents were of the highest purity available (Burdick and Jackson, Muskegon, MI and Mallincrodt Chemicals, Paris, KY).

PLFA Analyses

Sediment samples, as 75 g aliquots, were pulverized, homogenized, and extracted in a single phase, chloroform-methanol, organic solvent system [7], modified to include a phosphate buffer [67]. Total extractable lipid was then fractionated into lipid classes using silicic acid column chromatography [62]. The polar-lipid fraction was subjected to a mild alkaline methanolysis, whereby the polar-lipid fatty acids were transesterified into fatty acid methyl esters [62] for subsequent quantification and identification by gas chromatography/mass spectrometry [30].

Analysis of 16S Ribosomal RNA Gene Sequences

A standard chloroform-isoamyl alcohol extraction procedure [29] was used to isolate genomic DNA from selected bacteria cultured from Benson Ranch and Benson Springs on 5% TSA (see Results). Twenty ng of DNA was used as a template for polymerase chain reaction (PCR) amplification [46] of an ~1,500-base segment of the 16S ribosomal RNA (rRNA) gene. The PCR amplification primers were fD1 and rP2 [66].

The PCR amplification products were sequenced with an Applied Biosystems Model 373A DNA sequencer, using the "Taq DyeDeoxy™ Terminator Cycle Sequencing" method [3, 35]. The sequencing primer was primer G [42]. It typically yielded about 400 bases of usable sequence, corresponding approximately to positions 375 to 775 in the 16S rDNA nucleotide sequence for *Escherichia coli* [11]. The resulting sequences were evaluated in the context of the proposed secondary structure of the 16S rRNA molecule [22], and edited (when necessary) after examining the original output from the ABI sequencer. Each edited sequence was then analyzed with the Similarity Rank feature of the Ribosomal Database Project (RDP) [34], to determine the names of the 10 strains or species of bacteria in the RDP database (release 5.0; May 25, 1995) with the most similar 16S rRNA sequences. The resulting information was used to infer the most likely genus-level identity of each isolate (recognizing that confirmation of these identities would require additional analyses that were beyond the scope of this study).

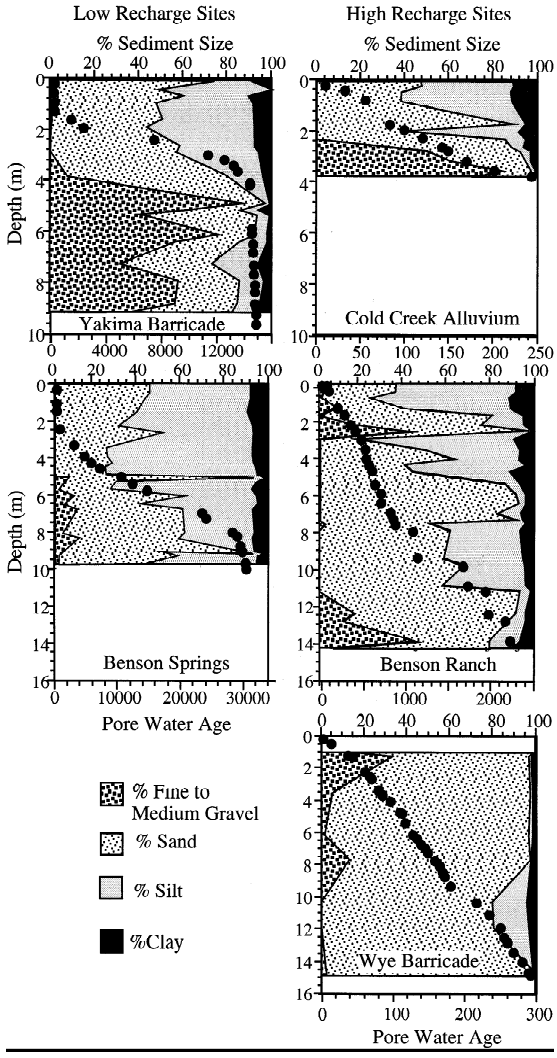

Fig. 2. Sediment size distribution and chloride pore water ages with depth at the five study sites.

Results

Hydrologic Characteristics of Field Sites

The pore water ages at the different study sites were calculated and are presented in Fig. 2. At the low-recharge sites, the upper limit on the pore water ages ranged from ~15,000 to 30,000 y. The upper limit at the Yakima Barricade site is consistent with the timing of late Pleistocene catastrophic floods, suggesting that vertical recharge from rainfall is very low at this site (0.01 mm y^{-1} by CMB). The Benson Springs

site is located above the high-water mark from the Pleistocene floods, and contains pore water ranging to ~30,000 y in age. The recharge rate at this site is slightly higher (0.02 mm y^{-1}). The older pore water ages reflect the lack of impact from the Pleistocene floods. The actual sediment ages are one to two orders of magnitude older than those of the pore water. The sediment ages at Benson Springs ranged from $\leq$100,000 y, in the shallow portions of the profile, to $\geq$2 million years at 9 to 15 m depth, whereas the sediment ages were >125,000 y between 10 and 15 m depth at Yakima Barricade (Bjornstad, unpub data). The sediment ages reflect the maximum age of microbial colonization; the pore-water age reflects the minimum age of colonization (*e.g.,* following transport of the microorganisms to their current depth).

At the high recharge sites, the pore water ages are generally less than a few hundred years old (Fig. 2). Although the age of pore waters at Benson Ranch ranged to 2500 y by CMB, a second tracer, ^{36}Cl, indicated 40-year-old pore water at depths of 12 to 14 m. The discrepancy between the two tracers indicates that preferential flow is occurring in response to the periodic run-on in this drainage. Preferential flow often occurs under saturated or near-saturated flow conditions [12, 40] (*e.g.,* periodic flooding from spring run-on) and, thus, is a possible recharge mechanism at both the Benson Ranch and Cold Creek Alluvium sites. In contrast, preferential flow does not occur under conditions of low water content below the root zone, as was observed at the Yakima Barricade, Benson Springs, and Wye Barricade sites. Although the pore water ages are <300 y at the Wye Barricade site, the gravimetric water contents are low (2 to 8%); they do not approach the saturated or near-saturated conditions required for preferential flow. The distinction between the flow mechanisms at the high-recharge sites is important. Water moves through the smallest pores by capillary force, under unsaturated flow conditions, as would occur at Wye Barricade. However, when preferential flow occurs at Benson Ranch or Cold Creek Alluvium, the largest pores become saturated, rapidly transmitting water through the vadose zone (*e.g.,* largely bypassing routes through the small pores).

The sediment size distribution (% gravel, sand, silt, and clay; Fig. 2) indicates that the high-recharge sites are fairly well matched with their corresponding low-recharge controls. Furthermore, the pore water ages, as determined by CMB, are not correlated with heterogeneity in the sediment size distribution (i.e., water flux, rather than sediment heterogeneity, primarily controls pore water age). Both Yakima Barricade (low recharge) and Cold Creek Alluvium (high recharge) have the highest silt content in the upper 2 m and high gravel content lower in the profile. The high gravel content hindered sample recovery with the hollow stem auger below 4 m at the Cold Creek Alluvium site. The Benson Springs (low recharge) and Benson Ranch (high recharge) sediments have similar clay contents, and are predominantly composed of sand and silt. Wye Barricade is predominantly composed of coarse to medium sand, and, unlike the other study sites, does not have a well-developed silt loam soil at the surface.

Microbial Biomass and Numbers

Analysis of ester-linked phospholipid fatty acids (PLFAs) and plate counts made on four media were used to determine microbial biomass and population size of culturable microorganisms, respectively, in each sample. The plate counts on 5% TSA are shown in Fig. 3; similar results were obtained with the other four media. PLFA values are also shown in Fig. 3. They were converted from pmol PLFA g^{-1} sediment to microbial cells g^{-1} sediment, based on published values for *Escherichia coli* of 5.9×10^{12} cells g^{-1} dry weight [57] and 100 μmol PLFA g^{-1} dry weight [67]. The *E. coli* conversion factor provides a useful working estimate of biomass, but it must be realized that different species contain differing amounts of PLFAs in their membranes. A number of environmental factors can influence the amount of PLFA produced by a particular species [5].

Plate counts and PLFA analyses were consistent with a sharp decrease in biomass with depth at all five study sites (Figs. 3 and 4). Plate counts ranged from 10^7 colony-forming units (CFU) g^{-1} sediment, in the shallowest samples, to below detection (approximately 10^2 CFU g^{-1}) in the deeper Benson Ranch, Benson Springs, and Wye Barricade samples. The decrease in culturable cell populations and biomass with depth was especially abrupt at the Wye Barricade site. The plate counts on all media for all samples taken below 0.85 m were below detection. PLFA values generally paralleled the plate counts and varied over an equally wide range, with depth. Because the great majority of viable microorganisms in environmental samples are not cultured on laboratory media, the PLFA approach detected microorganisms in many of the samples that were below the detection limit of the plating technique.

There was no correlation between the decrease in biomass with depth and the gravimetric water content (Fig. 3) or soil matric potential (data not shown) of the samples, especially at the Benson Ranch, Benson Springs, and Wye Barricade

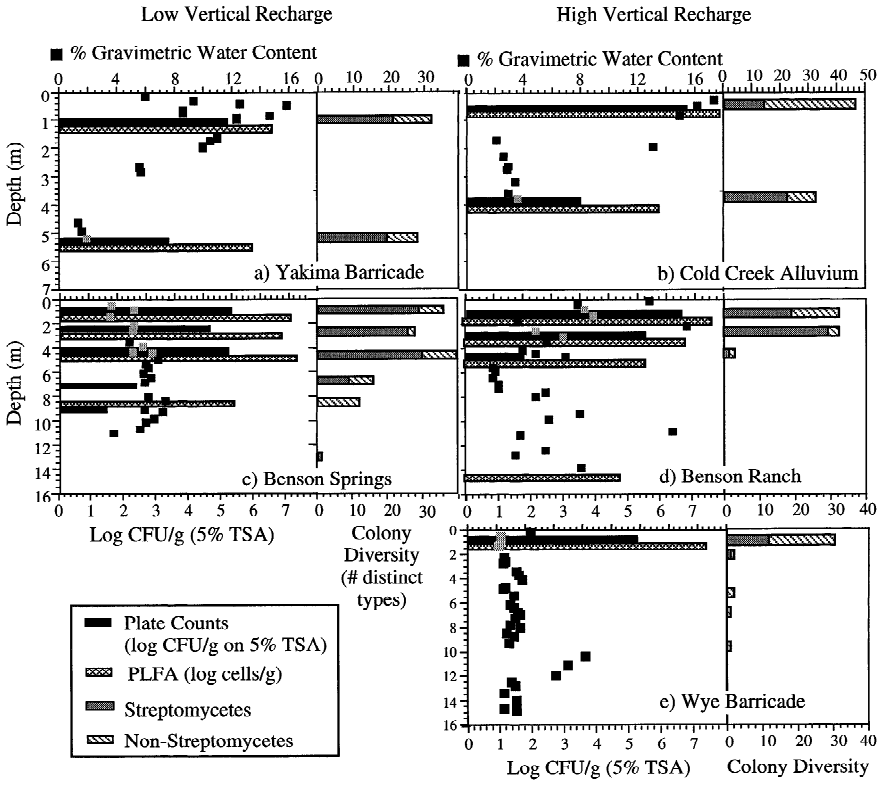

Fig. 3. Gravimetric water content (%), numbers of culturable microorganisms (CFU g^{-1}), and diversity of colony types plotted against depth at the five study sites. CFU, colony-forming units.

sites. Gravimetric water contents ranged from a low of 1.4%, at a depth of 5 m at the Yakima Barricade site, to a high of 17.4%, just below the surface at Cold Creek Alluvium. The moisture contents were generally above 2% (Fig. 3). The Benson Springs site was sampled in the fall, and the remaining sites were sampled in the spring. The effect of snowfall and spring melt was evident in the water contents of near-surface samples from the spring sites, whereas the effect of a summer drying period was evident in near-surface samples obtained from the Benson Springs site.

Diversity and Types of Culturable Aerobic Chemoheterotrophs

The numbers of distinct colony types appearing on the plates used for viable counts were a crude measure of diversity of the microbial populations in each sample (see Materials and Methods). The results for 5% TSA are shown in Fig. 3; similar results were obtained with the other three media. Between 25 and 40 types of culturable microorganisms (almost all bacteria) were recovered from most of the shallow samples at each site. The numbers of distinct colony types recovered decreased with depth at all sites. There were no obvious differences in colony diversity between high- and low-recharge sites.

The majority (55–90%) of the microorganisms recovered from most samples on all four plating media could be identified as streptomycetes, on the basis of their cell and colony morphological characteristics. To investigate the probable identity of the remaining isolates, the non-streptomycetes obtained from the Benson Springs and Benson Ranch samples on 5% TSA were examined in two ways: (i) analysis of 16S RNA gene (16S rDNA) nucleotide base sequences, and (ii) analysis of fatty acid methyl esters by the MIDI technique. The most likely genus-level identities of these strains, based on the results of a Similarity Rank Analysis that matched each 16S rDNA sequence to that of the most similar species in the Ribosomal Database Project (RDP release 5.0; [34]), are summarized in Table 1. Seventy-five percent of the isolates examined in this way were Gram-positive. Most of them could tentatively be assigned to the genus *Bacillus* (the rest were assigned to *Arthrobacter*). The Gram-negative isolates included members of the alpha-*Proteobacteria* (probable genera: *Azospirillum, Rhizobium,* and *Bradyrhizobium*) and the gamma-*Proteobacteria* (*Pseudomonas, Telluria,* and *Xanthomonas*). Thirty-seven percent of the isolates were not assigned to a particular genus by the MIDI analysis, either because their lipid patterns did not closely match any of those in the current database, or because there was insufficient growth for a valid analysis. For the remaining isolates, however, the most likely genus-level

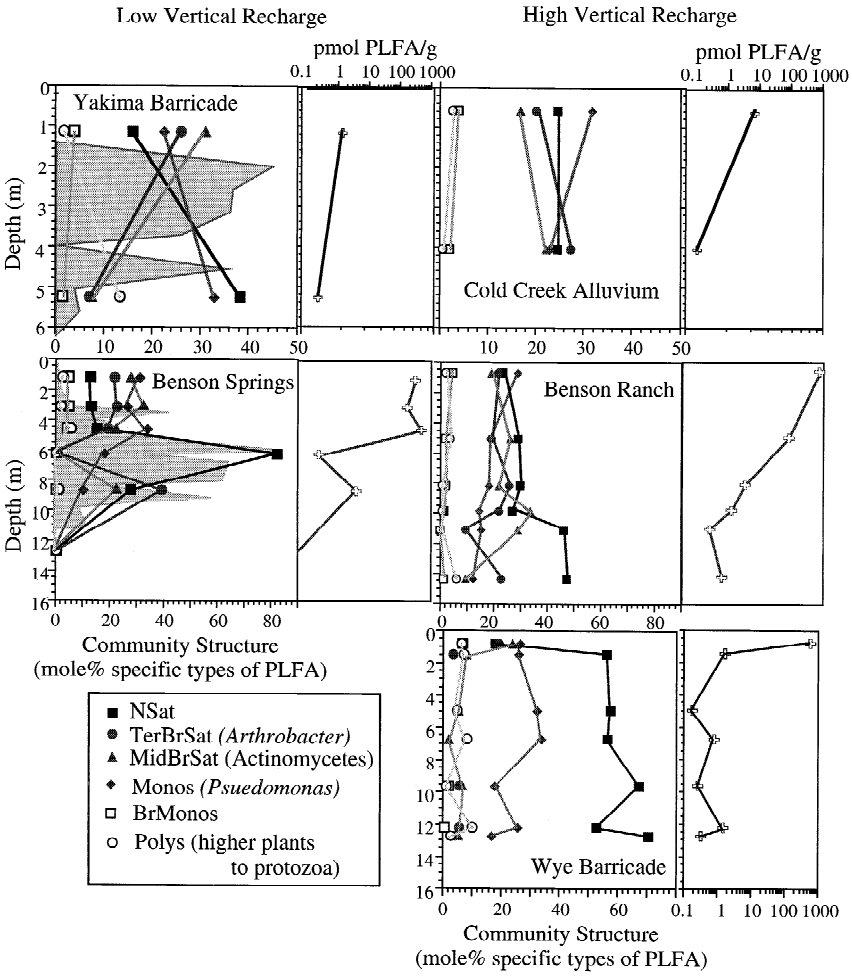

Fig. 4. Total PLFA and mole % for six specific types of PLFAs in samples from the Pasco Basin study sites. The shaded areas on the Yakima Barricade and Benson Springs graphs represent the location and shape of the chloride profiles in the pore waters.

identities obtained by MIDI analysis generally agreed with those obtained by sequence analysis (Table 1).

The percentages of isolates (from the Benson Springs and Benson Ranch sites) falling into major microbial groups (based on morphological characteristics for fungi and streptomycetes, and 16S rDNA sequence analysis for all others) are shown in Table 2. The percentages of streptomycetes isolated on 5% TSA increased sharply with depth at Benson Ranch (a high-recharge site), but decreased with depth at Benson Springs (a low-recharge site). This difference was due to the fact that a greater diversity of bacterial types, especially low-G+C Gram-positive (Similarity Rank analysis implied several different *Bacillus* species) and Gram-negative bacteria, were isolated from the deeper Benson Springs samples. Very few Gram-negative bacteria could be isolated from any of the Benson Ranch samples. The non-streptomycete isolates from the Yakima Barricade and Cold Creek sites were not characterized, but similar trends were observed regarding the percentage of streptomycetes recovered by plating. The percentage of streptomycetes was relatively constant with depth at the low-recharge Yakima Barricade site (68% and 73% at depths of 1.1 and 5.5 m, respectively), but increased sharply with depth at the high-recharge Cold Creek site (30% and 69% at 0.55 and 4.1 m, respectively).

Direct Microscopic Examination of In Situ Cell Morphologies

The flotation procedure used to determine the *in situ* cell morphologies of microorganisms showed that >95% of the cells detected in all samples were cocci or coccoid rods. Most of the rods were <1.5 μm in length and <1.0 μm in diameter. A few groups of cells that may have been microcolonies were seen in the shallowest samples from each site, but most cells occurred individually and did not appear to be dividing. No filamentous forms were detected in any of the samples, but cells the size of typical streptomycete spores [32, 36, 45, 58] were present in all of the samples. Except for the possible microcolonies found in the shallowest samples, there were no obvious differences in the cell morphologies observed in samples from different depths or from different sites.

Article 4, Table 1

Table 1. Probable identities of non-streptomycete 5% TSA isolates from the Benson Ranch and Benson Springs sites

Benson Ranch			Benson Springs		
Depth (m)	Sequence ID[a]	MIDI ID[b]	Depth (m)	Sequence ID[a]	MIDI ID[b]
0.55	*Bacillus (macroides)*	*Bacillus (megaterium)*	0.77	*Bacillus (pseudomegaterium)*	No ID[c]
	Arthrobacter (globiformis)	*Arthrobacter (oxydans)*		*Bacillus (lautus)*	No ID
	Arthrobacter (globiformis)	*Micrococcus (roseus)*		*Bacillus (macroides)*	No ID
	Bacillus (macquariensis)	No ID[c]		*Bacillus (macroides)*	*Bacillus (megaterium)*
	Bacillus (macroides)	*Bacillus (megaterium)*		*Bacillus (lautus)*	*Bacillus (filicolonicus)*
	Bacillus (macroides)	*Bacillus (megaterium)*		*Bacillus (macroides)*	No ID
	Bacillus (viscosis)	No ID		*Bacillus (macroides)*	*Bacillus (megaterium)*
	Bacillus (polymyxa)	No ID	2.7	*Bacillus (lautus)*	No ID
	Telluria (mixtra)	*Hydrogenophaga (pseudoflava)*		*Bacillus (macroccanus)*	*Bacillus (megaterium)*
	Bacillus (macroides)	*Bacillus (megaterium)*	4.9	*Bradyrhizobium sp.*	No ID
	Bacillus (macroides)	*Bacillus (megaterium)*		*Bradyrhizobium sp.*	*Pseudomonas (diminuta)*
	Bacillus (macroides)	*Bacillus (megaterium)*		*Arthrobacter (globiformis)*	*Arthrobacter (oxydans)*
	Arthrobacter (globiformis)	*Micrococcus (lylae)*		*Azospirillum sp.*	*Pseudomonas (putida)*
	Arthrobacter (globiformis)	*Micrococcus (roseus)*		*Bacillus (macroccanus)*	*Bacillus (megaterium)*
	Arthrobacter (globiformis)	No ID		*Rhizobium (huakuii)*	No ID
	Bacillus (macroides)	*Bacillus (megaterium)*		*Xanthomonas sp.*	No ID
5.3	*Azospirillum (brasiliensis)*	No ID		*Pseudomonas (putida)*	*Pseudomonas (putida)*
	Azospirillum (brasiliensis)	No ID		*Azospirillum sp.*	*Pseudomonas (putida)*
	Azospirillum (brasiliensis)	No ID	6.2	*Bacillus (pseudomegaterium)*	No ID
	Azospirillum (brasiliensis)	*Xanthobacter (agilis)*		*Bradyrhizobium sp.*	*Methylobacterium*
				Bacillus (subtilis)	*Bacillus (amyloliquifaciens)*
				Bacillus (macroides)	*Bacillus (megaterium)*
				Bacillus (pabuli)	No ID
				Bacillus (larvae)	No ID
				Bacillus (polymyxa)	*Bacillus (pabuli)*
			8.7	*Bacillus (pseudomegaterium)*	No ID
				Bacillus (larvae)	*Arthrobacter (globiformis)*
				Bacillus (pseudomegaterium)	*Arthrobacter (viscosus)*
				Bacillus (macroides)	*Bacillus (megaterium)*
				Bacillus (subtilis)	*Bacillus (brevis)*
				Bacillus (pseudomegaterium)	*Arthrobacter (viscosus)*
				Bacillus (pseudomegaterium)	No ID
				Bacillus (macroides)	*Bacillus (megaterium)*
				Bacillus (pseudomegaterium)	*Bacillus (sphaericus)*
				Rhizobium (huakuii)	*Bacillus (megaterium)*
				Bacillus (macroides)	No ID
				Bacillus (macroides)	*Bacillus (megaterium)*

[a] Most likely genus-level ID and (closest species in the RDP database), based on Similarity Rank Analysis. Species names are provided for information only and should not be taken as actual identities

[b] Closest genus and (species) in the MIDI database. Species names are provided for information only

[c] No ID = no match or insufficient growth to obtain ID by MIDI analysis

PLFA Analyses for Community Composition and Nutritional Status

Ester-linked PLFAs were extracted from each sample and analyzed to obtain information on microbial community composition that accounts for both culturable and nonculturable microorganisms. Mole percent values for six classes of PLFAs, some of which are characteristic for specific groups of organisms, are shown for all samples in Fig. 4.

The percentages of some groups of PLFAs varied between the two depths sampled at the Yakima Barricade (low-recharge) and Cold Creek Alluvium (high-recharge) sites, but they varied in different ways. The percentages of terminally-branched saturated fatty acids (characteristic of *Ar-*

Table 2. Percentages of microbial types[a] isolated from Benson Ranch and Benson Springs samples on 5% TSA

Sample depth (m)	Total no. of isolates	Fungi	Streptomycetes	Other high G+C Gram-positives	Low G+C Gram-positives	alpha-*Proteobacteria*	gamma-*Proteobacteria*
Benson Ranch							
0.55	41	2.4	58.5	12.3	24.4	0	2.4
5.3	41	0	90.2	0	0	9.8	0
8.1	2	0	100	0	0	0	0
Benson Springs							
0.77	36	0	80.6	0	19.4	0	0
2.7	28	0	92.9	0	7.1	0	0
4.9	40	2.5	75.0	2.5	2.5	12.5	5.0
6.2	16	0	56.3	0	37.5	6.2	0

[a] Fungi and streptomycetes determined by colony and cell morphological characteristics; all other types determined by analysis of 16S rRNA gene sequences

throbacter sp. and other Gram-positive bacteria) and mid-chain-branched saturated fatty acids (characteristic of actinomycetes) decreased markedly between the two depths at the low recharge site, but increased slightly between depths at the high recharge site. In contrast, the percentage of monoenoic fatty acids (characteristic of Gram-negative genera such as *Pseudomonas*) increased sharply with depth at the low recharge site, but decreased between the two depths sampled at the high-recharge site. These trends are reasonably consistent with the percentages of streptomycete bacteria recovered from the Yakima Barricade and Cold Creek Alluvium samples by plating.

Differences in how the mole percentages of general PLFA groups varied with depth were also noted at the Benson Springs (low-recharge) and Benson Ranch (high-recharge) sites (Fig. 4). The percentages of several classes of PLFAs varied markedly between 1 and 10 m at the low-recharge site, but remained relatively constant to a depth of 10 m at the high-recharge site. Moreover, the overall trend observed above was also observed at the Yakima Barricade and Cold Creek Alluvium sites. The combined percentages of two mid-chain, methyl-branched, saturated fatty acids characteristic of actinomycetes (although they have also been detected in *Desulfobacter* species) in the Benson Springs and Ranch samples are shown in Fig. 5. The percentages of these two fatty acids increased with depth at Benson Ranch (high recharge), even though total PLFA-detected biomass decreased. In contrast, the percentages of the actinomycete-associated fatty acids decreased with total biomass at the low-recharge site.

Additional differences between low- and high-recharge sites were noticed in the case of specific fatty acids synthesized primarily by Gram-negative bacteria (Fig. 6). The ratio of the cyclopropyl fatty acid, cy 19:0, to its monoenoic precursor, 18:1ω7c, increased with depth at the Cold Creek (high-recharge) site; it did not vary with depth at the Yakima

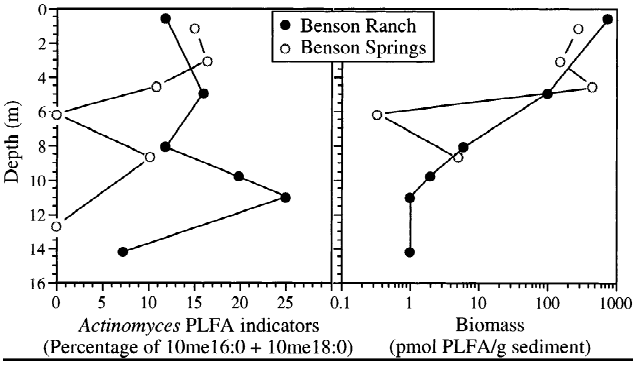

Fig. 5. Percentages of 10me16:0 and 10me18:0 fatty acids and total PLFA in samples from the Benson Ranch and Benson Springs sites.

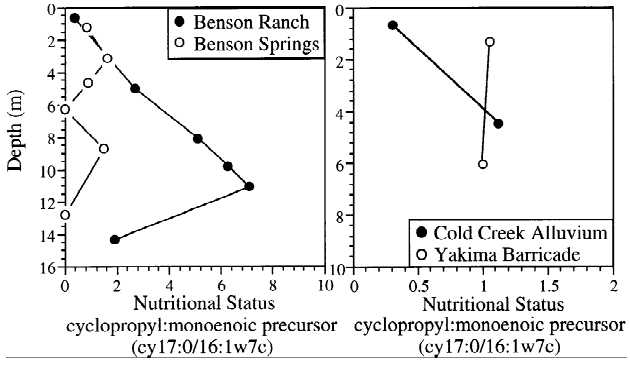

Fig. 6. Ratios of selected cyclopropyl fatty acids to their monoenoic precursors in samples from the Benson Ranch and Benson Springs sites.

Barricade (low-recharge) site. Similarly, the ratio of the cyclopropyl fatty acid, cy 17:0, to its monoenoic precursor, 16:1ω7c increased with depth at the Benson Ranch (high-recharge) site, it did not increase with depth at the Benson Springs (low-recharge) site.

At the Wye Barricade site, the mole % of mid-chain-branched, saturated fatty acids (typical of actinomycetes) dropped sharply between 0.85 and 1.46 m, and then remained quite low throughout the rest of the profile. The mole % of monoenoic fatty acids (characteristic of some Gram-negative genera) decreased only slightly with depth. However, the predominant PLFAs (50–70 mole %) were *n*-saturated fatty acids. They are produced by all types of bacteria, and, therefore, cannot be related to any particular group.

Discussion

Based on the chloride tracer data, the sample sites examined in this study can be classified as: (i) low-recharge control sites (Yakima Barricade and Benson Springs), where the likelihood of microbial transport is low or occurs over extremely long time periods; (ii) high-recharge sites, where preferential flow can short-circuit unsaturated flow processes, maximizing the potential for transport of microorganisms (Cold Creek Alluvium and Benson Ranch); and (iii) a high-recharge site where recharge occurs via unsaturated flow processes (Wye Barricade), so microorganisms would be transported through the smallest pore diameters (Fig. 2). Also limiting microbial transport at the low recharge sites is the high chloride concentration in the pore waters, which reached ~4000 mg l^{-1} at 2 m below the surface at Yakima Barricade and at 6 m below the surface at the Benson Springs site [38]. High ionic strength promotes sorption of bacteria through compression of the ionic double layers on both the cell and the binding surface [17, 48, 51, 53]. For example, Gannon et al. [17] found that only 1.5% of *Pseudomonas* sp. cells passed through a column saturated with a 0.01 M (570 mg l^{-1}) NaCl solution. In contrast to the low recharge sites, pore water chloride concentrations at the high recharge sites were generally below 100 mg l^{-1} [38]. These low chloride concentrations, coupled with periodic preferential flow processes, would promote transport by advection and dispersion, and lessen the effect of retardation processes such as filtration and adsorption. Depth-related trends in the microbial populations at the paired (high- and low-recharge) sites (Cold Creek Alluvium vs. Yakima Barricade and Benson Ranch vs. Benson Springs) are discussed first. We then examine and compare microbial trends at the high-recharge unsaturated flow site (Wye Barricade).

Plate counts (Fig. 3) and PLFA analyses (Fig. 4) indicated that microbial populations and biomass decreased by several orders of magnitude with depth at the four paired sample sites. The viable counts represent the numbers of culturable, aerobic (or facultatively anaerobic) chemoheterotrophs, only. The PLFA-based biomass estimates represent total (culturable and nonculturable) microbial populations. Decreases in microbial populations with depth in the vadose-zone have been reported previously [9, 26, 64], so the decrease seen in this study was not unexpected. The numbers of distinct colony types recovered by plating also decreased with depth at the Benson Ranch and Springs sites (Fig. 3), but these data do not necessarily imply a decrease in microbial diversity. Rather, the number of different colony types detected in the deeper samples was limited by the very low total number of colonies that appeared on the plates (the numbers of culturable organisms in those samples being only slightly above the detection limit of the plating procedure).

The percentages of streptomycetes recovered on 5% TSA (Fig. 3, Table 2; similar results for the other plating media) decreased or remained constant with depth at the two low-recharge sites (Yakima Barricade and Benson Springs), but increased with depth at the analogous high-recharge sites (Cold Creek Alluvium and Benson Ranch, respectively). Similarly, mole percents of fatty acids associated with the actinomycetes (which include the streptomycetes) decreased with depth at both low-recharge sites, while increasing or remaining constant at the high-recharge sites (Figs. 4 and 5). These results suggest that streptomycetes, which are quite abundant at the surface (typically $>10^7$ CFU g^{-1}), are being transported from the surface (to levels where the total numbers of microorganisms are substantially lower) when periodic run-on is flushed through the high-recharge sites.

Extensive examination of flotation films by epifluorescence microscopy failed to detect filamentous bacteria in any of the samples, so many of the abundant streptomycetes that were readily detected by plating and PLFA assays probably existed as spores. This is generally the case in soil environments [16]. Streptomycete spores might be very good candidates for passive vertical transport (advection processes) at the high-recharge sites, because they are relatively small (typically <1 μm in diameter [32, 36, 45, 58]), have an uncomplicated morphology (spherical or ovoid vs. filamentous [32]), and have a net negative surface charge [14]; charac-

teristics that would facilitate (or, at least, not hinder) their movement through the pores. Ruddick and Williams [45] found that the transport of actinomycete spores was primarily influenced by the structure of the pore surface, spore wettability, and the size of the pore spaces in columns packed with porous media ranging from silt to coarse sand.

Vertically transported streptomycete spores initially would not be adversely affected by harsher environmental conditions at greater depths (*e.g.,* lower moisture or nutrient levels), because they are reasonably resistant to desiccation and starvation [16]. Similarly, they would not have to compete with established microorganisms as long as they remained in the spore state. Thus, streptomycete spores could be expected to persist for relatively long periods, after being transported to the subsurface. If so, periodic transport down from the topsoil could easily account for the higher percentages of streptomycetes that were found in deeper samples at the high-recharge sites.

If streptomycete spores are being transported downward by water drainage at the two high-recharge sites, other forms may also be transported. Yet, the percentages of Gram-negative bacteria recovered by plating decreased with depth at the Benson Ranch (high-recharge) site and increased with depth at the Benson Springs (low-recharge) site. It must be realized, however, that plating detects only the numerically predominant forms of culturable microorganisms; thus, Gram-negatives may have existed in relatively low numbers at the high-recharge site. Indeed, the PLFA analyses detected lipids characteristic of certain Gram-negative genera (*e.g., Pseudomonas*) throughout the profile at Benson Ranch (Fig. 4). PLFA analyses also detected cyclopropyl fatty acids that are associated primarily with Gram-negative bacterial metabolism (Fig. 6).

Previous studies have indicated that bacteria are most affected by passive transport processes (*e.g.,* advection or filtration) in unsaturated sediment profiles [9, 26, 61]. If the Gram-negative populations are being passively transported at the high-recharge sites, they may display physiological changes in response to changing environmental conditions at depth. For example, it has been shown that the ratios of cyclopropyl fatty acids to their monoenoic precursors increase when Gram-negative cultures enter the stationary phase or are otherwise placed under environmental stress [21]. It can be hypothesized, then, that an increased ratio of cyclopropyls to their precursors in an environmental sample is an indication of physiological stress within the Gram-negative microbial community. PLFA analyses indicated that the ratios of specific cyclopropyl fatty acids to their monoenoic precursors increased markedly with depth at both high-recharge sites, but remained fairly constant with depth at both low-recharge sites (Fig. 6). Thus, the Gram-negative populations at the two high-recharge sites examined in this study display increasing amounts of physiological stress at greater depths, whereas those at the low-recharge sites do not.

Increased physiological stress at depth among Gram-negative bacteria at the high-recharge sites is a bit surprising in the sense that one might expect nutrient levels to be higher throughout the profile at the high recharge sites. On the other hand, if Gram-negative bacteria were being transported downward from the surface soil, they (unlike streptomycetes spores) would have to compete with established species and deal directly with more adverse conditions in the new environment. If these organisms were unable to adapt quickly, they might well be placed under physiological stress. In contrast, the Gram-negative bacteria found in samples from the low-recharge sites have probably been in place for long periods of time, and had, or developed, adaptations that allow them to function, and possibly thrive, in these environments. As a result, these organisms may not be under physiological stress.

PLFA analyses showed that the relative percentages of fatty acids representing specific groups of microorganisms (*e.g., Arthrobacter,* actinomycetes, and *Pseudomonas*) remained relatively constant to 10 m at Benson Ranch (high-recharge site), but fluctuated considerably over the same interval at Benson Springs (low-recharge site). Below 10 m, the total amounts of PLFA were so low that percentages of specific types may not be accurate. To some extent, similar results were observed with the culturable populations at these two sites, in that the types of organisms recovered varied more with depth at Benson Springs than at Benson Ranch (Table 2). The PLFA isolated from the profile at Benson Springs shows consistency in the zone above the chloride pulse (shaded area of Fig. 4). The top of the chloride pulse is usually considered the bottom of the root zone [38], as long as this depth is consistent with known root depths of native plant species. Due to water uptake by plants, the pore-water velocities may vary over two to four orders of magnitude [63] in the rooting zone. The shallow portion of the sediment profile is also the most susceptible to preferential flow from periodic intense rainfall. However, unless the amount of water is massive, as may occur in flooding, saturation of macropores cannot extend to significant depths. The unique water dynamics of the root zone may rapidly distribute microorganisms, as suggested by the uni-

formity in the total PLFA and PLFA profiles down to ~4 m at Benson Springs. The extraction procedure for PLFA analyses readily removes sorbed microorganisms, so enhanced sorption of microorganisms in the presence of high chloride concentrations should not have affected the PLFA measurements.

The greater uniformity of the fatty acids with depth at the high recharge site may be additional evidence that preferential flow processes at this site tend to distribute the community along the vertical profile. It has long been recognized that bacterial movement through macropores can be significant in unsaturated systems [55, 59, 64]. Advective transport of microorganisms would dominate under preferential flow. An extreme example of preferential flow likely occurred over much of the Palouse region of southeastern Washington state during the Pleistocene-Holocene transition, when multiple glacial floods inundated this area. In contrast to the relatively uniform distribution of fatty acids with depth at the high-recharge sites, it is possible that the low-recharge at the Benson Springs site has allowed distinct populations to develop and become established at each depth. Benson Springs is also above the surface elevation affected by the glacial floods.

The Wye Barricade site provided an interesting comparison to the other sites. The Wye Barricade has the highest *unsaturated flow* recharge rate of all the sites examined in this study. This high recharge results from the medium-to-coarse sands that dominate this site, absence of a developed soil horizon, and a low vegetative cover. In spite of the relatively high recharge, however, biomass in samples from below ~2 m at the Wye Barricade was extremely low (generally <2 pmol PLFA g^{-1}; Fig. 4). Consistent with the other high-recharge sites, the community profiles at Wye Barricade, from PLFA, showed little variation with depth, except for a sharp change at the most shallow depth. Unlike the other sites, normal saturates dominated the PLFA profiles at all depths. Although all microorganisms contain normal saturates, a predominance of normal saturates can be indicative of low membrane fluidity brought about by adverse environmental conditions. The concentration of PLFA in these samples was insufficient to determine nutritional stress, as was shown at the other sites (Fig. 6). Consistent with the low biomass by PLFA, and predominance of normal saturates (suggesting environmental stress), was our inability to culture viable microorganisms at all but the most shallow depths at Wye Barricade. Even though the sandy sediments at Wye Barricade contribute to the high recharge, the recharge, and, hence, moisture content, of these sediments fluctuates seasonally. Silts and clays, which tend to moderate moisture fluctuations, are very minor components in these sediments (Fig. 2). In addition, several factors limit organic nutrients: 1) low silt and clay, which tend to sorb organic nutrients, 2) low rate of organic nutrient input due to poor vegetative cover, and 3) lack of run-on to transport organic nutrients from adjacent areas that have more vegetative cover. These will result in physiological stress and contribute to our inability to culture microorganisms at this site.

Under unsaturated flow, water moves through the smallest pores by capillary pressure. Particles or microorganisms moving through these small pores will be subject to processes, such as filtration, to a greater degree than microorganisms that are advectively transported during preferential flow. This may explain the sharp decline in biomass and the rapid change in the PLFA community profile at shallow depths at Wye Barricade, while a more gradual decline in biomass occurs with a fairly constant community profile at Benson Ranch (high recharge, preferential flow). These results suggest that, under normal unsaturated flow processes, few microorganisms may be transported through deep vadose zones found in semi-arid regions such as the Pasco Basin.

In summary, passive microbial transport processes, such as advection and filtration, seem to dominate, and may best explain the collective results discussed above. Specifically, transport appears to occur primarily under conditions of preferential flow. At the high-recharge site not characterized by preferential flow, filtration processes may effectively prevent movement of most microorganisms to significant depths under unsaturated flow. Superimposed upon the passive transport processes are survival strategies employed by different genera. Streptomycetes and *Bacillus* species, form spores, facilitating both survival and advective transport processes. In contrast, the Gram-negative bacteria at the high-recharge sites show physiological stress that is likely associated with transport into the subsurface. At both of the low-recharge control sites, the microbial communities appear to be well-adapted to their low-nutrient environment.

References

1. Allison GB, Gee GW, Tyler SW (1994) Vadose-zone techniques for estimating groundwater recharge in arid and semi-arid regions. Soil Sci Soc Am J 58:6–14
2. Andrews JH, Harris RF (1986) r- and K-Selection and microbial ecology. Adv Microbial Ecol 9:99–147

3. Applied Biosystems, Inc (1992) Taq DyeDeoxy terminator cycle sequencing kit, user bulletin no. 901497, revision E. Applied Bisystems, Inc., Foster City, California
4. Baker VR (1978) Palcohydraulics and hydrodynamics of Scabland floods. In: Baker VR, Nummedal D (eds) The Channeled Scabland. Program Office of Space Sciences, National Aeronautics and Space Administration, Planetary Geology, Washington, DC, pp 59–80
5. Balkwill DL, Ghiorse WC (1985) Characterization of subsurface bacteria associated with two shallow aquifers in Oklahoma. Appl Environ Microbiol 50:580–588
6. Bengtsson G (1991) Bacterial exopolymer and PHB production in fluctuating ground-water habitats. FEMS Microbiol Ecol 86:15–24
7. Bligh EC, Dyer WJ (1959) A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37:910–917
8. Bone TL, Balkwill DL (1986) Improved flotation technique for microscopy of in situ soil and sediment microorganisms. Appl Environ Microbiol 51:462–468
9. Breitenbeck GA, Yang H, Dunigan EP (1988) Water-facilitated dispersal of inoculant *Bradyrhizobium japonicum* in soils. Biol Fertil Soils 7:58–62
10. Brockman FJ, Payne W, Workman DJ, Soong A, Manley S, Hazen TC (1995) Effect of gaseous mitrogen and phosphorus injection on in situ bioremediation of a trichloroethylene-contaminated site. J Hazard Mater 41:287–298
11. Brosius J, Palmer ML, Kennedy PJ, Nolier HR (1979) Complete nucleotide sequence of a 16S ribosomal gene from Escherichia coli. Proc Natl Acad Sci USA 75:4801–4805
12. deSmedt F, Wierenga PJ (1984) Solute transfer through columns of glass beads. Water Resour Res 20:225–232
13. DOE (1995) Estimating the cold war mortgage: The 1995 baseline environmental management report, DOE/EM-0230, US Department of Energy, Washington, DC
14. Douglas HW, Ruddick SM, Williams ST (1971) A study of the electrokinetic properties of some actinomycete spores. J Gen Microbiol 63:289–295
15. Dullien FAL (1992) Porous media fluid transport and pore atructure, 2nd ed. Academic Press, Inc., New York
16. Ensign JC (1978) Formation, properties, and germination of Actinomycetes spores. Ann Rev Microbiol 32:185–219
17. Gannon J, Tan Y, Baveye P, Alexander M (1991) Effect of sodium chloride on transport of bacteria in a saturated aquifer material. Appl Environ Microbio 57:2497–2501
18. Gannon JT, Manilal VB, Alexander M (1991) Relationship between cell surface properties and transport of bacteria through soil. Appl Environ Microbiol 57:190–193
19. Gee GW, Bauder JW (1986) Particle-size analysis. In: Klute A (ed) Methods of Soil Analysis: Part 1. Physical and mineralogical methods, 2nd Ed. American Society of Agronomy. Soil Science Society of America, Madison, Wisconsin, pp 405–407
20. Griffin DM, Quatl G (1968) Movement of bacteria in moist particulate systems. Aust J Biol Sci 21:579–582
21. Guckert JB, Hood MA, White DC (1986) Phospholipid ester-linked fatty acid profile changes during nutrient deprivation of *Vibrio cholerac:* increases in the *trans/cis* ratio and proportions of cyclopropyl fatty acids. Appl Environ Microbiol 52:794–801
22. Gutell RR, Larsen N, Woese CR (1994) Lessons from an evolving rRNA: 16S and 23S rRNA structures from a comparative perspective. Microbiol Rev 58:10–26
23. Harvey RW, George LH, Smith RL, LeBlanc DR (1989) Transport of microspheres and indigenous bacteria through a sandy aquifer: Results of natural- and forced-gradient tracer experiments. Environ Sci Technol 23:51–56
24. Harvey RW, Kinner NE, MacDonald D, Metge DW, Bunn A (1993) Role of physical heterogeneity in the interpretation of small-scale laboratory and field observations of bacteria, microbial-sized microsphere, and bromide transport through aquifer sediments. Water Resour Res 29:2713–2721
25. Hinchee RE, Wilson JT, Downey DC (1995) Intrinsic bioremediation. Battelle Press, Columbus, Ohio
26. Huysman F, Verstracte W (1993) Water-facilitated transport of bacteria in unsaturated soil columns: Influence of cell surface hydrophobicity and soil properties. Soil Biol Biochem 25:83–90
27. Jenneman GE, McInerney MJ, Knapp RM (1985) Microbial penetration through nutrient-saturated berea sandstone. Appl Environ Microbiol 50:383–391
28. Jenneman GE, McInerney MJ, Crocker MF, Knapp RM (1986) Effect of sterilization by dry heat or autoclaving on bacterial penetration through Berea sandstone. Appl Environ Microbiol 51:39–43
29. Johnson JL (1981) Genetic characterization. In: Gerhard P, Murray RGE, Costilow RN, Nester EW, Wood WA, Krieg NR, Phillips GB (eds) Manual of Methods for General Bacteriology. American Society for Microbiology, Washington, DC, pp 450–472
30. Kieft TL, Ringelberg DB, White DC (1994) Changes in ester-linked phospholipid fatty acid profiles of subsurface bacteria during starvation and desiccation in a porous media. Appl Environ Microbiol 60:3292–3299
31. Last GV, Rohay VJ (1993) Refined conceptual model for the volatile organic compounds-arid integrated demonstration and 200 West Area carbon tetrachloride expedited response action, PNL-8597. Pacific Northwest Laboratory, Richland, Washington
32. Locci R, Sharples GP (1984) Morphology. In: Goodfellow M, Mordarski M, Williams ST (eds) The Biology of the Actinomycetes. Academic Press, New York, p 544
33. Lombard KH, Borthen JW, Hazen TC (1994) The design and management of system components for in situ methanotrophic bioremediation of chlorinated hydrocarbons at the Savannah River Site. In: Hinchee RE (ed) Air Sparging for Site Remediation. Lewis Publishers, Boca Raton, Florida, pp 81–96
34. Maidak BL, Larsen N, McCaughey MJ, Overbeek R, Olsen GJ, Fogel K, Blandy J, Woese CR (1994) The ribosomal database project. Nucleic Acids Res 22:3485–3487

35. McBride LJ, Koepf SM, Gibbs RA, Salser W, Mayrand PE, Hunkapiller MW, Kronick MN (1989) Automated DNA sequencing methods involving polymerase chain reaction. Clin Chem 35:2196–2201
36. McVittic A (1974) Ultrastructural studies on sporulation in wild-type and white colony mutants of *Streptomyces coelicolor*. J Gen Microbiol 81:291–302
37. Mullineaux DR, Wilcox RE, Ebaugh WF, Fryxell R, Rubin M (1977) Age of the late major scabland flood of eastern Washington, as inferred from associated ash beds of Mount St. Helens set S. Geol Soc America, Abstracts with Programs 9: 1105
38. Murphy EM, Ginn TR, Phillips JL (1996) Geochemical estimates of paleorecharge in the Pasco Basin: Evaluation of the chloride mass-balance Technique. Water Resour Res 32:2853–2868
39. Myers CW (1979) Geologic studies of the columbia plateau: A status report, FIIO-BWI-ST-4. Rockwell Hanford Operations, Richland, Washington
40. Nkedi-Kizza P, Bigger JW, v. Genuchten MT, Wierenga PJ, Selim HM, Davidson JM, Nielsen DR (1983) Modeling tritium and chloride 36 transport through an aggregated oxisol. Water Resour Res 19:691–700
41. Norris RD, Hinchee RE, Brown R, McCarty PL, Simprini L, Wilson JT, Kampbell DH, Reinhard M, Bouwer EJ, Borden RC, Vogel TM, Thomas JM, Ward CH (1994) Handbook of Bioremediation. Lewis Publishers, Ann Arbor, Michigan
42. Reeves RH, Reeves JY, Balkwill DL (1995) Strategies for phylogenetic characterization of subsurface bacteria. J Microbiol Meth 21:235–251
43. Reynolds PJ, Sharma P, Jenneman GE, McInerney MJ (1989) Mechanisms of microbial movement in subsurface materials. Appl Environ Microbiol 55:2280–2286
44. Riley RG, Zachara JM, Wobber FJ (1992) Chemical contaminants on DOE lands and selection of contaminant mixtures for subsurface science research, DOE/ER-0547T. US Department of Energy, Washington, DC
45. Ruddick SM, Williams ST (1972) Studies on the ecology of Actinomycetes in soil V. Some factors influencing the dispersal and adsorption of spores in soil. Soil Biol Biochem 4:93–103
46. Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York
47. Schaefer CE, Arands RR, v. d. Sloot HA, Kosson DS (1995) Prediction and experimental validation of liquid-phase diffusion resistance in unsaturated soils. J Contam Hydrol 20:145–166
48. Scholl MA, Mills AL, Herman JS, Hornberger GM (1990) The influence of mineralogy and solution chemistry on the attachment of bacteria to representative aquifer materials. J Contam Hydrol 6:321–336
49. Semprini L, McCarty PL (1991) Comparison between model simulations and field results for in-situ biorestoration of chlorinated aliphatics: Part 1. Biostimulation of methanotrophic bacteria. Ground Water 29:365–374
50. Semprini L, McCarty PL (1992) Comparison between model simulations and field results for in-situ biorestoration of chlorinated aliphatics: Part 2. Cometabolic transformations. Gound Water 30:37–44
51. Sharma MM, Chang YI, Yen TF (1985) Reversible and irreversible surface charge modifications of bacteria for facilitating transport through porous media. Colloids Surfaces 16:193–206
52. Sharma PK, McInerney MJ, Knapp RM (1993) In situ growth and activity and modes of penetration of *Escherichia coli* in unconsolidated porous materials. Appl Environ Microbiol 59: 3686–3694
53. Shonnard DR, Taylor RT, Hanna ML, Boro CO, Duba AG (1994) Injection-attachment of *Methylosinus trichosporium* OB3b in a two-dimensional miniature sand-filled aquifer simulator. Water Resour Res 30:25–36
54. Smibert RM, Krieg NR (1981) General characterization. In: Gerhardt P, Murray RGE, Costilow RN, Nester EW, Wood WA, Krieg KR, Phillips GB (eds) Manual of Methods for General Microbiology. American Society for Microbiology, Washington, DC
55. Smith MS, Thomas GW, White RE, Ritonga D (1985) Transport of *Escherichia coli* through intact and disturbed soil columns. J Environ Qual 14:87–91
56. Stone WJ (1984) Recharge in the Salt Lake coal field based on chloride in the unsaturated zone. Open File Report 214. New Mexico Bureau of Mines and Mineral Resources
57. Stratford DC (1977) An Atlas of Medical Microbiology: Common Human Pathogens. Blackwell Scientific, Edinburgh
58. Strunk C (1978) Sporogenesis in *Streptomyces melanochromogenes.* Arch Microbiol 118:309–316
59. Thomas GW, Phillips RE (1979) Consequences of water movement in macropores. J Environ Qual 8:149–152
60. Thomas JM, Ward CH (1994) Introduced organisms for subsurface bioremediation. In: Norris RD, Hinchee RE, Brown R, McCarty PL, Simprini L, Wilson JT, Kampbell DH, Reinhard M, Bouwer EJ, Borden RC, Vogel TM, Thomas JM, Ward CH (eds) Handbook of Bioremediation. Lewis Publishers. Ann Arbor, Michigan, pp 227–244
61. Trevors JT, vanElsas JD, VanOverbeek LS, Starodub ME (1990) Transport of a genetically-engineered *Pseudomonas fluorescens* strain through a soil microcosm. Appl Environ Microbiol 56:401–408
62. Tunlid A, Ringelberg D, Phelps T, Low C, White DC (1989) Measurement of phospholipid fatty acids at picomolar concentrations in biofilms and deep subsurface sediments using gas chromatography and chemical ionization mass spectrometry. J Microbiol Methods 10:139–153
63. Tyler SW, Walker GR (1994) Root zone effects on tracer migration in arid zaones. Soil Sci Soc Amer J 58:25–31
64. vanElsas JD, Trevors JT, vanOverbeek LS (1991) Influence of

soil properties on the vertical movement of genetically-marked Pseudomonas fluorescens through large soil microcosms. Biol Fertil Soils 10:249–255

65. vanOverbeek LS, Eberl L, Givskov M, Molin S, v. Elsas JD (1995) Survival of, and induced stress resistance in, carbon-starved *Pseudomonas fluorescens* cells residing in soil. Appl Environ Microbiol 61:4202–4208
66. Weisberg WG, Barns SM, Pelletier DA, Lane DJ (1991) 16S ribosomal DNA amplification for phylogenetic study. J Bacteriol 173:697–703
67. White DC, Davis WM, Nickels JS, King JD, Bobbie RJ (1979) Determination of the sedimentary microbial biomass by extractible lipid phosphate. Oecologia 40:51–62
68. Wong PTW, Griffin DM (1976) Bacterial movement at high matric potentials—I. In artificial and natural soils. Soil Biol Biochem 8:215–218
69. Yates MV, Yates SR (1991) Modeling microbial transport in the subsurface: A mathematical discussion. In: Hurst CJ (ed) Modeling the Environmental Fate of Microorganisms. American Society for Microbiology, Washington, DC, p 292