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Factors Limiting Microbial Growth and Activity at a Proposed High-Level Nuclear Repository, Yucca Mountain, Nevada

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As part of the characterization of Yucca Mountain, Nev., as a potential repository for high-level nuclear waste, volcanic tuff was analyzed for microbial abundance and activity. Tuff was collected aseptically from nine sites along a tunnel in Yucca Mountain. Microbial abundance was generally low: direct microscopic cell counts were near detection limits at all sites (3.2×10^4 to 2.0×10^5 cells g^{-1} [dry weight]); plate counts of aerobic heterotrophs ranged from 1.0×10^1 to 3.2×10^3 CFU g^{-1} (dry weight). Phospholipid fatty acid concentrations (0.1 to 3.7 pmol g^{-1}) also indicated low microbial biomasses; diglyceride fatty acid concentrations, indicative of dead cells, were in a similar range (0.2 to 2.3 pmol g^{-1}). Potential microbial activity was quantified as $^{14}CO_2$ production in microcosms containing radiolabeled substrates (glucose, acetate, and glutamic acid); amendments with water and nutrient solutions (N and P) were used to test factors potentially limiting this activity. Similarly, the potential for microbial growth and the factors limiting growth were determined by performing plate counts before and after incubating volcanic tuff samples for 24 h under various conditions: ambient moisture, water-amended, and amended with various nutrient solutions (N, P, and organic C). A high potential for microbial activity was demonstrated by high rates of substrate mineralization (as much as 70% of added organic C in 3 weeks). Water was the major limiting factor to growth and microbial activity, while amendments with N and P resulted in little further stimulation. Organic C amendments stimulated growth more than water alone.

Yucca Mountain, Nev., has been selected as a potential site for the United States' high-level nuclear waste repository. The purpose of underground disposal is to protect the safety and health of the public by limiting or preventing radioactive wastes from reaching the accessible environment. A thick unsaturated zone in a remote arid region was selected as a candidate repository site because of the limited potential for radionuclide transport. The suitability of Yucca Mountain tuff is being studied as part of the Yucca Mountain Site Characterization Project to determine the probability that Yucca Mountain can retain high-level wastes for a minimum of 10,000 years. In addition to geologic and hydrologic issues, the effects of microorganisms on transport of radioactive wastes are a major concern. Biogeochemical processes in the vicinity of nuclear waste repositories can be categorized as those occurring in (i) the near-field environment, i.e., the highly disturbed area surrounding the waste, and (ii) the far-field environment, i.e., distant areas not affected by repository construction or by the physical and chemical conditions of the repository itself. Near-field concerns include biocorrosion of containers, biogenic gas production, and biologically mediated redox reactions (18, 24, 30). The study presented here focused on the far-field environment, where the potential interactions between microorganisms and radioactive wastes are more subtle than in the near field. Far-field microbial populations are expected to be smaller, due to lower nutrient availability; nonetheless, even low rates of microbial activity in the far field may significantly alter radionuclide transport, given the extensive time and distance constraints placed on repository performance.

Recent research has demonstrated the nearly ubiquitous presence of microorganisms in subsurface environments (6, 8, 11, 13, 23, 29), including unsaturated volcanic tuff similar to that of Yucca Mountain (14, 15, 19, 20, 33). Subsurface bacteria have the potential to influence radionuclide transport through sediments and fractured rocks (18, 24, 25, 35). Solute transport may be retarded in the presence of microorganisms by sorption to microbial cells and their exopolysaccharides (2, 3) or through microbially mediated redox reactions that form insoluble precipitates (26, 27). Alternatively, microorganisms may accelerate the transport of wastes by chelation (28), by pH changes that increase radionuclide solubilities, by redox reactions that generate soluble species (3), and by affecting the colloid transport of sorbed radionuclides (4, 18).

The purposes of this study were (i) to quantify microorganisms in Yucca Mountain tuff, (ii) to determine their potential for mineralization of organic substrates, and (iii) to identify factors that may limit microbial growth and activity in Yucca Mountain tuff.

MATERIALS AND METHODS

Yucca Mountain. Yucca Mountain is approximately 125 km northwest of Las Vegas, Nev., on the western boundary of the Nevada Test Site. The mountain is a ridge oriented north-south and is composed of tuff, formed by ashflow and ashfall deposition from successive volcanic eruptions 9 to 13 million years ago

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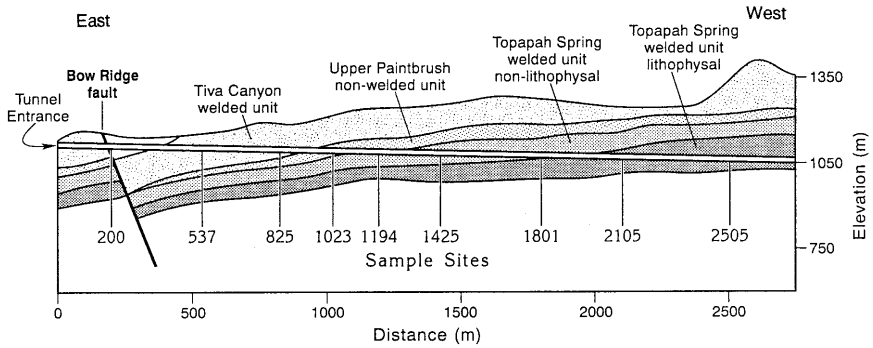

FIG. 1. Diagram of Yucca Mountain showing the ESF tunnel, major geologic formations, and sample collection sites.

(5). Thick layers of welded tuff, a hard, heat-consolidated volcanic rock, are separated by thinner layers of bedded tuff, a nonwelded, less consolidated material. The proposed repository is approximately 275 m above the water (1) and 300 m below the ridge line of Yucca Mountain in the Topopah Spring member of the Paintbrush Tuff, a welded tuff formation.

As part of the site characterization of Yucca Mountain, an exploratory study facility (ESF) was initiated in early 1995 by drilling a 7.6-m-diameter tunnel into the eastern face of Yucca Mountain with a tunnel-boring machine (TBM). The TBM is 140 m long and has 48 carbon steel cutting discs, a conveyor system to expel rock material, and machinery to lay railroad tracks. The TBM was chosen over blasting techniques because it causes less fracturing of the rock material. Also, it is electrically driven, thereby avoiding hydrocarbon emissions in the tunnel (25).

Sample collection. During the period of our study (February to November 1995), the TBM crossed one fault and passed through three geologic units, extending the ESF tunnel from the entrance to the formation proposed for waste disposal (Fig. 1). Samples were collected from nine locations, immediately after the entire length of the TBM passed by each sample location. On average, 1 month passed between the time the rock was uncovered by the cutting head of the TBM and the time when a sample could be collected. To prepare each site for collection, a 1.0-m^2 area was excavated horizontally into the north wall of the ESF tunnel to a depth of approximately 0.5 m with a jackhammer. As a test to determine whether microorganisms were carried from the rock surface into the underlying tuff during subsequent excavations, a randomly selected 10-cm^2 area within the 1.0-m^2 surface was painted with 1.0-μm-diameter fluorescent styrene beads (Interfacial Dynamics, Portland, Oreg.) as surrogate microbial tracers. A sample of this microbead-painted rock was collected. Rock was then removed to a depth of approximately 1.0 cm from the entire face of the 1.0-m^2 area with sterile chisels and a handheld pneumatic hammer. The 1.0-m^2 area was divided into four quadrants, and material from each quadrant was collected into plastic freezer bags (Ziplock heavy duty; Dow Brands, Indianapolis, Ind.) with sterile chisels (handheld or pneumatic). We tested this type of freezer bag for sterility by pressing the internal surfaces of bags onto R2A agar (Difco) and by pouring liquid 10% PTYG broth (22) into bags. Plates and bags containing broth were incubated at 28°C for up to 1 month. No growth appeared on plates or in the broth within the bags. Twice as much rock was collected from one quadrant (randomly selected prior to sampling); rock from this quadrant was mixed and divided into two separate plastic bags. A second randomly selected quadrant was designated to be either autoclaved (2 h, 15 lb/in^2, 121°C) or spiked with a lab culture of *Pseudomonas fluorescens*, thereby serving as a negative or positive control, respectively. Thus, five samples were collected at each location: three quadrants, a duplicate of one of these quadrants, and the fourth quadrant, which was an autoclaved (or spiked) control. Exceptions were the site at 1,023 m from the tunnel entrance, for which six samples (five "live samples" and one autoclaved control) were collected, and the site at 2,505 m, where three live samples were collected. A sample from beneath the original 10-cm^2 microbead-painted surface was also collected at each site and examined for microbeads by UV epifluorescence microscopy; none was detected in any samples. Lithium bromide was added as a tracer to all fluids used for tunnel boring procedures, so that contamination from the drilling process could be monitored. Again, no evidence of the tracer was found in the samples.

Samples were divided into three aliquots: (i) samples for lipid analyses, shipped overnight on dry ice to the University of Tennessee; (ii) samples for direct counts, plate counts, and radiorespirometry, shipped overnight on ice to the New Mexico Institute of Mining and Technology; and (iii) samples retained at the University of Nevada, Las Vegas, for nutrient-limitation and plate-count experiments.

Sample preparation. Samples shipped to the New Mexico Institute of Mining and Technology were crushed aseptically in HEPA-filtered air hoods using a combination of a hydraulic press and mechanical hammering and grinding. The hydraulic press first crushed the rock to an approximately 1- to 3-mm particle size, and then pounding (with a hammer) and grinding (with a stainless-steel rod in a cast-iron pan) further reduced the particle size to 250 to 500 μm. Similar crushing procedures, but without a hydraulic press, were used at the other institutions.

Cell enumeration. Direct microscopic counts of microbes from crushed rock samples were performed by acridine orange staining and epifluorescence (20, 21). The minimum level of detection was 3.2×10^4 cells g^{-1}. Culturable counts were performed with spread plates (R2A agar; Difco). Crushed rock (1.0 g) was vortex mixed in 10 ml of 0.1% $Na_4P_2O_7$ (pH 8.0) for one min, and a 10-fold dilution series was prepared in phosphate-buffered saline (1.18 g of Na_2HPO_4, 0.223 g of $NaH_2PO_4 \cdot H_2O$, and 8.5 g of NaCl liter of H_2O^{-1}). Triplicate plates were made for each sample and incubated at 22°C for 1 month. The minimum level of detection for cultured counts was 10 CFU g^{-1} [dry weight].

Fatty acid analyses. Extraction and quantification of phospholipid fatty acids (PLFAs) were performed as described by Kieft et al. (22). Lipids were extracted in phosphate-buffered chloroform and separated on a silicic acid column. PLFAs were transesterified to fatty acid methyl esters, which were then separated and quantified by gas chromatography-mass spectrometry. Diglyceride fatty acids (DGFAs) were recovered by spotting the chloroform fraction onto a thin-layer chromatography plate (60 A, 250 μm thick; Whatman, Clifton, N.J.) and were quantified as previously described (22).

Potential for microbial growth. To determine factors limiting microbial growth, crushed tuff samples were amended with water and with various nutrient solutions in separate treatments. Control subsamples were left in a dry state; amended samples were made into 1:10 (wt/vol) slurries with water or nutrient solutions. Nutrient solutions consisted of (i) 10 mM potassium phosphate (P amendment); (ii) 12.5 mM ammonium nitrate (N amendment); and (iii) 3.0 mM (each) sodium pyruvate, sodium acetate, and glucose (C amendment). All nutrient solutions were adjusted to pH 8.0. Plate counts were performed before and after 24-h incubation (room temperature, approximately 24°C) of ambient-moisture crushed rock and crushed rock slurries. Dilutions were spread onto R2A agar. Colonies were counted after the plates were incubated for 2 weeks at room temperature. Growth was quantified as the change in CFU g^{-1} during the 24-h incubation of the crushed rock or rock slurry.

Radiorespirometry. Laboratory microcosms were used to determine potential microbial activity and to determine factors that might limit the growth of microbes. Ten grams of crushed rock sample was added to sterile 60-ml serum vials. To each vial, 10 μl of either [U-^{14}C]glucose (1.28 mM, 3.7 kBq), [U-^{14}C]acetate (1.28 mM, 3.7 kBq), or [U-^{14}C]glutamic acid (1.26 mM, 3.7 kBq) (all >99% radiopure; Sigma Chemical Co., St. Louis, Mo.) was added. For each radiolabeled substrate, four conditions were tested: (i) crushed rock at ambient moisture (amended only with the 10 μl of radioactive substrate solution); (ii) crushed rock amended with water to a water potential of approximately −0.03 MPa, as described in Kieft et al. (21); (iii) crushed rock amended with a nutrient solution (0.1 mM NH_4Cl, 0.05 mM K_2HPO_4) (a volume equal to the volume of water in condition ii); and (iv) crushed rock poisoned with 3.7% formaldehyde (a volume equal to the volume of water in condition ii). Microcosms were also set up with a 100-fold-greater amount of glucose (unlabeled, 116.5 mM) added along with the radiolabeled glucose (1.28 mM, 3.7 kBq) to test if our standard concentrations (1.25 mM) were sufficient to stimulate metabolism by the microbes. As a negative control, volcanic tuff samples were combusted at 550°C for 3 h, followed by crushing of the rock and analysis of glucose mineralization activity. Triplicate vials were prepared for each treatment. The vials were incubated at 22°C for 3 weeks. Radiolabeled CO_2 was captured in alkaline traps and quantified by liquid scintillation counting, as previously described (21).

Statistical analysis. Percent mineralization data were arcsine transformed to make variances independent of means (34). Mean values of cells g^{-1}, CFU g^{-1}, and percent mineralization for each site were calculated with individual data from the four noncontrol samples collected from three quadrants, i.e., $n = 4$. The two subsamples collected from the same quadrant were treated as two distinct samples because the sample was not crushed prior to division into subsamples

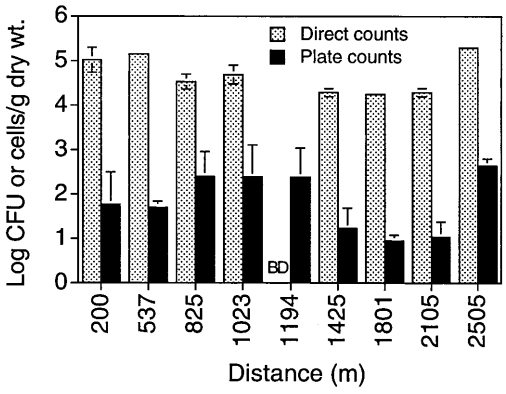

FIG. 2. Direct microscopic counts and plate counts (mean ± standard error [SE]) for samples collected from nine sites at various distances from the ESF tunnel opening (BD, below detection).

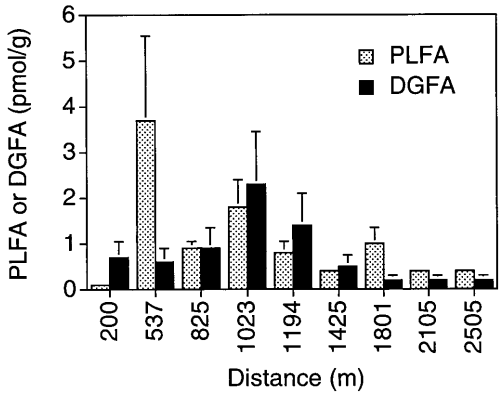

FIG. 3. PLFA and DGFA concentrations (mean ± SE) for samples collected from nine sites at various distances from the ESF tunnel opening.

(instead each subsample consisted of intact rocks up to 10 cm in diameter) and because variability in microbiological parameters between subsamples within a quadrant was as great as variability among the three quadrants. Correlations between parameters were tested by the Pearson product moment method. Analysis of variance (ANOVA) was used on ^{14}C-substrate mineralization data to compare samples by site, treatment, and substrate. Nested ANOVA was performed to determine the variance of samples within a site. Growth potentials (differences in plate counts after 24-h incubation) were also compared by ANOVA. The main effects of site and treatment were tested. Post hoc comparisons of treatment effects were made with Tukey tests. Analyses were performed with Systat version 5.2 software (Systat Inc., Evanston, Ill.).

RESULTS

Cell enumeration. Total microscopic counts and plate counts were generally low. The average counts ranged from below the limit of detection (3.2×10^4 cells g^{-1}) to 3.2×10^5 cells g^{-1} (dry weight) (Fig. 2). The CFU g^{-1} (dry weight) of crushed rock ranged from the limit of detection, 10 CFU g^{-1}, to 10^3 CFU g^{-1} (Fig. 2). Samples from 825 and 1,023 m had the highest CFU g^{-1}. These two locations were in less consolidated, more porous bedded tuff. Samples from the harder and more consolidated welded tuff in the Tiva Canyon and Topopah Spring units showed the lowest CFU g^{-1} (dry weight). One exception was at 2,505 m, with 630 CFU g^{-1} (dry weight). The numbers of organisms cultured on R2A agar were orders of magnitude less than the total number of cells found at the sites. Nearly all colonies on R2A agar appeared to be bacterial; fungi were not evident. Negative combusted controls typically showed no CFU g^{-1}. The samples from 1,801 m and 2,105 m spiked with *P. fluorescens* had 2.0×10^5 and 1.8×10^6 CFU g^{-1}, respectively.

PLFAs and DGFAs. PLFA concentrations (a measure of microbial biomass) were generally low, ranging from 0.1 to 3.7 pmol g^{-1} (Fig. 3). This corresponds to approximate cell abundances of 5.9×10^3 to nearly 5.9×10^5 cells g^{-1} (assuming approximately 5.9×10^4 cells pmol of PLFA^{-1}) (25). DGFAs (a measure of dead cells) showed the same general range of values (0.2 to 2.3 pmol g^{-1}).

Potential for microbial growth. The effects of site and treatment on increases in culturable cells during a 24-h incubation of rock were both highly significant ($P < 0.0005$ for each factor). Incubation of crushed tuff samples under ambient moisture (dry) conditions increased the number of CFU g^{-1} in four of eight cases; incubation of tuff in slurries (H_2O) increased the average number of CFU g^{-1} in all cases (Fig. 4). Increases in culture counts during 24-h incubation of slurries ranged from 3×10^4 to 2×10^8 CFU g^{-1}, depending on the site of collection and on the slurry conditions. Amendments of the slurries with N or P did not result in significantly greater increases in culturable cells compared to the water treatment alone ($P < 0.0005$). Organic C amendments significantly increased cultured populations compared to P solution amendments ($P = 0.011$) and N solution amendments ($P = 0.047$).

Radiorespirometry. Glucose mineralization (Fig. 5A) ranged from 10 to 45% of added substrate. Overall, glucose mineralization was significantly greater in microcosms with added water than in ambient microcosms ($P < 0.001$). However, moisture amendment had no significant effect in samples from the Tiva Canyon welded unit ($P = 0.362$). The elevation in activity by moisture amendment was most pronounced in the less consolidated material of the Upper Paintbrush nonwelded unit (825 and 1,023 m) and at the Bow Ridge Fault (200 m). The lowest activities were seen in formations with the more consolidated welded tuff, i.e., in the Tiva Canyon welded unit (537 m) and the upper and lower Topopah Spring welded units (1,194 m and beyond). Amendment of glucose microcosms with nitrogen and phosphorus did not stimulate activities ($P > 0.95$). Formaldehyde-poisoned controls showed minimal glucose mineralization (<2% in 3 weeks). Addition of 100-fold-greater concentrations of unlabeled glucose (116 mM) to microcosms along with standard concentrations of radiolabeled glucose (1.28 mM) yielded no significant stimulation of activity ($P > 0.5$) (data not shown). Negative controls with combusted tuff showed <1% mineralization of radiolabeled glucose.

Glutamate mineralization data approximated those of glucose (Fig. 5B). Percent mineralization ranged from 10 to 60%. Moisture amendment generally stimulated activity ($P < 0.001$), except in the Tiva Canyon welded unit ($P = 0.573$). As with glucose, glutamate mineralization activities were highest in the Upper Paintbrush nonwelded unit (825 and 1,023 m) and at the Bow Ridge Fault (200 m). The lowest activities were seen in the Tiva Canyon welded unit (537 m) and the Topopah Spring welded units (1,194 m and beyond). The samples from 1,194 and 1,425 m showed an increase in glutamate mineralization with added nitrogen and phosphorus ($P < 0.018$), indicating possible inorganic nutrient limitation at that location. Poisoned controls showed little mineralization (mean, <3%).

Percent mineralization of ^{14}C-labeled acetate was generally higher than that for glucose or glutamate (Fig. 5C). Samples from sites containing welded tuff showed generally high rates of mineralization, with the exception of the site at 2,105 m. Overall, water was the major factor limiting acetate mineral-

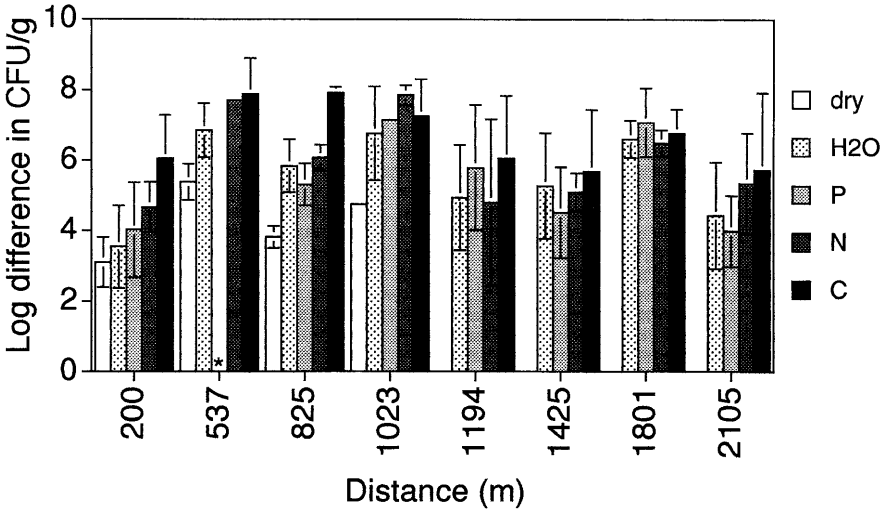

FIG. 4. Log differences in plate counts (log CFU g^{-1}) performed before and after 24-h incubation of Yucca Mountain tuff samples as crushed dry rock (dry) or as crushed rock slurries. Slurries were made with water alone (H_2O), with a 10 mM potassium phosphate solution (P), with a 12.5 mM ammonium nitrate solution (N), or with 3.0 mM (each) sodium pyruvate, sodium acetate, and glucose (C). The bars show means ± SEs. *, no data.

ization activity ($P < 0.001$), while nitrogen and phosphorus were not limiting ($P = 1.0$).

Correlation coefficients for percent mineralization of the three substrates under moisture-amended conditions were positive and significant (r, 0.707 to 0.962; P, 0 to 0.033). ANOVA showed that all two-way interactions were significant: conditions by substrate ($P < 0.001$), conditions by distance ($P < 0.001$), and substrate by distance ($P < 0.05$). Higher-order interactions were folded into the error term. Variances of the four noncontrol samples within each site were significant ($P < 0.001$).

DISCUSSION

Plate count, direct count, and PLFA data indicate a low abundance of microorganisms in Yucca Mountain tuff. The low numbers of culturable aerobic heterotrophs in Yucca Mountain samples is consistent with previous findings for unsaturated tuff (14, 15, 19, 20). Enrichments for anaerobes carried out by other investigators on the same samples were negative (32). The finding of evidence for dead cells (DGFAs) in nearly as high amounts as live cells (PLFAs) is not surprising in a deep subsurface environment where cells may have undergone long-term sequestration and nutrient deprivation.

The distribution of microbial abundance and activities within the ESF shows consistent patterns. In general, samples from faults or samples from less consolidated, nonwelded, or bedded tuffs (e.g., Bow Ridge Fault or the Upper Paintbrush nonwelded unit, respectively) had greater microbial numbers and activities than those from harder, more consolidated welded tuffs (Tiva Canyon and Topopah Spring welded units). From past hydrologic studies (1) it is known that the Upper Paintbrush nonwelded unit has the highest porosity, the highest water flow rate, and, from our studies, the largest moisture content as a percentage of weight. The Upper Paintbrush nonwelded unit also had the highest CFU g^{-1} (dry weight) and the highest rates of mineralization of any site tested at Yucca Mountain.

Although populations of microorganisms in the Yucca Mountain tuff are small, they showed a high potential for metabolic activity and growth in laboratory microcosms. Incubation of crushed tuff for only 1 day resulted in significant increases in populations of organisms that could grow on R2A agar. In some cases, such an increase was evident even without the addition of moisture. Such increases have been observed in other cases in which subsurface material, including volcanic tuff, has been stored in the laboratory (12, 16, 17). It is impossible to determine from our experiments whether increases in culture counts are due primarily to growth or to a change in the physiological status of existing cells such that they can be cultured on R2A agar. In either case, a tremendous potential for increase in microbial activity is evident.

Water appears to be the primary factor limiting microbial growth and activity in the unsaturated volcanic tuff. The addition of water alone resulted in highly significant increases in culturable counts and increases in ^{14}C-labeled substrate mineralization compared to non-water-amended treatments. The addition of N or P solutions did not result in significantly greater culturability or mineralization activities when compared with the addition of water alone. This suggests that N and P are present in significant quantities in situ. However, given sufficient time or organic C amendments, inorganic nutrients could become limiting. The significance of water as the major factor limiting microbial activity at Yucca Mountain was demonstrated by large differences in mineralization between ambient and moist conditions. However, ambient microcosm conditions may not reflect actual in situ conditions at Yucca Mountain because the rock is desiccated by the venting of the ESF tunnel. Tunnel air and dust are replaced by outside air at an approximate rate of one tunnel volume every 15 to 20 min. Additional desiccation occurred during rock crushing under the flow of HEPA-filtered air.

Microcosm experiments typically yield rates of microbial activity that are orders of magnitude higher than in situ rates (7, 31); this is undoubtedly the case for our experiments as well. However, microcosm experiments are useful as tests for potential microbial activity and to determine conditions that stimulate activity. In the case of the proposed repository, moisture inputs would likely increase microbial activity; addition of organic carbon along with water would likely further stimulate activity.

The origins of the bacteria in the volcanic tuff of Yucca

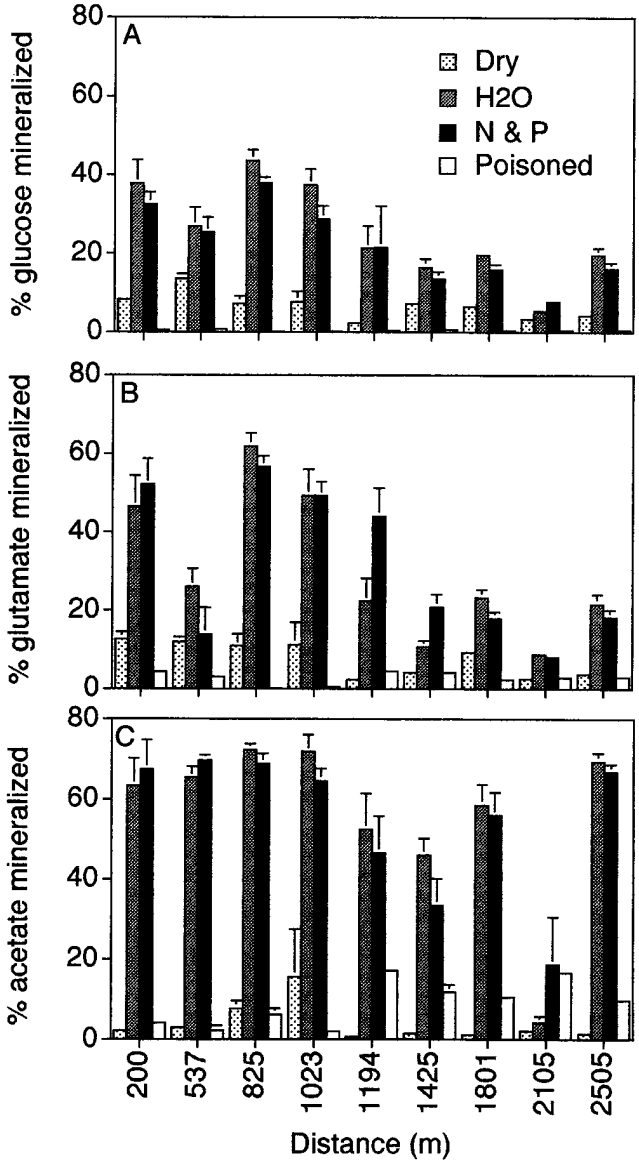

FIG. 5. Percent mineralization of ^{14}C-labeled organic substrates to $^{14}CO_2$ during 3 weeks of incubation of crushed Yucca Mountain tuff in microcosms. Microcosms consisted of tuff at ambient moisture (Dry), tuff moistened to field capacity with water alone (H_2O), tuff moistened with a solution of 0.1 mM NH_4Cl and 0.05 mM K_2HPO_4 (N & P), and a poisoned control sample moistened with 3.7% formaldehyde (Poisoned). The labeled substrates were glucose (A), glutamate (B), and acetate (C). The bars show means ± SEs.

Mountain are uncertain. Since temperatures in molten ashflows can reach 500 to 1,000°C (10), colonization must have occurred after tuff deposition 9 to 13 million years ago. Transport of microbes into the rock presumably occurred in a water phase, either as diffusion through thin water films under unsaturated conditions or by advection. Porewater ages in the more welded tuffs at Yucca Mountain are estimated to be between 5×10^5 and 7×10^5 years (9); thus, indigenous bacteria probably arrived at least that long ago. Microorganisms recovered from Yucca Mountain may have been introduced during tunnel construction; however, evidence suggests that most are indigenous to the formations. The absence of LiBr and fluorescent microbead tracers in our samples indicates that sampling was aseptic. As further evidence for these microorganisms being indigenous, we cultured very few fungi, even though the air circulated through the ESF could be expected to have many fungal spores. Fungi are seldom found in subsurface samples (6) and so serve as serendipitous tracers. When samples were taken from a tunnel site without prior excavation, we found significant growth of fungi in our culture plates; these were likely introduced after drilling. It is also unlikely that rock processing in the laboratory introduced contaminants. Our negative control (combusted tuff that was crushed by our standard procedure) showed <1% mineralization of radiolabeled glucose and plate counts that were below detection.

The indigenous rock bacteria quantified in these experiments confirm earlier findings of bacteria in volcanic tuff (14, 15, 19, 20, 33) and are also of importance in considering the far-field transport of radionuclides. Our experiments demonstrated that the Yucca Mountain ESF environment can be conducive to the growth and activity of microorganisms.

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