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Influence of Media on Measurement of Bacterial Populations in the Subsurface

Numbers and Diversity

ANTHONY V. PALUMBO,* CHUANLUN ZHANG, SHI LIU, SHIRLEY P. SCARBOROUGH, SUSAN M. PFIFFNER, AND TOMMY J. PHELPS

*Environmental Sciences Division, Oak Ridge National Laboratory,** Oak Ridge, TN 37831-6038*

ABSTRACT

To examine the factors contributing to microbial heterogeneity, the US Department of Energy Subsurface Science Program has initiated research at a Coastal Plain site near Oyster, VA. Sediments at the site are composed of unconsolidated, fine to coarse beach sands and gravel. The influence of microbiological culture media on the measurement of colony-forming units (CFU) and microbial diversity was examined in this study. Two low-nutrient media formulations (i.e., dilute peptone, tryptone, yeast extract, and glucose medium [PTYG] and a soil extract medium) gave equivalent estimates of CFU (maximum of about 6.5×10^5 CFU/g). However, a higher nutrient medium, organic nutrients with brain-heart infusion (N/BHI), gave lower numbers than the dilute PTYG. A medium used to enumerate fungi also gave significantly lower counts than the dilute PTYG medium. Although the correlations were highly significant among total CFU numbers measured with the different types of media, we found few significant correlations in several indices of microbial diversity, and the correlation coefficients were below 0.25. Thus, CFU was a relatively robust measurement, but microbial diversity was not consistent between the media. Species richness was highest with the higher nutrient N/BHI medium, and N/BHI may be a better choice for a study of diversity as determined by colony morphology.

Index Entries: Diversity; CFU; bioremediation; media, subsurface; microbiology; fungi.

*Author to whom all correspondence and reprint requests should be addressed.

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INTRODUCTION

Heterogeneity in subsurface environments may play an important role in the fate of bioremediation activities, but the extent and effects of physical, chemical, or biological heterogeneity in subsurface sediments are largely unknown. We have been examining the distribution and diversity of bacteria in a shallow subsurface site. Examining heterogeneity requires the analysis of large numbers of samples. Thus, relatively simple methods for analysis of diversity are required if diversity is to be examined spatially and temporally. Although other methods for measuring microbial diversity are being developed, none are yet as amenable to analysis of numerous samples as are distinctions based colony morphology.

The classic method of determining the structure, and thus the diversity, of a community is to count the organisms in environmental samples. These data can be used to determine properties of the community, such as species richness and diversity. This approach works well for most organisms and has been used successfully for microorganisms by examining the morphology of colony-forming units CCFU (e.g., *1,2*). As pointed out by Atlas, there are problems estimating microbial diversity based solely on colony morphology *(3,4)*. Nevertheless, diversity indices based on colony morphology units are relatively simple measurements and yield information on the diversity of the microbial community.

Microbial diversity and biomass have been examined in many subsurface environments, including shallow aquifers *(5)*, deep coastal sediments *(6)*, and deep arid sites *(7)*, using measurements of CFU and colony morphology. Several different media types have been used in these studies, and low-nutrient media are often selected to maximize bacterial counts. However, use of low-nutrient media could limit the ability to distinguish among colony types (because of reduced pigmentation) *(8)*—potentially reducing the measured diversity. One common low-nutrient medium is a dilute version of peptone, tryptone, yeast extract, and glucose (PTYG) used in many US Department of Energy (DOE) Subsurface Science Program (SSP) studies (e.g., *6,9*). Although several studies have shown that PTYG generally gives as high or higher total CFU as do other types of media, few studies have compared the effect of media composition on observed diversity of colony morphology.

In this study, we composed types of media for assessing microbial numbers and diversity in a shallow coastal aquifer. For comparison with the PTYG formulation used in many DOE-sponsored SSP investigations, we chose a richer medium containing organic nutrients with brain-heart infusion (N/BHI) *(10)* and a soil extract medium *(8)* made using soil from the surface of the site.

METHODS

Media

The dilute PTYG medium *(11)* was prepared by adding glucose (0.1 g), yeast extract (0.1 g), peptone (0.05 g), tryptone (0.05 g), magnesium sulfate 7-hydrate (0.60 g), calcium chloride dehydrate (0.07 g), and Bacto-agar (17 g) to 1 L of filtered distilled water and autoclaving for 30 min. The dilute PTYG medium, most of which was poured into plates, was prepared at Oak Ridge National Laboratory (ORNL). Additional medium (several liters) was sterilized in 1-L bottles and transported to the site. Plates were poured on site from this stored medium after the agar was melted in a microwave oven.

The soil extract medium was made using a modification of a published procedure *(8)*. A 1:2 suspension of surface soil from the sampling site in distilled water was autoclaved for 2 h, after which the liquid extract was clarified by centrifugation and filtration. The final medium was prepared by adding 20% of the liquid extract to 80% distilled water and together 15 g of purified agar/L. The medium was autoclaved on-site, and plates were poured in a laminar flow hood in the mobile laboratory.

The N/BHI medium was prepared by combining nutrient agar (23 g) with brain heart infusion broth *(10)* (5.0 g/L) and glucose (1.0 g). One liter of the final medium contained peptone (6.3 g), beef extract (3.0 g), glucose (1.3 g), sodium chloride (0.7 g), disodium phosphate (0.3 g), and infusions from calf brains (27 g) and beef heart (34 g).

Low-pH (4.8) Bacto-mycological medium *(10)* was prepared using Bacto-soytone (10 g) plus Bacto-dextrose (10 g) and Bacto-agar (15 g)/L of medium.

Site

All samples were obtained from the proposed site for the DOE SSP heterogeneity and bacterial transport field studies. The site (Fig. 1) near Oyster, VA., was chosen for heterogeneity and proposed bacterial transport experiments. The site is owned by the Nature Conservancy, and is operated and maintained by investigators from the University of Virginia. The soil is a Typic Hapludult (i.e., coarse loam) and is underlaid by sediments of the Coastal Plain physiographic province. The transport experiments will take place in the Butler's Bluff Member of the Nassawadox Formation; many samples used in this study were from that formation. The formation consists of unconsolidated, fine to coarse beach sands and gravels that are generally clean and well sorted *(12)*.

Sampling

Using techniques developed as part of the DOE SSP, we obtained core samples from steam-cleaned Lexan core liners with split spoon coring tools and a hollow-stem auger system *(13)*. To ensure that retrieved materials were representative of the subsurface and were not compromised by surface soils, sidewall slough, or drilling activities, microsphere tracer was used for quality control during collection of most of the cores. Following removal from the split spoon, the Lexan liners containing the core were cut into sections with a flame-sterilized hacksaw. A fresh face of the sectioned core was pared away with a flame-sterilized spatula. After paring, the spatula was resterilized and used to collect the centermost section of the core for bacterial enumeration and diversity determination. Ten to 150 g of sediment material were placed into sterile Whirl-pak bags and transported on ice to the mobile laboratory. Within the mobile laboratory, samples were processed in a laminar flow high-efficiency particulate air-filtration unit.

Seventy nine samples were obtained from 10 wells (Fig. 1) for this media comparison in August of 1994. Three to five cores were obtained from each well: D2 (five), D3 (four), E1 (five), E2 (five), E3 (four), F1 (four), F2 (three), F3 (three), T1 (five), and T2 (four). From each core, one to five samples were obtained for microbiological investigations.

Viable Cell Counts and Diversity

We estimated numbers of viable bacteria and fungi, or CFU in the subsurface sediments, using published plate count procedures *(8)*. Briefly, 10-g subsamples

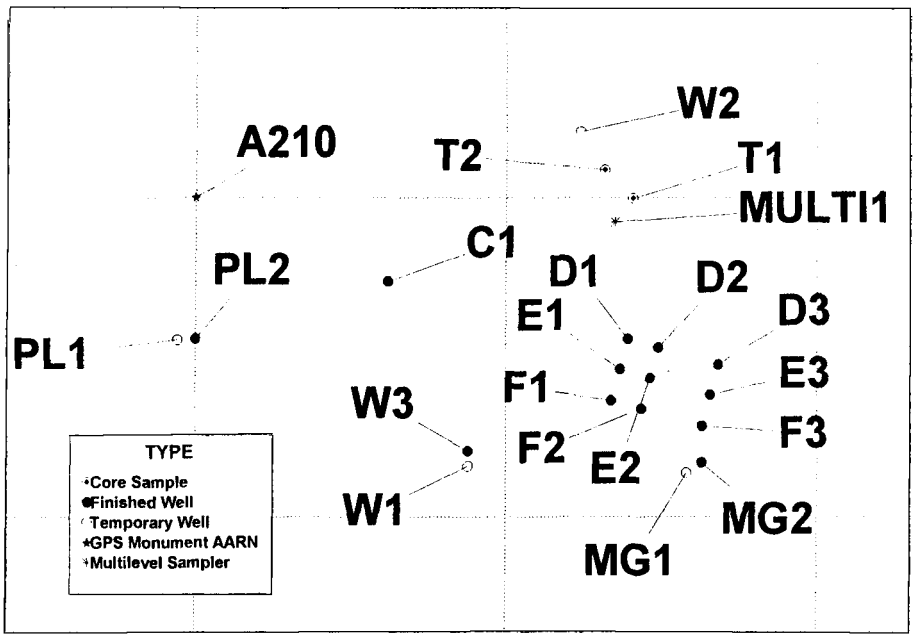

Fig. 1. Map of the study site showing the locations of the wells sampled during August 1994 at the Oyster site (D2, D3, E1, E2, E3, F1, F2, F3, T1, and T2). Also shown are some wells placed in June (D1, MG2, PL2, C1, W2, W3) and other preliminary drilling placed prior to June 1994. Grid lines are spaced at 100-m intervals, and north is to the top of the figure.

were suspended into 100 mL of a sterile pyrophosphate buffer and mixed in a Waring blender. Serial dilutions of the blended samples were made into phosphate buffer and, along with aliquots from the original pyrophosphate, were plated onto the different types of media. Samples for dilute PTYG, soil extract medium, and some fungal plates were processed on site within 3–12 h after sample collection in the mobile laboratory. Other samples were shipped to The University of Tennessee for plating within 48 h after sample collection on N/BHI and additional fungal media. Plates were incubated aerobically for up to 4 wk at room temperature (22–25°C) and evaluated periodically for CFU and colony morphology.

Using published procedures *(14)*, we described colony types based on differences in colony morphology *(8)*. Using the formula (Table 1) in Odum *(15)*, we calculated indices of evenness, richness, number of species, Shannon-Weaver, and dominance.

Data were analyzed using Excel 5.0 and Sigma Plot for Windows on a ZEOS 486 computer. The 95% confidence interval was used to test significance.

RESULTS AND DISCUSSIONS

Total CFU Numbers

CFU numbers on the dilute PTYG medium used for these comparisons varied over a range of 1.42×10^2–6.4×10^6. The $\log_{10}$ of CFU enumerated on the different types of media was significantly correlated with $r^2 > 0.58$. The overall mean CFU of the 79 samples taken during this period was 6.7×10^5 *(16)*. An exponential decrease in bacterial numbers with depth was noted *(16)*. In two shallow aquifers in Okla-

Table 1

Diversity Indices Used for Media Comparisons

Index	Abbreviation	Calculation
No. of species	s	= number of colony types
Shannon-Weaver	H	$= -\sum (n_i/N) \log(n_i/N)^a$
Evenness	e	= H/log S
Richness	d	= S -1/ log N
Dominance	c	$= \sum (n_i/N)_2$

[a]N = number of individual colonies, n_i = number of individuals in i^{th} colony type.

homa, similar but narrower ranges for total aerobic CFU were observed on dilute (5%) PTYG agar, 6.0×10^3–3.5×10^6 *(8)*. As with our N/BHI data, a richer medium, undiluted PTYG, gave lower numbers (range of 6.0×10^3–3.5×10^6) in the Oklahoma study *(8)*. Ranges for the Oklahoma study may have been narrower because of the smaller number of samples examined. The range of numbers observed in this study was similar to that reported for Eastern Coastal Plain sediments (i.e., <400 m in depth) *(8)*.

More variability was observed in the relationship between CFU numbers on the dilute PTYG medium and the N/BHI medium than between the PTYG and the soil extract media (Fig. 2). The correlation (r^2) for the regression of $\log_{10}$ counts on the N/BHI and PTYG media was 0.578. The coefficient (r^2) for the regression of counts on the soil extract to the PTYG was much higher (0.893). Linear regression r^2 values for nontransformed data were lower for the PTYG and N/BHI regression and higher for the PTYG and soil extract regression. In comparison with the counts on dilute PTYG, the counts on the N/BHI medium tended to be lower. Most of the points fell below the line of equal counts (solid line on Fig. 2) on a plot of log counts on the two types of media. The regression line (dotted line on Fig. 2) was below the equal count line, indicating a bias toward the PTYG medium. The intercept of the regression was at 1.74 on the log plot, again indicating higher counts on the PTYG medium. These results indicate that soil extract and dilute PTYG media revealed similar CFU, whereas the more nutrient-rich N/BHI did not facilitate CFU formation by a significant portion of the subsurface microbial community.

In contrast to the results with the N/BHI medium, a comparison of the counts on PTYG and counts on the soil extract media showed more similarity. The line of equal counts (solid line on Fig. 2B) on a plot of log counts of the media was much closer to the regression line and only slightly biased toward the PTYG medium. The slope of the PTYG soil extract regression was more nearly equal to one (0.951) than the slope for the regression between PTYG and N/BHI (0.713). Also, the majority of the points on the plot of PTYG and N/BHI fall below the line of equal counts. Thus, the slopes and location of the points indicate nearly equal counts on the PTYG and soil extract media, but not N/BHI.

Counts on the lower pH fungal medium were, as expected, lower than those on the PTYG medium (Fig. 3), but appeared to be related to the total counts, since there was a significant regression coefficient ($r^2 = 0.386$, $n = 55$) between counts on the two media. The pattern of logarithmic decrease in bacterial numbers with depth was also observed for fungi. The fungal CFU were lower than bacterial CFU at the surface, but the slope of the decrease was about the same as that for the bacteria *(16)*.

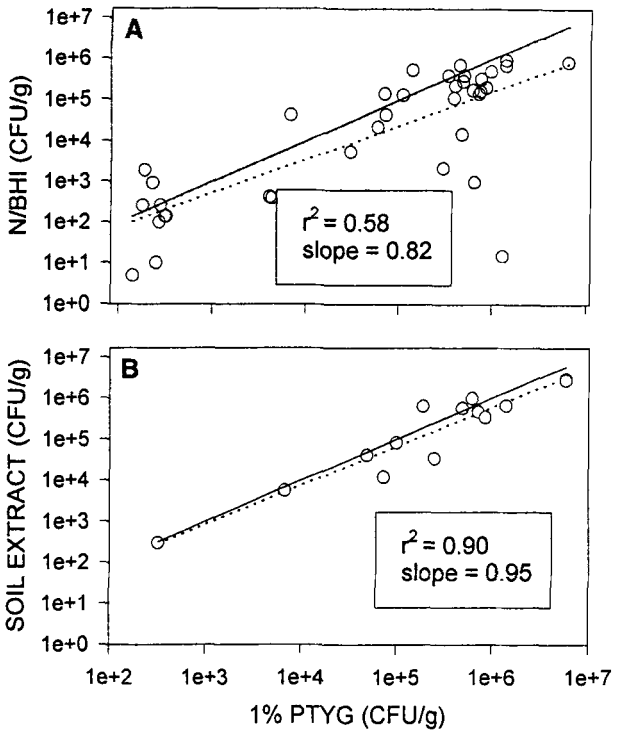

Fig. 2. Comparison of number of CFU on different media. The solid line is the calculated regression between the two types of media. The dashed line is the line representing equal numbers on the two types of media. A. Comparison between CFU on dilute PTYG and N/BHI media B. Comparison of CFU on dilute PTYG and soil extract media.

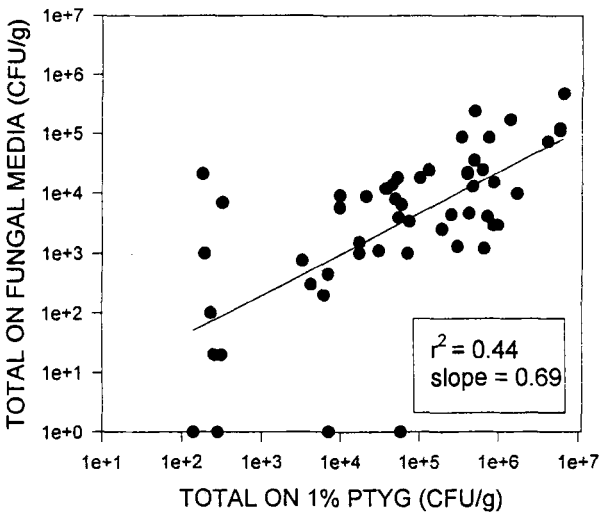

Fig. 3. Comparison of number of CFU on dilute PTYG and the fungal medium.

Table 2
Results of Paired *t*-Test Comparison of Diversity Indices for the Dilute PTYG and the Soil Extract Media[a]

Diversity index	Mean PTYG	Mean N/BHI	*t*	Probability of greater *t*	Person correlation
Evenness	0.705	0.815	-3.15	<0.1%	-0.026
Richness	3.04	2.74	0.67	NS	0.114
No. of species	7.64	6.42	1.31	NS	0.137
Shannon-Weaver	0.601	0.647	-1.05	NS	-0.157
Dominance	0.221	0.272	-1.69	NS	0.043

[a]Total number of samples is 14.

Diversity

Diversity measured on the subsets (Tables 1 and 2) used in the media comparisons (i.e., $n = 34$ N/BHI and $n = 17$ with soil extract) was not appreciably different from that measured on the whole data set ($n = 79$). Mean values for all of the samples taken during this sampling period were 8.05 for number of species, 0.25 for dominance, 3.21 for richness, 0.553 for Shannon-Weaver, and 0.631 for evenness *(16)*. Thus, these comparisons appear to have been made on a representative subsample of the diversity measurements made at the site.

Of the several measures of diversity calculated, only the number of species and the dominance index were similar across the three types of media, and only evenness differed in both comparisons. No significant difference was noted between number of species on the dilute PTYG and the soil extract media (Table 1) or between the PTYG and the N/BHI (Table 2). The mean number of species observed in the Oklahoma study *(8)* varied among different media, although very limited numbers of samples were compared in these earlier studies. Numbers of different colony types on undiluted PTYG ranged from 1 to >12 colony types, 1–9 on dilute PTYG, and 6 to >10 on a sediment extract agar (similar to the soil extract agar) for nine samples *(8)*. The number of species on PTYG for our August data set at Oyster (i.e., $n = 79$) appeared to decrease at high levels of CFU and high levels of phosphorus *(16)*. In the Oklahoma study, numbers of species decreased with depth and saturation on some media types, but not others *(8)*. In our study, phosphorous also may be related to microbial respiration activity at the site *(17)*. Also, we noted no significant difference between dominance on the dilute PTYG and the soil extract media (Table 1) or between the PTYG and the N/BHI (Table 2). The evenness index was significantly different between both the dilute PTYG and the soil extract media (Table 2) and the PTYG and the N/BHI (Table 3). An important significant difference was between the richness index on dilute PTYG and N/BHI. This index takes into account both the number of colony types and the total number of colonies observed. The richness index clearly suggests that the nutrient-rich N/BHI medium allowed for better expression of diversity as judged by colony morphology.

For richness and Shannon-Weaver indices, we observed more similarities between the soil extract and the dilute PTYG (Table 2) than between the PTYG and the N/BHI (Table 3). Again the results indicated that the two lower nutrient media, although giving higher total counts, may not be as good in eliciting information on

Table 3
Results of Paired *t*-Test Comparison of Diversity Indices for the Dilute PTYG and the N/BHI Media[a]

Diversity index	Mean PTYG	Mean N/BHI	*t*	Probability of greater *t*	Person correlation
Evenness	0.621	0.780	-4.4	<0.1%	0.076
Richness	3.61	5.10	-4.09	<0.1%	0.217
No. of species	8.88	8.94	-0.08	NS	0.168
Shannon-Weaver	0.564	0.724	-3.8	<0.1%	0.062
Dominance	0.237	0.278	-1.06	NS	-0.100

[a]Total number of samples is 34.

microbial diversity as the N/BHI medium. Despite the lack of a significant difference in number of species and dominance among the media types, the correlations among them were quite poor (Tables 2 and 3). The highest correlations were with species richness and number of species on dilute PTYG and N/BHI and on PTYG and soil extract media. The highest variances were seen in the number of species, dominance, and species richness. Variance for the number of species (i.e., 15.86 for dilute PTYG in the 34 samples used compared with N/BHI at 8.6) was quite large and differed among the media types. Variances of Shannon-Weaver and evenness were generally small (i.e., <0.05 % of the means) and were similar among the different types of media.

Although more sophisticated tools are available for measuring microbial diversity, interesting relationships between microbial community structure and properties can be elucidated using colony morphology and indices of diversity (e.g., *16*). Other methods requiring bacterial growth, including measurements of multiple substrate utilization (e.g., *18*), identification of strains to genus level *(19)*, calculation of a functional evenness index *(20)*, and physiological and nutritional versatility indices *(21)*, have been used. However, most of these methods require tremendous effort, since many tests must be run for each colony to be identified or characterized.

Molecular methods are being developed to examine microbial community diversity without the requirement for bacterial growth. The analyses of 5S and 16S rRNA sequences have been used in relatively simple systems (e.g., *22,23*). Other molecular methods, such as reassociation kinetics, approaches using PCR techniques, and restriction-fragment-length polymorphism, are also being developed (e.g., *24–26*). These molecular procedures are not yet practical for large numbers of samples required for the geostatistical analysis of spatial and temporal heterogeneity of microbial communities.

We used the colony morphology technique, despite its limitations, because of its relative ease of use on many samples and its history of use in microbiological studies. For subsurface communities, this approach may be valid. In a recent paper, Haldeman and Amy examined colony morphology as a measure of recoverable diversity and concluded that "colony morphology can provide an accurate basis on which to define recoverable diversity" *(27)*. In a study of deep vadose zone paleosols, Brockman et al. showed significant differences in several diversity indices based on colony morphology on dilute PTYG between several different paleosols *(9)*.

In our examination of media types, we found that using a high-nutrient medium (N/BHI) has advantages in measuring diversity over using the dilute PTYG, which has the advantage in measuring total counts. Use of the low-nutrient media derived from site soil has no advantages over the dilute PTYG or the high-nutrient media. The effort in preparing the low-nutrient site-specific media, together with the potential difficulty in comparing across sites, argues for use of more generic low-nutrient media, such as the dilute PTYG in assessing total colony counts. The reduction in numbers observed with higher-nutrient H/BHI medium argues against using it for total colony counts. As hypothesized, evidence indicates that better expression of differences may be seen in colony morphology on the higher-nutrient N/BHI medium. Thus, for studies where diversity is a primary concern, use of a higher nutrient medium, such as N/BHI, is advisable. Complementing dilute enumeration media with nutrient-rich media for diversity studies substantiates CFU enumerations while providing increased insight into microbial diversity and heterogeneity.

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