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Potential for Microbial Growth in Arid Subsurface Sediments†

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ABSTRACT

To determine if bacterial populations could be increased for bioremediation of contaminants, the microbial growth arising in 200 h from the solid material from the vadose zone at an arid site after addition of water and nutrients was determined. Initial bacterial populations and activities at the site were very low. Bacteria grew in the interbed sands and silts with the higher carbon contents (0.08–1.1%), but did not respond in the basalts and interbed material with lower carbon content (undetectable). In longer term studies, to 30 d, there was evidence for bacterial activity in less than 25% of either the basalts or interbed material.

Index Entries: Arid; bacteria; bioremediation; paleosol; particulate organic carbon.

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INTRODUCTION

The Department of Energy is faced with remediation of organic contaminants at many arid sites in the western region of the United States (e.g., the Idaho National Engineering Laboratory [INEL] and the Pacific Northwest Laboratory [PNL] Hanford site in the state of Washington). There are numerous organic contaminants in the subsurface at these sites that could possibly be biodegraded.

Compared to more humid regions, the bacterial populations in the subsurface at many arid sites are quite sparse. There is a host of studies from humid sites showing relatively large populations of active bacteria at depth in the subsurface (e.g., *1–4*). At many arid sites much of the contamination is located within the deep vadose zones. The low numbers and activities of bacteria in the vadose zone at arid sites (*5,6*) raises questions related to bioremediation of contaminants at these sites.

In situ bioremediation at arid sites will require both the presence of bacteria that can degrade the contaminant and the knowledge of limiting factors necessary to stimulate activity of the bacterial population (e.g., addition of water or water and nutrients). Although bacteria that can degrade organic contaminants have been isolated from subsurface environments at arid sites (*7,8*), there is limited information on the potential for the bacteria present in these deep vadose zones to respond to attempts to stimulate their *in situ* degradative activity. To a large degree, it has not yet been determined what limits bacterial growth in the deep subsurface. It is possible that either inorganic nutrients or organic carbon may be limiting bacterial growth, and the specific limiting factors will probably change with site, depth, and so on. In some subsurface systems particulate organic carbon (POC) has been shown to influence bacterial growth (*9*).

The purpose of this research was to determine the potential for stimulating the bacterial activity, growth, and organic carbon incorporation and mineralization in the arid deep vadose zone by simple addition of water or water plus nutrients. Numbers and activities of bacteria in the vadose zone were determined and sediment characteristics measured. After assaying the initial population and activity, the ability of organic carbon in the deep subsurface to support bacterial growth (with supplements of water or water plus nutrients) was examined.

METHODS

Sites and Samples

The primary study site was at INEL, 68 km from Idaho Falls, ID, atop the basalt flows of the Eastern Snake River Plain (*10*). The vadose zone at this site consists primarily of large basalt flows with limited sedimentary

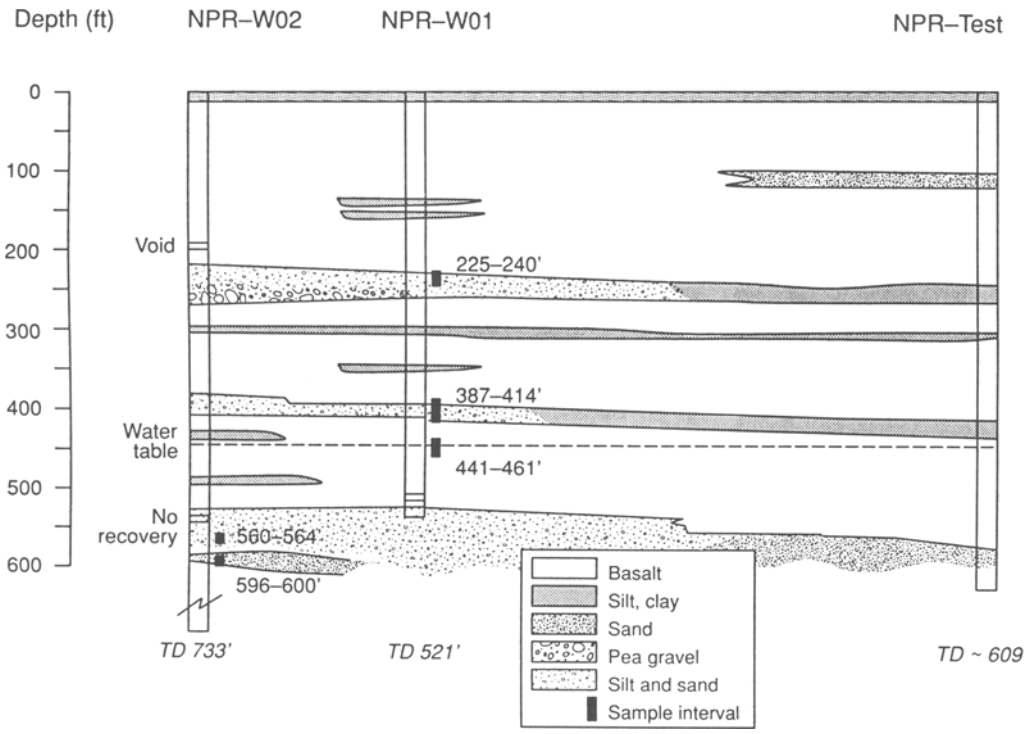

Fig. 1. Stratigraphic section of the drilling location at INEL.

interbeds (Fig. 1). Details of the site stratigraphy, drilling techniques, sample handling, and sample processing are given elsewhere (*10*).

Cores were obtained from two wells using techniques that minimized cross-contamination. Argon was used as a coring fluid to depths of 560 ft. Of the samples used for this study, one was obtained from the unconsolidated surface sediment, approximately half were obtained from basalts and half from interbeds. Samples were processed at the site and assigned a sample number based on the depth, in feet, from which the sample was obtained. For consistency all depths are given in feet below the surface. Because of sample and time limitations many analyses were made on only a subset of the samples and the numbers and types of samples used are given for each of the specific measurements (*see below*). All subsurface samples were obtained from the deep vadose zone (below 200 ft). One basalt sample was obtained from below the water table. A sample that had been spiked with a bacterial culture was shipped to us as a blind positive control. Two surface samples and a spiked sample obtained from a second arid site at PNL were used in some of the comparisons.

Sediment Characteristics

Total particulate carbon in the sediment was measured prior to the incubations, and the leached dissolved organic carbon (DOC) in the water was measured after the incubation. A Carlo Erba Model NA1500 CNS analyzer was used for measurements of particulate carbon, nitrogen, and sulfur. DOC was measured using an OI Corporation total organic carbon analyzer.

Initial Microbial Biomass and Activity in Sediments

Colony forming units (CFU) were measured and used as an index of the initial presence of bacteria in the subsurface material. Serial dilutions with duplicate counts at each dilution were used for measurements of CFU. The bacteria were grown on a dilute medium (*11*) with 10 mg/L each of peptone, trypticase, yeast extract, and glucose with Noble Agar and trace minerals (including selenium and molybdate), dilute vitamin mix, bicarbonate (10 m*M*), and phosphate buffer (2 m*M*). All solutions and additions were made up with Milliq.

The initial microbial activity was assessed in several types of incubations using radiolabeled organic compounds. Measurement included acetate incorporation into lipids, acetate mineralization, and glucose mineralization. Incubations of the sediment sample (2.0 g and 1 mL of sterile distilled water) were made within 72 h of core acquisition. All incubations were made aerobically in the dark at ambient laboratory temperatures which were close to the *in situ* temperatures of 21–27°C.

Acetate incorporation into lipids was measured using 5.0 μCi ($\approx 1.1 \times 10^7$ DPM) of 1,2,3-^{3}H-acetate (sodium salt) (New England Nuclear, Boston, MA), at a specific activity of 3.3 Ci/mmol, added to sediment aliquots. Samples were taken over time (0, 2 h, 8 h, 1 d, 3 d, and 10 d), inhibited with 3.0 mL of a phosphate-buffered chloroform-methanol solution, and frozen at −20°C for later extraction by a modified (*12*) chloroform-methanol method (*13*) and analysis (*14*). Initial rates were calculated based on earliest linear time points and appropriate controls were used to ensure that only acetate incorporated into lipids, not acetate bound to soil, was measured. Later time points were used in assessing the potential for stimulation of activity with water addition (*see next section*).

Glucose mineralization and acetate mineralization were assessed using 2.0 μCi ($\approx 4.44 \times 10^6$ DPM) of 1-^{14}C-acetate at a specific activity of 56 mCl/mmol^{-1} or U-^{14}C-glucose at a specific activity of 2.8 mCl/mmol (Amersham Corp., Braunschweig, Germany) added to sediment aliquots and incubated from 0 h to 30 d in 25-mL crimp-top tubes. At designated time points tubes were inhibited with 0.5 mL of 2.0*M* sodium hydroxide, and were acidified with 0.5*M* HCl 1 h prior to analysis. A GC-gas proportional counter (Packard 417) was used to quantify $^{14}CO_2$ production, and rates were cal-

culated based on initial slopes of $^{14}CO_2$ evolution (*15*). As with acetate incorporation, later time points were used in assessing the potential for stimulation of activity with water addition (*see below*).

Response to Addition of Water and Water with Nutrients

Two methods were used to assess the potential for stimulation of the bacterial population. Stimulation by addition of water was tested in long-term activity experiments (to 30 d). Stimulation by addition of water with inorganic nutrients was tested in short-term growth experiments (to 200 h). The effect of addition of water was assessed using the longer term time points from the glucose mineralization, the acetate mineralization, and the acetate incorporation assays (*see above*). The effect of water and nutrient addition was assessed by measuring changes in bacterial numbers in sediment incubations in water containing nutrients.

Changes in rates from initial rates in the linear region in activity measurements to higher rates in incubations up to 30 d are noted as indicative of the potential for increased rates of activity. A change in slope of greater than three times is used as an indication of a significant change in activity owing to the water addition.

The potential for stimulation of the microbial population and the factors controlling bacterial populations in sediments at this site were examined. Growth of bacteria after additions of water and mineral nutrients (including nitrogen, phosphorous, and trace elements) to the solid material was used to determine the ability of indigenous bacteria to grow on the carbon present. Changes in numbers of bacteria in short-term incubations with water and nutrient addition were followed. Portions (1.5 cm^3) of samples from different depths at the site were incubated for up to 200 h in the mineral salt medium. The microcolon enumeration method (*16,17*) was used to estimate changes in microbial populations during incubations. Bacteria were grown and enumerated on nutrient agar (Difco, Detroit, MI). In some samples no growth was observed during the incubation period. A nutrient and trace element mixture (*18*) was used for all nutrient additions. The samples to which water and nutrients were added were mixed gently on an orbital shaker.

Statistical Analysis

Calculations of means and standard deviations use the limit of detection for all values below the limit. In analysis of CFU per gram of sediment, initial rates of glucose mineralization and initial rates of acetate incorporation there were five samples from basalts and six from interbeds. There were three surface samples used for comparisons and two spiked samples except for acetate incorporation for which there was only one spiked sample.

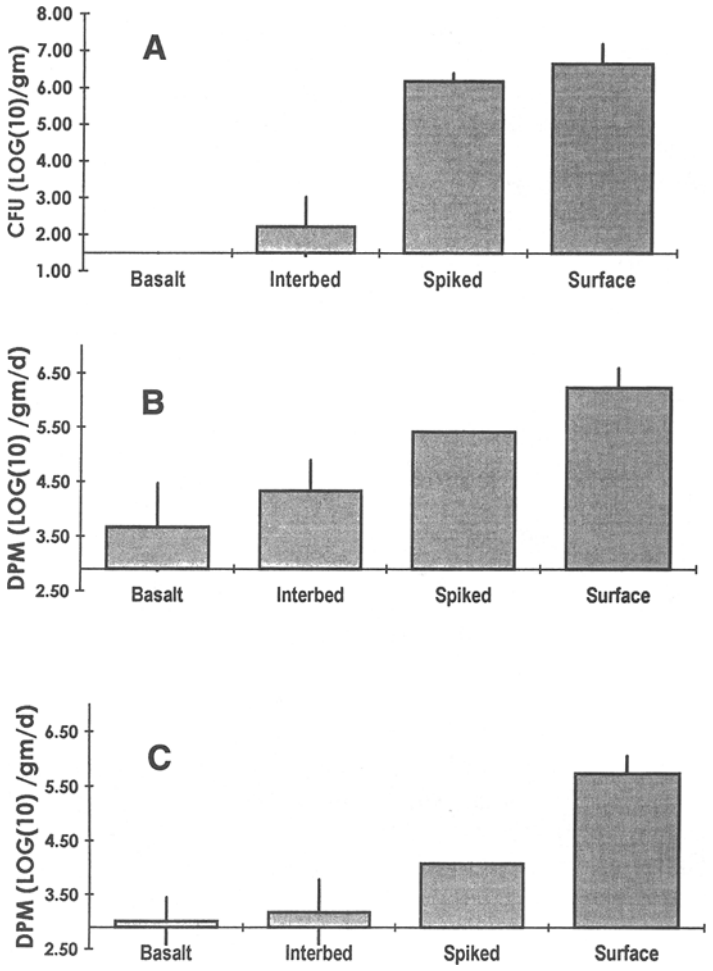

Fig. 2. Mean (±1 SD) of microbiological characteristics for basalts and interbed material from INEL, and spiked and surface samples from INEL and PNL. The horizontal axis is plotted at the limit of detection. **A**. CFU per gram of sediment (all basalt samples were below the limit of detection). **B**. Initial rates of glucose mineralization. **C**. Initial rates of acetate incorporation.

RESULTS

Initial Microbial Characterization of Sediments

The microbial biomass in the basalts and interbeds was very low compared to the surface samples and the spiked samples (Fig. 2A). CFUs were undetectable in all five of the deep vadose zone basalt samples. Even among the six interbed samples, CFUs were not detected in three of the samples. Among the interbed samples that contained detectable bacteria the mean concentration was 7.21×10^2 CFU/g, many orders of magnitude below the concentrations in the surface samples (7.57×10^6 CFU/g).

Table 1
Depth of Sample, Organic Carbon in Leachate Before and After 200 h Incubation, Carbon, Nitrogen, and Sulfur Content in Sediment Samples and Type of Sample[a]

Depth, ft	DOC, before	DOC, after	Carbon, %	Nitrogen, %	Sulfur, %	Type
surface	2.295	NA	1.110	0.023	0.010	ss
240	0.723	0.744	0.091	0.000	0.000	ib
394	0.745	1.446	0.000	0.000	0.000	b
396	0.731	1.248	0.000	0.000	0.000	ib
396.6	0.616	1.305	0.006	0.000	0.000	ib
401	0.657	1.0472	0.021	0.000	0.000	ib
560	0.600	0.803	0.084	0.000	0.000	ib
spiked	1.000	NA	0.000	0.000	0.000	b

[a] Sediment types included basalts (b), interbed material (ib), and a surface soil (ss).

The pattern among the types of samples for glucose mineralization (Fig. 2B) and acetate incorporation (Fig. 2C) was similar to that observed for the CFU data. The lowest activity levels were observed in the samples from the basalts. There were marginally higher levels for acetate incorporation in the interbed samples and a larger difference in glucose mineralization levels between the basalts and the interbed material. Surface samples were again more than 100 times higher for both acetate incorporation and glucose mineralization.

Sediment Characteristics

There were relatively subtle differences in sediment particulate carbon and leachate DOC between the basalts and the interbed material. The basalts did tend to have lower carbon content than did the interbeds, but all samples had extremely low carbon content (Table 1). Nitrogen and sulfur were detected only in the surface sample (Table 1). Also, there was little relationship between particulate organic carbon or the amount of leachate DOC and the depth of the sample (Fig. 3). The spiked sample was enriched in DOC compared to the other vadose zone sediment samples. The highest leachate DOC was seen in the surface sample. In all cases, additional DOC was leached from the sediments during the 200 h incubations. There were consistently higher levels of DOC at the conclusion of these experiments than was present at their initiation (Table 1).

Response to Addition of Water and Water with Nutrients

The addition of water to the basalt and interbed samples resulted in long-term stimulation (greater than three times the initial rate of activity) of glucose mineralization, acetate incorporation, or acetate mineralization in few of the assays. Of the five basalt samples only one of the samples

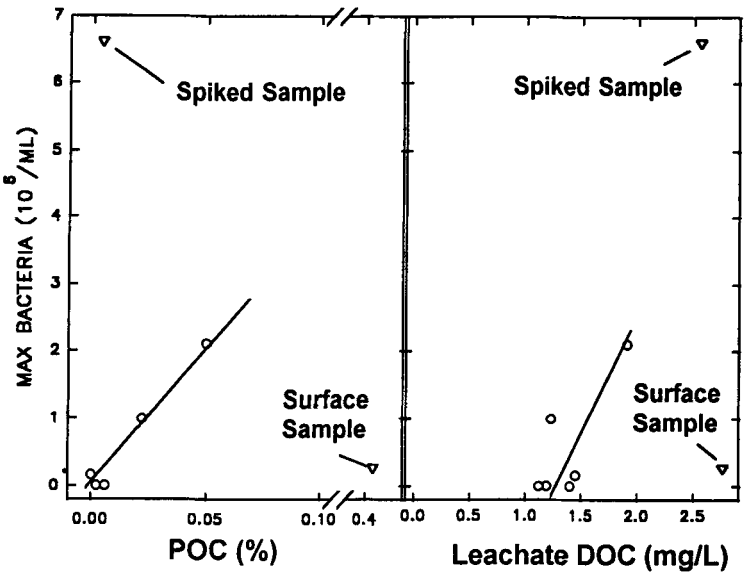

Fig. 3. Depth vs sediment particulate organic carbon and amount of DOC leached during short-term incubations.

(228 ft) showed activity stimulation. Of the 14 activity assays done on the basalts, only three showed stimulation. Similar proportions apply to the interbed sediments. Of the seven interbed sediments, three of the samples (231, 443, and 560 ft) showed an activity stimulation. Again, of the 14 activity assays done on the interbeds only three showed stimulation. Thus, in these long-term experiments addition of water alone resulted in long-term stimulation in fewer than 25% of the assays.

The addition of water plus nutrients resulted in detectable growth (to a maximum of 2.1×10^6 cells/mL in the unspiked vadose zone samples) in four of the seven sediment samples assayed including both the spiked and the surface samples. The amount of growth in the unspiked vadose zone sediments was not correlated with depth of the sample ($r^2 = 0.168$).

The maximum numbers of bacteria resulting from the short-term incubations (Fig. 4) were better correlated to the carbon content of the samples ($r^2 = 0.97$) than to the amount of DOC in the leachate from the incubations ($r^2 = 0.61$). The highest carbon content (0.08–1.1%) in the solid material was in the four samples that exhibited growth. No growth was evident in the three samples having the lowest carbon content (0.063% to undetectable).

DISCUSSION

The CFU numbers and the pattern of large differences in CFU between surface and subsurface samples are similar to those seen in the few published studies of bacteria in these arid vadose zone sediments (*5,6*). Inter-

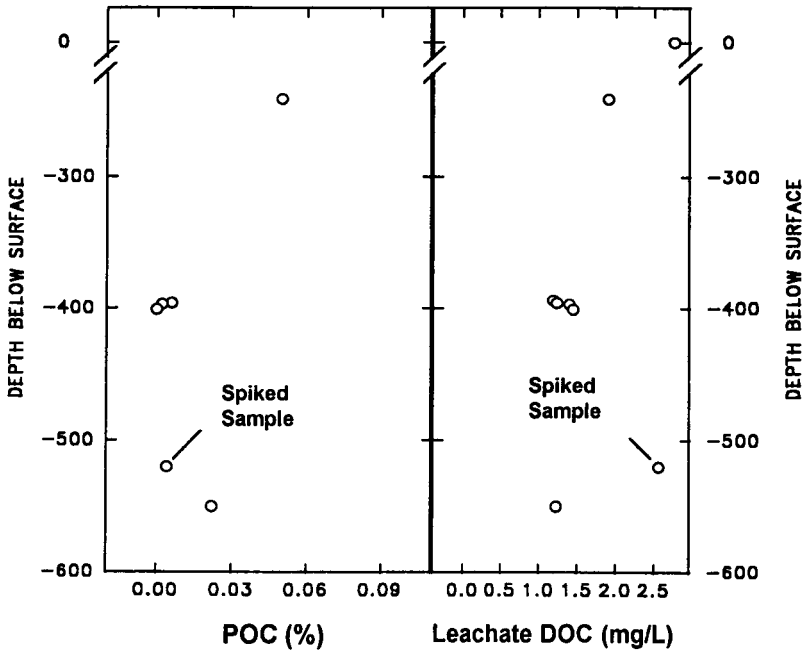

Fig. 4. Maximum number of bacteria observed in short-term incubations vs sediment particulate organic carbon and amount of DOC leached during short-term incubations.

beds, some of which have been interpreted to be paleosols, appear to have higher initial numbers of bacteria and higher initial rates of activity than do the basalts. However, levels of activity in both are quite low when compared to samples from more humid regions (e.g., *1,2,19*). The spiked sample was clearly identifiable by the higher DOC content in the leachate and the high bacterial number in the suspensions.

Colwell (*5*) found that surface sediments with a particulate organic carbon content of 0.26% had greater than 10^5 CFU/g and subsurface samples, with organic carbon contents of 0.05–0.17%, had less than 10^2 CFU/g with no CFU detected in one sample. The very low POC levels Colwell reported were similar to samples we analyzed in that all had total carbon levels below 0.06%. Brockman et al. (*6*) found similar CFU levels (1.59–2.81 log CFU/g) in paeosols processed immediately after collection from the Hanford site in Washington. In Colwell's study CFU decreased with depth, but there were even fewer samples (three) than in our study. Our results show that the influence of depth once below the surface soil is not as great as the influence of the POC content. In our study, subsurface samples were significantly lower than surface samples in CFU, as in Colwell's, and the maximum number of bacteria observed during short-term experiments was higher at the surface. However, once into the subsurface there was not a continuous decrease in the growth potential, but rather a dependence on the POC content.

POC levels were correlated with the number of bacteria resulting from growth on the sedimentary material after addition of water plus nutrients. Neither the carbon content or the amount of leached DOC strongly correlated to the depth of the sample. In more humid environments, POC is thought to be an important controlling factor in microbial biomass and activity. McMahon and Chapelle (*9*) have postulated that in South Carolina coastal sediments, anaerobic fermentation in low-permeability formations (e.g., clay-rich horizons) converts POC to low-molecular-weight organic acids such as formate and acetate. Levels of DOC in the more transmissive formations remain low because of the slow rate of diffusion of the organic acids out of the clay into the aquifer, and respiration of these simple acids in more oxygenated zones (*9*). In these well-oxygenated low-organic-carbon arid environments it is not likely that anaerobic processes play a significant role. However, the potential importance of the subsurface carbon is evident from our experiments.

Surprisingly, the amount of DOC in the leachate from the incubations did not show as high a correlation with the maximum number of bacteria resulting from growth in the sediment suspensions as did the POC content. There may be sample-to-sample differences in the bioavailability of the carbon. This would result in a buildup of refractory organic carbon, giving higher final DOC concentrations in some samples. There are differences in bioavailability of DOC in different fractions of surface water (*20,21*) and there have been some studies of the bioavailability of carbon in groundwater (*17,22*). Because of the small volumes of material used in our experiments and the low DOC concentrations, it was not possible to fractionate the leachate water to determine if there were differences in the type of DOC among the leachates.

Both the long-term activity assays, where only water was added, and the short-term growth assays, where both nutrients and water were added, show the potential for stimulation of the bacterial populations. In the long-term assays, increases in activity with water addition do not indicate differences between basalt and interbed material. Only the upper-most basalt sample (228 ft) and the interbed sand sample from below the water table (560 ft) exhibited a stimulation. Thus, none of the basalts in the deep vadose zone exhibited a stimulation with the addition of water. Within the deep vadose zone only 2 of 10 samples (basalt and interbed) showed a stimulation by the water experiment. There appeared to be better success in stimulating the populations in the short-term experiments with the addition of water plus nutrients. Four of the seven samples tested showed potential for stimulation of growth with addition of nutrients and water. Accordingly, there is potential for manipulations that are designed to increase growth and rate of activity to succeed, particularly if carbon sources (such as organic contaminants) are present. In our experiments, after addition of water plus nutrients, carbon may have been limiting. Consequently at sites where carbon is present as an organic contaminant,

addition of water and nutrients may serve to significantly stimulate the population.

A cautionary note is the lack of growth in some low carbon samples. Evidently populations were so sparse in these samples that our 200-h experiments were not long enough to stimulate population growth to the extent that any bacteria could be detected. However, at sites affected by organic contamination, bacteria may be transported to the site by the same mechanisms as the contamination. These bacteria could serve as a source of degradative populations.

There is evidence for the presence of degradative bacteria in these arid sediments. Isolations from subsurface sediments at INEL and Hanford have yielded bacteria that can degrade several contaminants (*7,8*). Isolates from this site at INEL have been screened for their ability to degrade toluene and xylene. Several isolates from this site that are capable of oxidizing and/or growing on these substrates have been identified (*7*).

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