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Microbial Transport, Survival, and Succession in a Sequence of Buried Sediments

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Abstract

Two chronosequences of unsaturated, buried loess sediments, ranging in age from <10,000 years to >1 million years, were investigated to reconstruct patterns of microbial ecological succession that have occurred since sediment burial. The relative importance of microbial transport and survival to succession was inferred from sediment ages, porewater ages, patterns of abundance (measured by direct counts, counts of culturable cells, and total phospholipid fatty acids), activities (measured by radiotracer and enzyme assays), and community composition (measured by phospholipid fatty acid patterns and Biolog substrate usage). Core samples were collected at two sites 40 km apart in the Palouse region of eastern Washington State, near the towns of Washtucna and Winona. The Washtucna site was flooded multiple times during the Pleistocene by glacial outburst floods; the Winona site elevation is above flood stage. Sediments at the Washtucna site were collected from near surface to 14.9 m depth, where the sediment age was ~250 ka and the porewater age was 3700 years; sample intervals at the Winona site ranged from near surface to 38 m (sediment age: ~1 Ma; porewater age: 1200 years). Microbial abundance and activities declined with depth at both sites; however, even the deepest, oldest sediments showed evidence of viable microorganisms. Same-age sediments had equal quantities of microorganisms, but different community types. Differences in

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community makeup between the two sites can be attributed to differences in groundwater recharge and paleoflooding. Estimates of the microbial community age can be constrained by porewater and sediment ages. In the shallower sediments (<9 m at Washtucna, <12 m at Winona), the microbial communities are likely similar in age to the groundwater; thus, microbial succession has been influenced by recent transport of microorganisms from the surface. In the deeper sediments, the populations may be considerably older than the porewater ages, since microbial transport is severely restricted in unsaturated sediments. This is particularly true at the Winona site, which was never flooded.

Introduction

The biosphere is now known to extend to thousands of meters below land surface, and microorganisms have been found in a wide variety of subsurface environments [6, 11, 14, 22, 26–28, 33]. Microbial life in subsurface environments raises the question of how they arrived there. Two explanations, not mutually exclusive, can be posited: (1) Microbes were deposited at the time of geologic deposition, and (2) microbes have been transported to the subsurface since geologic deposition [33, 38]. In saturated zones with high groundwater flow rates, transport may dominate, as has been noted in Atlantic coastal plain aquifers of the southeastern United States [38]. In subsurface environments with low groundwater flow rates, e.g., in clay-rich aquitards and in thick, unsaturated zones of arid and semiarid regions with low moisture recharge rates, physical filtration inhibits the transport of surface organisms [7]. In these relatively static subsurface environments, the microbial communities are most likely derived from populations of microorganisms that have been buried for hundreds to thousands of years (in some cases, since the time of geologic deposition). The age of the microbial community can be constrained by the age of the geologic material (oldest possible) and the porewater age (youngest possible) [37]. In some cases, microbiological and geological data lead one to conclude that a particular microbial community is derived from populations that existed at the time of geologic deposition, for instance in deep Cretaceous shale sediments of the San Juan Basin in New Mexico, where at least a portion of the community appears to date from the original marine sediment community [17]. Similarly, microbes in clay-rich lacustrine sediments of the Ringold Formation in the Columbia Basin of eastern Washington are thought to be derived from Miocene bacteria that were originally present in lake sediments [18].

The changes that occur in microbial communities over geologic time can be considered to form an ecological succession. In relatively static, diffusion-dominated subsurface systems, where colonization by immigrant bacteria is negligible, succession occurs primarily through selection of populations capable of reproduction and/or long-term starvation survival. Reconstruction of such a microbial succession through geologic time requires combining tools of microbiology and geology. Two chronosequences of buried soils (paleosols) were investigated. A chronosequence is a set of soils that are comparable in climate, organisms, topography, and parent material, but that differ with respect to time [24, 42, 50]. In this study, the time factor is represented by a range of times since sediment deposition; this type of chronosequence has been defined as a fully time-transgressive chronosequence without historical overlap [50]. The sediment profiles in this research are loess sediments consisting of mostly silt, the Palouse, in eastern Washington State. They were deposited during similar climatic periods that promoted aeolian processes. Once deposited, however, the exposed soils may have been subjected to climatic variations. Deposition times of these sediments range from modern to >1 million years before present. Soils developed in these sediments and were subsequently buried by further aeolian deposition. The objectives of this study were (1) to determine the relative importance of microbial survival and transport to the succession of microbial communities that has occurred in these sediments since the time of burial and (2) to determine patterns of microbial abundance, activities, and community composition in chronosequences at two sites that have had different moisture inputs.

Materials and Methods

Sites

Two sites located in the Channeled Scabland region of eastern Washington State were chosen for study (Fig. 1). This is an area of silt loess deposits that overlie basalt bedrock. Portions of the loess were eroded and channeled by brief, cataclysmic floods during the Pleistocene, when the ice dams that formed Glacial Lake Missoula were breached. Approximately 10–50 of these floods occurred dur-

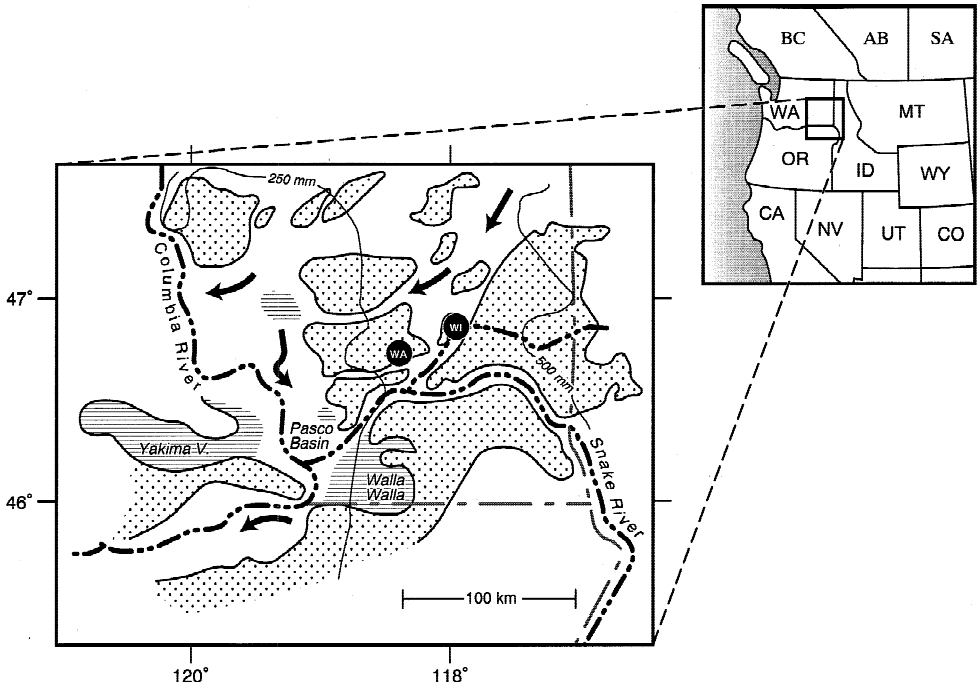

Fig. 1. Locations of Washtucna (WA) and Winona (WI) boreholes in eastern Washington State (after McDonald and Busacca, 1992). Stippled areas indicate Palouse loess, arrows indicate flows of Pleistocene floodwaters, and horizontal lines indicate valleys where floodwater sediments were deposited. Annual precipitation contours (250 mm and 500 mm) are also shown.

ing the last glaciation [51]; most of these floods were not large enough to affect the Washtucna site. Our sites were near the towns of Washtucna and Winona. Each is located near a road cut where the sediments have been described previously by McDonald and Busacca [34–36]. The Washtucna site of our study was adjacent to the WA-9 site of McDonald and Busacca [34]; the Winona site was adjacent to the WIN-1 site of McDonald and Busacca [36]. The exposed road cuts provided stratigraphic controls for the adjacent drilling sites of this study. The Washtucna site was first cultivated (dryland wheat) between 1890 and 1900; dryland wheat farming at the Winona site has been continuous since approximately 1940. The native plant community in this semiarid region is shrub-steppe [43].

A moisture gradient exists in the region: annual precipitation means are 28 cm at Washtucna and 33 cm at Winona. Surface elevations are 469 m at the Washtucna site and 503 m at the Winona site. This difference in elevation is the cause of differences in Pleistocene flooding effects. The Washtucna site underwent multiple catastrophic floods during the last glaciation; the Winona site lies above the maximum flood level, as indicated by geomorphology and the projected water surface profile of Baker and Numendal [4]. Thus, the Winona site was not flooded and probably remained unsaturated throughout that period. Both sites are at the crests of hills, so runon and ponding are unlikely, precluding saturated water recharge since the last Pleistocene floods. Properties of the buried soils indicate that the climate has remained arid to semiarid throughout the >1 million-year period of deposition [12].

Sampling

Samples were collected by coring, using a hollow-stem auger, split-spoon sampler technique. Cores (8.9 cm diam × 76 cm length) were collected in sterile Lexan liners. Fluorescent carboxylated microbeads (1 µm diam) were added to the open borehole after collection of the first core and thereafter at 7.6 m intervals (i.e., every 10th core). These microbeads served as tracers for potential microbiological contamination of subcores [16, 40]. Cores were sealed in the Lexan liners with sterile plastic end caps, in the field, and returned to Pacific Northwest National Laboratory (PNNL) in Richland, WA for further processing. Lexan liners were opened and the cores were processed in a sterile transfer hood in the flow of HEPA-filtered air. Samples for chloride measurements were collected at ~10-cm intervals. Samples for microbiological analyses were collected from subcores after the outer 0.5 cm had been aseptically pared away. Microbiology samples were collected from subcore intervals of 20 to 50 cm; each was designated by the depth at the top of the subsample interval. These samples, ~1000 g each, were mixed and subsampled into sterile plastic bags (NASCO Whirl-Pak, Ft. Atkinson, WI). Subsamples were sent on ice, by overnight delivery, to other labs for microbiological analyses. Samples intended for lipid analysis were sent to the University of Tennessee. Samples for fluorescent microbead tracers were obtained from the tops of cores that were collected immediately after adding beads to the borehole and from subcore material approximately 10 cm below the tops of these cores. Tracer beads were quantified by epifluorescence microscopy. The concentration of beads at the tops of cores collected immediately following tracer bead addition ranged from 2.4×10^7 to $5.8\ 10^7$ beads g^{-1}; concentrations of beads in the subcores were all below detection ($<3 \times 10^4$ beads g^{-1}).

Stratigraphy

The sediments at both sites are relatively well sorted, largely consisting of silt and very fine sands (Fig. 2). The loess stratigraphy at

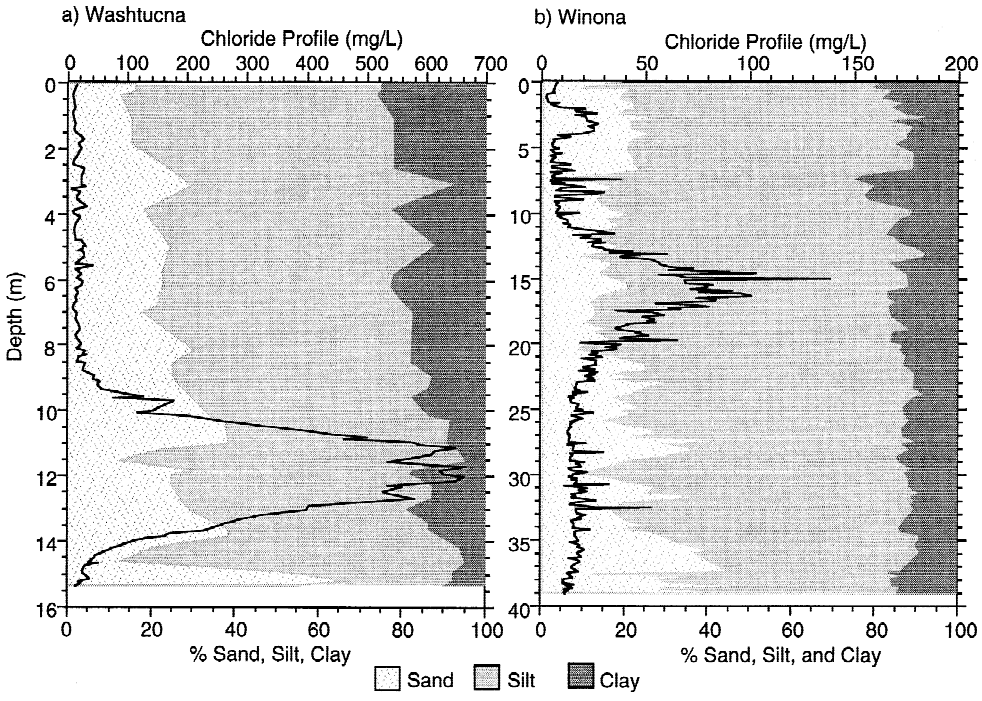

Fig. 2. Percents sand, silt, and clay in sediments, with chloride profile superimposed as black lines, in mg Cl liter^{-1}, as a function of depth at the Washtucna **(a),** Winona **(b)** sites.

each site appears to be vertically continuous without major unconformities (large breaks in the time record or sediment deposition resulting from erosion or truncation by catastrophic loss to erosion or flooding). The presence of buried soils between loess layers indicates variable rates of sediment deposition (soil formation during periods of slow deposition), but the vertical succession of loess deposition is largely uninterrupted. The surface soils have been plowed to a depth of approximately 1 m. The underlying loess contains multiple buried soils, each representing variable lengths of soil formation at the land surface before burial by younger loess. Typical features of soil development include: pedogenic calcium carbonate in disseminated and segregated forms, soil structure, reddish brown accumulation of iron oxides, and evidence of burrowing by soil invertebrates (e.g., earthworms, cicadas). The shallowest of the well-developed burial soils is the Washtucna Soil, previously characterized by McDonald and Busacca [35, 36]. Formation of the Washtucna Soil occurred ~15,000 to 50,000 years ago. It is bracketed between two volcanic ash layers (tephra), erupted from Mount Saint Helens (~300 km to the west). The shallower tephra is the Mount Saint Helens set S, which was deposited 13,000 to 15,000 years ago; the deeper tephra is the Mount Saint Helens set C, deposited 40,000 to 50,000 years ago [8, 36]. Individual loess layers at the Washtucna site are generally thicker than those at the Winona site, reflecting the fact that Washtucna is closer to the source of sediments in the vicinity of the Pasco Basin, to the southwest [13, 36]. The deepest sediments sampled at the Winona site are fluvial sediments, containing basaltic clasts and thinly stratified sands and silts, deposited by giant glacial outburst flooding in the Channeled Scabland. The underlying basalt layer was encountered at a depth of 39 m at Winona, which was consistent with estimated depths from the geologic map.

The ages of the sediments were estimated using (1) radiocarbon dating of tephra layers, (2) estimated rates of soil development, and (3) paleomagnetic orientation. Estimated soil development rates were based on the degree of soil development within well-dated sections of loess (e.g., the Washtucna soil). These rate estimates were compared with the degree of soil development for each buried soil at the Washtucna and Winona sites. Cumulative ages were determined for the top of each buried soil, yielding age estimates for the entire thickness of loess sediment. A paleomagnetic reversal was detected at 29.4 m in the Winona borehole, corresponding to an age of about 790 ± 20 ka [25].

Chemical and Physical Analyses

The profiles of anion, dissolved inorganic carbon, and dissolved organic carbon concentrations in the soil water were determined at 0.1-m intervals over the depth of the borehole at each site. Bulk density and gravimetric water contents were determined on each sample. Anions were leached from the sediment by mixing ~10 g sediment with 20 g of purified water (Milli-Q, Millipore, Bedford, MA) in a glass centrifuge tube, shaking overnight, and removing the sediment by centrifugation. The supernatant was filtered through a 0.22-μm MF-Millipore filter (Millipore). Major anions were measured using ion chromatography (Dionex, Sunnyvale, CA). Dissolved organic and inorganic carbon concentrations were quantified in the same leachates, using a Dohrman carbon analyzer (Dohrman, Rosemount Analytical, Santa Clara, CA). The concentration of the anions in the soil water was estimated using the gravimetric water content [37]. Very little natural chloride, nitrate, sulfate, or phosphate was present in these predominantly siliceous sediments; however, some of these species, such as phosphate, may exist in precipitated or complexed forms in the natural environ-

ment. Leachable organic and inorganic carbon does not necessarily reflect the total carbon in these sediments, but, rather, the concentrations of carbon available to the microorganisms if sufficient water is present. Sediment size distributions were determined using standard sieving and hydrometer methods [20].

Determination of Recharge Rates

Atmospheric tracers provide site-specific recharge rates and are most successful in arid and semiarid environments [1, 21, 37]. The chloride mass balance method provides long-term average recharge rates by applying a mass-balance argument on the chloride ion, which comes naturally from the atmosphere. The difference between the chloride concentration in the soil water and the atmospheric input concentration is due to evapotranspirative enrichment. Therefore, the chloride concentration in pore water is inversely proportional to the flux of water through sediments. Recharge is determined by the relationship

$$J_R = (Cl_o/Cl_{sw})^* p \quad (1)$$

where J_R is the net downward residual flux (e.g., recharge in cm year^{-1}), Cl_o is the average atmospheric chloride concentration in local precipitation and dry fallout (mg liter^{-1} or equivalent units of g m^{-3}), Cl_{sw} is the average chloride concentration in the soil water (mg liter^{-1}), and p is the average annual precipitation (cm year^{-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:

$$tq_{Cl} = {}_i(Cl_i z_i \rho_b) \quad (2)$$

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), and ρ_b is the bulk density (g m^{-3}). The term ($Cl_i z_i \rho_i$) represents the total chloride mass of the peak in g m^{-2} over depth interval z_i.

Direct Microscopic Counts

Direct counts were performed using 4′,6-diamidino-2-phenylindole (DAPI) as described by Schallenberg et al. [47]. Sediment (4.0 g) was homogenized in 45 ml filter-sterilized 0.1% sodium pyrophosphate buffer (pH 7.5), and allowed to settle for 1 min. The supernatant was filtered onto 0.2 µm pore-size black polycarbonate filters (Nucleopore, Pleasanton, CA). Filters were treated for 1 h with a filter-sterilized solution containing 0.5% formaldehyde and 10 µg DAPI ml^{-1}. Stained slides were placed onto microscope slides and examined using epifluorescence microscopy. Cells were counted on ten randomly chosen fields, unless <10 cells field^{-1} were detected. In this case, 30 fields were examined.

Plate Counts

Counts of culturable heterotrophic microorganisms were made on R2A agar (Difco), as previously described [22].

Anaerobe Enumerations

Culturable anaerobic bacteria were quantified by a three-tube, most-probable number method, using medium MS [9] amended for counting fermentative bacteria and iron-reducing bacteria. For fermentative bacteria, the medium was amended as described by Boone et al. [9]; for iron-reducers, the medium of Boone et al. [10] was used. Iron reduction was considered a potential terminal electron-accepting process, given the presence of iron oxide coatings on sediment particles [12]. At least three dilutions, containing 0.001, 0.01, and 0.1 g sediment, were tested for each sediment; for the shallower sediments, dilutions containing 0.0001 and 0.00001 g sediment were also tested. Each MPN assay was run in triplicate. Tubes were incubated at 30°C for 85 days. Growth was detected microscopically. Microscopy is more reliable than iron analysis for detecting iron-reducing bacteria in MPN tubes, because abiotic iron reduction can occur during long incubations, leading to false positives (D.R. Boone, unpublished data).

Phospholipid Fatty Acid (PLFA) and Diglyceride Fatty Acid (DGFA) Analyses

Samples for PLFA and DGFA analyses were removed and frozen at −70°C immediately after subcoring. Extraction and quantification of PLFA and DGFA were performed essentially as described by Kieft et al. [30]. Fatty acid designations were as described by Ringelberg et al. [44].

[^{14}C]Glucose Mineralization

Ten g sediment ea was added to sterile 60 ml serum vials containing a stopper with an attached alkaline trap (Kontes 882320-0000, Vineland, NJ). To each vial, 10 µl of D-[U-^{14}C]glucose (1.28 mM, 3.7 kBq, >99% radiopure, Sigma Chemical Co., St. Louis, MO) was added. Sterile water was mixed into the sediment to a water potential of ~−0.03 MPa, as previously described [27]. Vials were sealed with a sterile stopper with attached trap containing 300 µl of 0.3 N NaOH plus a ~2.5 × 0.635 cm piece of rolled Whatman #1 chromatography paper (as a wick) [27]. Triplicate mineralization microcosms were also prepared for each sample. Three poisoned control vials, in which the sediment was moistened with a 3.7% formaldehyde solution instead of water, were set up for each sample. Vials were incubated at 22°C. At intervals of 1, 3, 5, 7, 11, and 14 days, the chromatography paper and alkaline solution in each vial were replaced. The chromatography paper and alkaline solutions were added to scintillation vials containing 10 ml Scintiverse BD liquid scintillation cocktail (Fisher Scientific, Pittsburgh, PA). Radioactivity was measured in a liquid scintillation counter (Packard Tri-Carb 460 CD). The sample CPM was compared to input CPM for each substrate, to determine the percent mineralization. Percent mineralization in poisoned controls was subtracted from percent mineralization in live samples, yielding the amount of biological mineralization. Lag times and rates of [^{14}C]glucose mineralization were estimated using nonlinear curve fitting, as de-

scribed by Brockman et al. [11]. Nonlinear curve fitting was performed using Systat 5.2 for Macintosh (Systat, Inc., Evanston, IL).

[^{3}H]Glucose Uptake

In preparation for [^{3}H]glucose uptake assays (and enzyme assays), sediment samples were crushed with a sterile mortar and pestle. They were then strained through a sterile, stainless steel, 500-µm pore-size sieve. Crushed and sieved sediment (0.1 g each) was placed into sterile 2.0 ml microcentrifuge tubes. For each sediment sample, three replicate vials were used. Additionally, sediment in one control vial was treated with 0.1 ml 0.1 M $HgCl_2$. To each vial, 0.1 ml D-[5, 6-^{3}H]glucose (27 nM, 7.3 kBq, >95% radiopure, DuPont NEN, Boston) solution in artificial porewater was added. The artificial porewater contained 20 mg $MgSO_4 \cdot 7H_2O$, 0.19 mg $Al_2(SO_4)_3$, 0.55 mg $Na_2BO_4O_7$, 14 mg $CaSO_4$, 28 mg $Na_2SiO_3 \cdot H_2O$, 13.5 mg KNO_3, 33 mg $CaCl_2 \cdot H_2O$, 170 mg $NaHCO_3$, and 0.05 mg $FeSO_4 \cdot 7H_2O$ liter^{-1} of deionized water. Vials were incubated at 24°C for 24 h, after which 1.5 ml of a 1 g liter^{-1} unlabeled glucose solution was added. The unlabeled glucose solution diluted the radioactive glucose solution such that further uptake of labeled glucose was negligible. The sediment was vortex mixed and centrifuged (4 min, 13,000 × g). The pellet was washed twice in artificial pore water and treated with 0.2 ml tissue solubilizer (Solvable, DuPont NEN, Boston), at 50°C, for 1 h. Scintillation cocktail (1.5 ml, Atomlight, DuPont NEN, Boston) was added to the mixture, which was then centrifuged. Radioactivity was quantified using a Beckman model LS 100 liquid scintillation counter. Results are reported with the poisoned control values subtracted.

Enzyme Activity

Enzyme activity was measured using a 4-methylumbelliferone (4-MUF)-labeled substrate. The 4-MUF is linked to the substrate by an ester bond that is enzymatically cleaved, releasing fluorescent 4-MUF [15, 23]. For our assays, β-glucosidase activity was measured using 4-methylumbelliferyl glucoside (Sigma). One-gram aliquots of crushed and sieved sediment (prepared as for [^{3}H]glucose uptake) were placed into scintillation vials. Ten ml 0.25 mM β-glucoside in artificial porewater was added. The vials were incubated, with shaking, for 5 days at 24°C. Fluorescent 4-MUF was quantified in a fluorimeter with an excitation wavelength of 355 nm and an emission wavelength of 455 nm. Triplicate subsamples, plus one poisoned control, were run for each sample. Poisoned controls received 0.1 ml 0.1 M $HgCl_2$. Results are reported with the poisoned control values subtracted.

Metabolic Diversity

Microbial metabolic diversity in sediments was quantified using Biolog GN plates (Biolog, Inc., Hayward, CA), according to the method Garland and Mills [19] devised to analyze environmental samples. Biolog plates were inoculated with the same cell suspensions used for the direct microscopic counts. Plates were incubated aerobically at 24°C for 7 days. Color development, indicating use of a particular carbon source, was quantified using a Vmax Kinetic Microplate Reader (Molecular Devices, Sunnyvale, CA).

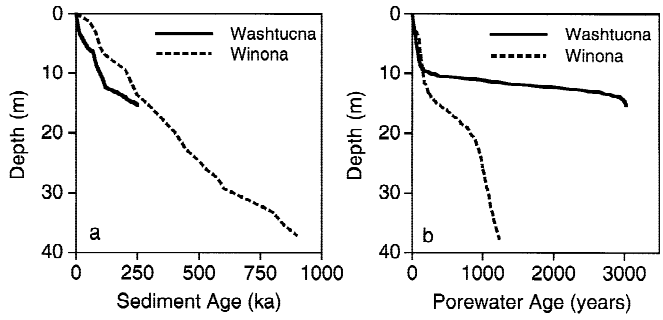

Fig. 3. Sediment age (a) and porewater age (b) as a function of depth.

Statistical Analyses

Spearman rank correlations were performed, using Systat version 5.2 software (Systat Inc., Evanston, IL) to determine significance of microbiological trends with depth and correlations among microbiological data.

Results

The sand, silt, clay analyses (Fig. 2) show a relatively uniform distribution of particle sizes with depth. The Winona site has a somewhat smaller percentage of sand, and greater percent silt, than the Washtucna site. This site is farther from the source of sediment (the Pasco Basin) and is expected to have a greater proportion of fine particles. Sediment size distribution is a good indicator of the structural heterogeneity of the profiles. For example, large shifts in the grain size distribution may indicate regions of preferential flow in the sediment profile, or, at the other extreme, hydraulic barriers to flow that often occur at caliche layers in arid regions. The consistency in sediment sizes with depth promotes greater uniformity in the water flux through the profile. Therefore, when the chloride profile is superimposed over the sediment size distribution (Figs. 2a, b), it is evident that the accumulation of chloride at depth is not an artifact of the physical structure of the sediments.

Sediment ages increased with depth to ~250 ka at 14.9 m at the Washtucna site and to ~1 million years at 38 m depth at the Winona site (Fig. 3a). The porewater ages are considerably younger: 3000 y at 14.9 m at the Washtucna site and 1300 years at 38 m depth at the Winona site (Fig. 3b). The long-term average recharge rates at the Washtucna and Wi-

nona sites are 0.1 cm year^{-1} and 0.8 cm year^{-1}, respectively, calculated using the chloride mass balance method. It is important to note, however, that agricultural practices have greatly altered the unsaturated flow rates at these sites [21]. The chloride profile accumulates at the bottom of the root zone, because of evapotranspirative enrichment. The chloride profiles at Washtucna (peak at ~12 m) and Winona (peak at ~15 m) are too deep to be considered part of the root zone. A more logical explanation is that the change in land use (from natural undisturbed vegetation to yearly cultivation) over the last century has increased the flux of water through the system by eliminating root extraction of water during the wet winter months, thereby convecting the chloride peak downward. This results in relatively young porewater ages from the surface soil to 9 m depth (Washtucna) or 12 m depth (Winona).

Patterns of porewater chemistry differed between the two sites. Leachable inorganic carbon peaked at 5 m depth (3500 mg liter^{-1}) and declined to ~200 mg liter^{-1} at 15 m in the Washtucna borehole; it ranged from 100 to 2000 mg liter^{-1}, without a depth pattern, at the Winona site. Leachable organic carbon was highest near the surface of both sites (~3000 mg liter^{-1}). It showed a second peak near 30 m (2000 mg liter^{-1}) in the Winona borehole. Leachable sulfate was low at both sites (<100 mg liter^{-1}), except for high values (~4,000 mg liter^{-1}) at about 10 m depth in the Washtucna borehole. Leachable phosphate concentrations were low (<100 mg liter^{-1}), except at a depth of 3–4 m in the Washtucna borehole. There, they peaked at ~300 mg liter^{-1}. Leachable nitrate concentrations were relatively high (50–200 mg liter^{-1}) in the top 5 m in the Winona borehole, but were low at greater depth, and were low (<50 mg liter^{-1}) throughout the Washtucna borehole.

Microbial biomass, as indicated by total PLFA and direct microscopic counts, declined by several orders of magnitude with depth (and also with sediment and porewater ages) at both sites (Figs. 4a and 5). Similar patterns were observed for counts of culturable aerobic heterotrophs (Fig. 5) and for percents of [^{14}C]glucose mineralization and ^{3}H-labeled glucose uptake during 24 h sediment incubation (Fig. 6). The abundance of culturable anaerobes was also highest in the shallowest sediments: 2,100 fermenters g^{-1} and 240 iron reducers g^{-1} were cultured from 0.3 m depth at Washtucna; >240,000 fermenters g^{-1} and 460 iron reducers g^{-1} were cultured from the 0.5-m sample at Winona. Fewer than 10 fermenters g^{-1} and <3 iron reducers g^{-1} were cultured from deeper sediments at both sites. The ratio of dead cells (indicated by DGFA) [30, 53] to live cells (PLFA) increased

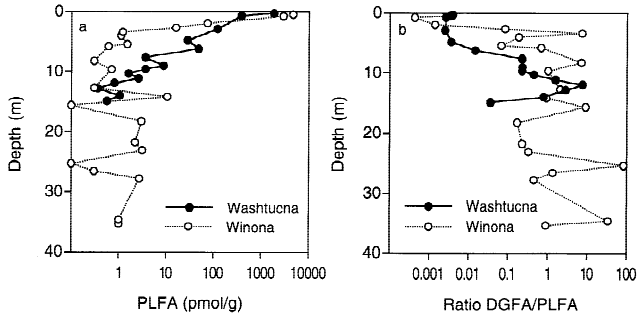

Fig. 4. PLFA (a) and ratio of DGFA to PLFA (b) as a function of depth. Data points represent averages of two analyses.

with depth and age at both sites, though it declined with depth in the 12- to 15-m interval at the Washtucna site (Fig. 4b). The percentage of cells that were culturable in heterotrophic plate counts declined from 1.35% at the shallowest sample to 0% in the deepest sediment at Washtucna; the percent culturable cells declined from 1.5% near the surface to 0.0003% in the 38 m sample at the Winona site. The rates of [^{14}C]glucose mineralization to CO_2, calculated using time course measurements over three weeks' incubation, declined with sediment depth and ages, whereas the lag times until [^{14}C]glucose mineralization commenced increased with depth (Fig. 7). β-Glucosidase activity, measured in sediments incubated 5 days, declined with depth over a 2- to 3-order of magnitude range (Fig. 8). When incubated in the presence of chloramphenicol, β-glucosidase activity declined with depth even more sharply.

Although the total PLFA data indicate that the magnitudes of the biomasses were approximately equal in samples

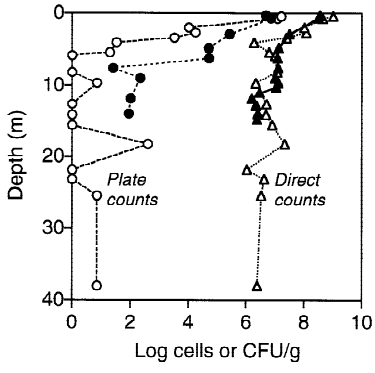

Fig. 5. Direct counts (triangles) and plate counts (circles) as a function of depth. Washtucna (closed symbols); Winona (open symbols). Points lying directly on the *y*-axis represent plate counts that were below detection (<3 CFU g^{-1}), i.e., no growth on any plates.

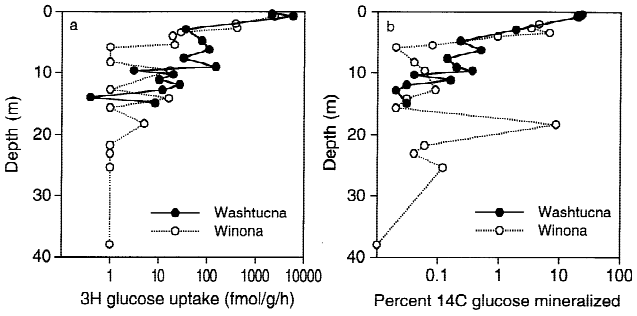

Fig. 6. Percent [^{14}C]glucose mineralized (a) and amount of [^{3}H]glucose uptake (b) as a function of depth in 24-h incubations. Data points represent averages of triplicate analyses.

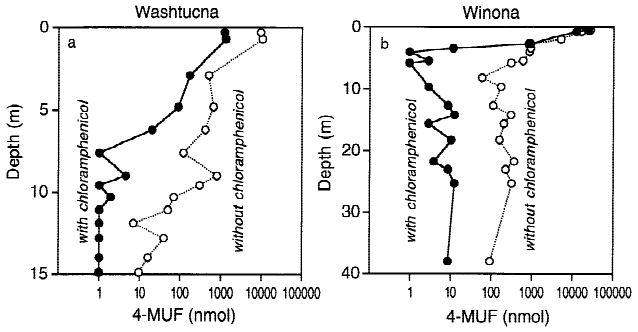

Fig. 8. Production of fluorescent 4-methylumbelliferone from 4-methylumbelliferyl β-D-glucoside by β-glucosidase in sediments incubated for 5 days with and without chloramphenicol. Washtucna (a); Winona (b). Data points represent averages from triplicate microcosms.

of equivalent sediment age at the two sites (Fig. 4a), the PLFA profiles indicated that the microbial communities at the two sites differed markedly from each other in their compositions (Fig. 9). In the Washtucna borehole, membrane lipids representative of several functional groups, including high G + C gram-positive bacteria, e.g., *Arthrobacter* (terminally branched saturated PLFA), actinomycetes (mid-branched saturated PLFA), gram-negative bacteria (monounsaturated PLFA), and microeukaryotes (polyunsaturated PLFA), were found throughout the depth profile. The Winona sediments showed a decline in the percentage of each of these functional groups over the same depth interval. The most striking difference between the two sites was in the percentage of polyunsaturated PLFA. These microeukaryotic lipid biomarkers were present throughout the Washtucna profile and nearly absent from the Winona profile. Percentages of terminally branched, saturated PLFA (gram-positive bacterial biomarkers) also differed considerably between sites, with the greater percentages occurring in the Washtucna sediments.

Microbial metabolic diversity, indicated by the number of carbon substrates that could be oxidized in the Biolog assay, declined with depth at both sites (Fig. 10). Nearly all of the 95 substrates were used by microbes in the shallowest sediments. In the deeper sediments, the few substrates that were utilized were a subset of those used by microbes in the shallower sediments.

The following microbiological data were negatively correlated with depth at both sites ($P < 0.05$): PLFA, direct microscopic counts of total cells, plate counts, [^{3}H]glucose

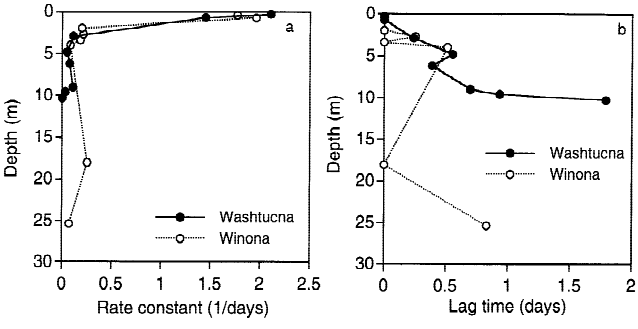

Fig. 7. [^{14}C]Glucose mineralization: rate constant (a) and lag time (b) as a function of depth.

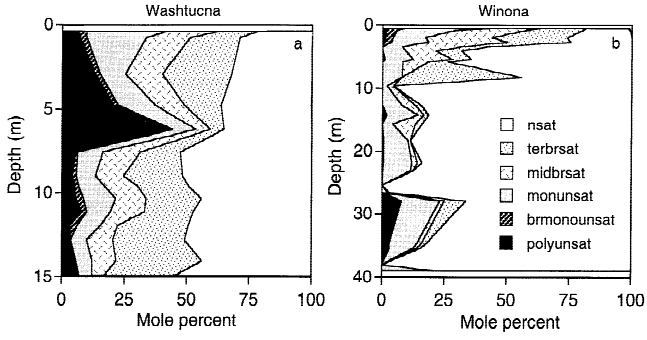

Fig. 9. Mole percents of signature PLFA classes (representing different functional groups of microorganisms) as a function of depth at two sites in the Palouse region of eastern Washington State. Polyunsaturated PLFA (polunsat) for microeukaryotes; branched monounsaturated PLFA (brmonounsat) for sulfate-reducing and iron-reducing bacteria; normal monounsaturated PLFA (monounsat) for typical gram-negative bacteria (such as *Pseudomonas*); mid-chain branched saturated PLFA (midbrsat) for actinomycetes; terminally branched saturated PLFA for typical gram-positive bacteria (such as *Arthrobacter*); normal saturated PLFA (nsat), which are ubiquitous and not indicative of any single group of microorganisms. Washtucna (a), Winona (b). Data points represent averages of duplicate analyses.

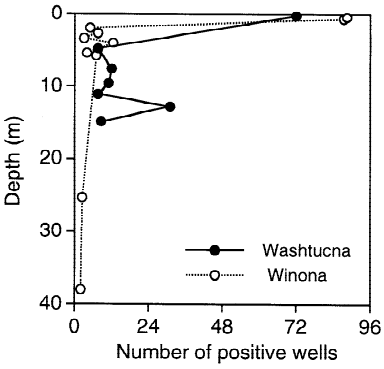

Fig. 10. Biolog assays: Number of positive wells (absorbance$_{590\ nm}$ > 0.1) as a function of depth. Data points represent the average from two Biolog plates.

uptake, and percent [^{14}C]glucose mineralization (24-h). Significant positive correlations ($P < 0.05$) were found between all possible pairs of these microbiological measurements, at both sites.

Discussion

Characterizing the abundance, activities, and physiological states of subsurface microbes requires a suite of microbiological assays, as noted by Chapelle [14], specifically pertaining to vadose zones. Although the results of different assays frequently conflict [14], the results of this study showed remarkably consistent patterns, as indicated by correlation analysis. Microbial biomasses and activities declined sharply with depth at both sites. This simple pattern of monotonic, continuous decrease in microbial abundance with depth reflects the uniform nature of these sediments. They are alike in texture and mineralogy, with differences occurring primarily in sediment and porewater ages. Many of the correlations between microbiological assays were expected. For example, agreement between measures of total biomass and total cells (e.g., PLFA concentrations and direct microscopic counts: $r = 0.916$, $P < 0.001$ and $r = 0.541$, $0.02 < P < 0.05$ at Washtucna and Winona, respectively) has been observed previously [5]. Direct counts and culturable counts were also correlated, although they differed by several orders of magnitude. This is a common occurrence in natural environments, including the subsurface [26–28, 32, 45]. The decline in the ratio of cultured to total cells with depth corresponded to an increase in the ratio of DGFA to PLFA. The DGFA/PLFA data suggested that at least a portion of the discrepancy between total and cultured cells was attributable to dead cells. Viable, but nonculturable, cells are likely also important; this is supported by the potential for metabolic activity in samples from which cells were not cultured.

Microbial activity (glucose uptake, glucose mineralization, and enzyme induction) data closely follow the microbial abundance (PLFA, direct count, and plate count) patterns in these sediments, as indicated by significant correlations between assays. Results from 24-h radiorespirometry incubations demonstrate potential metabolic activity by microbes present in the sediment samples at the time of collection. The longer-term incubations demonstrate a potential for growth, as well as for transforming a substrate. The increase in lag times with sediment depth and age shows the increased time required for microbial growth and/or enzyme induction in the older sediments. Smaller populations of cells that have been dormant for longer intervals require correspondingly longer times to respond to substrate addition [3]. This is also evident in the slower substrate mineralization rates in the older sediments. While glucose is probably not an important source of energy in these sediments, it does provide a useful, sensitive means of detecting viable microorganisms and quantifying potential metabolic activities in subsurface environments [11, 26–28, 32, 39]. The 5-day β-glucosidase activity data demonstrate the importance of growth and/or enzyme induction in metabolic response to substrate addition. The differences in response between the samples incubated without chloramphenicol and those incubated with chlorampehnicol show the amount of de novo enzyme synthesis. In the deeper, older sediments (i.e., those buried for longer than approximately 100 ka), the extant β-glucosidase activity was negligible.

The pattern of declining microbial biomass and activity with depth can be explained by transport of microorganisms from the surface and/or long-term microbial survival in situ. Transport from the surface, combined with attenuation over distance and time due to sorption, filtration, and cell death, would lead to just such a curve. Starvation survival of indigenous soil microorganisms following soil burial would yield the same pattern. The pattern of microbial biomass and activities with depth resembles the pattern one obtains over time when bacteria are starved in the laboratory [2, 31]. The relative importance of transport and survival can be assessed with respect to porewater and sediment ages, as well as geohydrological characteristics of the vadose zone.

The minimum time required for transport of microorganisms from the surface to a particular depth interval is the porewater age at that depth interval. The relatively young ages (approximately 100 years or less) of the groundwater to

depths of 9 m at the Washtucna site and 12 m at the Winona site suggest a strong transport component. Dryland farming, wherein the land is kept fallow for extended periods to minimize evapotranspiration, may have accelerated recharge in these shallow sediments during the past 100 years [21]. Thus, downward transport of bacteria may have made immigration the dominant influence on microbial succession in the shallow sediments of these sites.

In the deeper sediments, long-term survival of indigenous populations may have been a greater influence on microbial succession than transport. Microorganisms tend to move more slowly than water in a porous medium, particularly under unsaturated conditions [7]. Vadose zone bacteria are thought to be transported in water films that move by capillary forces during unsaturated flow. As water contents decrease, capillary forces retain water in pores of diminishing size. Under these conditions, bacterial transport is severely limited by physical filtration and attachment to particle surfaces [7], as well as attachment to gas–water interfaces [52]. The rate of microbial transport by unsaturated flow processes has been negligible at similar sites in southeastern Washington State [7]. Thus, the microbial communities at depth may be much older than the porewater ages. The living bacteria detected in the deepest sediments may even be close descendants of original surface soil populations (closer in age to the sediments than the porewaters). It is unlikely that individual cells have survived in the oldest sediments since deposition; however, cells may have replicated relatively few times since burial. Physical constraints on nutrient availability likely limited cell proliferation. Generations lasting several centuries, which have been estimated for subsurface sediments [29, 41], may be adequate to enable long-term persistence of a population.

The availability of exogenous nutrients affects the ability of microorganisms to persist over geologic time. The leachable DOC data give some measure of the quantity (but not the quality) of carbon sources for heterotrophic metabolism. Patterns of DOC were very different at the two sites; however, they appear not to be reflected in the microbial biomass and activity patterns at both sites. While DOC declined with depth at the Washtucna site, the Winona site showed less variation. Severson et al. [48] reported a decline in both heterotrophic plate counts and total organic carbon with depth at a site in the Palouse region near Pullman, Washington. They attributed this pattern to downward transport of surface-soil-derived organic carbon, as well as slow depletion of buried organic matter in the paleosols. Given the unsaturated nature of these sediments, downward transport of easily metabolized organic energy sources is unlikely. Microbes are, therefore, probably surviving and replicating sporadically on relatively recalcitrant organic matter in these paleosols. Dead cells may also be a source of nutrients. The increased ratio of DGFA to PLFA with depth indicates a greater proportion of dead cells in the deepest sediments. However, physical access to this and other sources of energy and nutrients is extremely limited in unsaturated sediments [26]. Patterns of leachable anion concentrations (NO_3^-, SO_4^{2-}, and PO_4^{3-}) did not coincide with microbial biomass and activity patterns; thus, they appear not to be a major factor in overall microbial distribution at depth. However, chemical characterization of the sediments was not exhaustive; these and other chemical features of the sediments are undoubtedly important, especially at the scale of individual pores. The differences between sites in leachable anions likely reflect differing moisture inputs.

PLFA profiles show patterns of change in the community profiles with depth and sediment age. If the deepest subsurface communities represent the survivors from original soil communities, then the patterns with sediment age indicate which microbial groups can persist for at least 1 million years and which groups declined in biomass or disappeared. Finding polyunsaturated fatty acids, indicative of eukaryotes, at depth is surprising, since microeukaryotes are thought to occur in the subsurface only in aquifers with relatively young groundwater [6, 49]. At the Washtucna site, the eukaryotic PLFA signature is found in the highest proportions in the shallower, 0–10 m depths, where relatively recent microbial transport is likely. Fungal spores may have been transported to these depths within the past 100 years. Eukaryotic signature PLFA were rare at the Winona site, except in some very deep samples (34 to 38 m). Although the mole percents of PLFAs in these deep sediments were in the 3–8% range, the actual quantities were minuscule. PLFA signature biomarkers, indicative of gram-negative bacteria (such as *Pseudomonas*) and of gram-positive bacteria (such as *Arthrobacter*) were present in sediments that have been buried for nearly 1 million years. The differences in community profiles within sediments of similar ages may be the result of spatial heterogeneity in microbial communities; for example, soil microbial communities at sites 40 km apart may have differed significantly from each other and may remain different in buried sediments. Alternatively, the differences in community structure that are evident between the two boreholes may be due primarily to differences in moisture patterns

(higher precipitation and recharge rate at the Winona site, and exclusively unsaturated recharge at the Winona site vs intermittent saturation by Pleistocene flooding at the Washtucna site).

Rothfuss et al. [46] used a similar approach in investigating microbial survival in a sequence of lake sediments. Microbial transport to deeper strata was limited by the high clay content. Microbial abundance declined exponentially from the shallowest, youngest lake sediments to the deeper, older sediments. Microbes in 6–7 m deep, 8,900-year-old sediments consisted entirely of endospores. In the present study, several of the deepest, oldest sediments showed evidence of a greater microbial diversity. They contained PLFA representative of three or four different major taxonomic groups of microorganisms, including ones that do not form endospores. Vegetative gram-positive and gram-negative bacterial populations have evidently persisted for a minimum of 1,000 years, probably much longer in the deepest, oldest sediments.

Although the sediments sampled in this study include ones that underwent pedogenesis following deposition, as well as ones that underwent less extensive soil development, the microbiological data do not reflect differences between these two categories of sediments. This may be explained by the complexity of these slowly aggrading sediments. Soil development occurred with previously buried soils as underlying parent material, resulting in a sequence of superimposed soil profiles [35]. Sediments at all depths were once exposed at the surface and were subjected to some degree of soil development before burial. Most of the sediment sequence now consists of B horizons, with only subtle differences to distinguish them from A or C horizons. The surface A horizons of these profiles have lost nearly all of their original organic content. They superficially resemble B horizons [12, 35]. Thus, the effects of different soil horizon types on the remanent microbial community are overshadowed by the influence of time since burial.

In summary, microbial communities in the shallower sediments (<9 m at Washtucna, <12 m at Winona) appear to have been strongly influenced by relatively modern transport of microorganisms from the surface. In the deeper sediments, the surviving communities may be considerably older than the porewater. Dryland farming has altered the rates of recharge and has increased the potential for downward transport of bacteria into the shallower sediments. Bacteria in the deepest, oldest sediments may be relatively close descendants of bacteria present in the soil at or near the time of sediment burial. Differences in microbial community composition between the two sites may be attributable to differences in moisture input, including differing rates of precipitation and effects of Pleistocene flooding.

Acknowledgments

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