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# Environmental factors affecting distribution and abundance of bacteria, fungi and protozoa in subsurface sediments of the Upper Atlantic Coastal Plain, USA

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## ABSTRACT

Exploratory statistical analyses of microbiological, hydrological and geochemical data for samples from four boreholes drilled into Upper Atlantic Coastal Plain sediments near the Savannah River Site, SC, showed highly significant correlations between bacterial abundance (AODC and CFU) and hydraulic conductivity (K). Sediment texture variables (% sand (S), % silt, % clay (C), and S/C) were strongly interrelated with K and, therefore, also correlated with bacterial abundance. AODC did not correlate with the concentrations of dissolved inorganic nitrogen (DIN) or dissolved organic carbon (DOC) in pore water. CFU also did not correlate with DIN, but a negative relationship was found between the CFU and DOC for sandy sediments, suggesting that microbial activity may control pore water DOC concentration. In some, but not all boreholes, AODC and CFU correlated negatively with the pore water concentrations of metals and positively with pH. Protozoan abundance correlated strongly with AODC and CFU in the two boreholes closest to the recharge areas for their major aquifers. It also correlated with sediment texture variables, but not with K. Fungal abundance did not correlate with the abundance of other microbial types when data from individual boreholes were considered; however it did correlate with both bacterial and protozoan abundance when data from all four boreholes were combined. There was no relationship between fungal abundance and either K or sediment texture. We conclude from this study that hydraulic conductivity and sediment texture are closely related master variables that predict bacterial population density in these subsurface sediments. Metal concentrations and pH are less consistent in their predictive value. Neither N nor C appear to be growth limiting factors. Protozoan abundance was at least partly controlled by factors regulating bacterial populations, but the environmental factors regulating fungal populations are not clear.

# INTRODUCTION

Until recently the terrestrial subsurface was not recognized as a habitat for microorganisms, much less an ecosystem. Instead, it was generally assumed that little, if any, life existed in subsurface regions beneath the root zone (1). Petroleum and mineral exploration industries had reported microbes in deep geological formations bearing petroleum and sulfur (19, 12, 4), but because aseptic sampling techniques were not routinely employed, these reports were not given much credence by microbial ecologists. In the early 1980's, concerns about groundwater pollution led to renewed interest in investigating the potential for biological activity at depth in terrestrial systems. Subsequently, several research groups obtained subsurface sediments using aseptic coring techniques. The results of these earlier studies showed clearly that diverse and active communities of bacteria, and even fungi and protozoa, can be found in aquifer sediments, even at depths of several hundred meters (8). Thus, the ground work was laid for a new subfield of ecology focused on subsurface microbial processes.

The objectives of subsurface microbial ecology are similar to those of general ecology: (1) description of the abundance, distribution and activity of resident populations, in this case bacteria, fungi and protozoa, and (2) identification of environmental controls on these populations. Considerable progress has been made towards describing where and in what densities microbes are found in aquifer systems, although many more sites must be examined before broad generalizations can be made. By contrast, little research has been directed towards the more difficult task of identifying environmental controls.

There are two basic approaches used by ecologists to identify controlling environmental factors: (1) Samples can be collected from the environment under study, and their biological, chemical and physical characteristics quantified. The data then can be analyzed statistically for correlations between populations densities and environmental factors. (2) Samples can be studied in laboratory or field experiments in which a single environmental variable is manipulated while others remain constant. The response to a single variable will reveal whether it is a controlling factor. The two approaches are best used in concert, as each has its own strengths and weaknesses. Correlation analysis suffers from its inability to establish causality. Often it is unclear which of two related variables is independent. Worse, two variables may correlate not because they influence one another, but because they are both related to a third independent factor. Through stringent controls, laboratory experiments may provide reasonable proof of cause and effect. However, experimental conditions often differ so much from those in situ that extrapolation of laboratory results to the environment is risky.

The most intensive study of deep subsurface microbiology to date has been the Deep Probe Project of the U.S. Department of Energy (DOE). In 1986, three boreholes were drilled through Upper Atlantic Coastal Plain sediments on DOE's Savannah River Site (SRS), Aiken, SC, to depths between 210-280 m. Two of these boreholes, P29 and P28, were drilled within 3 km of one another, while P24 was drilled ~10 km to the Southeast and down dip relative to the prominent aquifers (see Sargent and Fliermans (16) for site descriptions). A fourth hole, C10 was drilled in 1988 and penetrated the ground to a depth of 530 m. It was located outside the SRS, ~20 km Southeast of P24 . In this paper, we have used statistical correlation analyses to explore the relationships between microbial population densities and the physical and chemical properties of samples from the four boreholes. Despite evidence that drilling fluids penetrated some samples, and may have compromised their pore water chemistry (7), the pore water chemistry data gathered during the Deep Probe study are included in our analyses. We believed it was better to seek correlations in less than perfect data than to ignore these data all together.

## MATERIALS AND METHODS

The population density measurements included in the analysis were those measured in our laboratory at Cornell University, using methods described previously (17): viable protozoa and fungi (from most probable number techniques), total bacteria (acridine orange assays employed were assumed to be at the limit. Standard tables were used to determine the statistical significance ($p < 0.05$) of each r value based on the number of data pairs available for analysis. Variables for individual borehole profiles and for individual geological formations across the four boreholes were compared using two-tailed t-tests (again with $p < 0.05$).

## RESULTS

### Microbial Distribution and Abundance

Visual inspection of the vertical profiles for distribution of bacteria in each of the four boreholes (Fig. 1) suggested that bacterial density does not decrease progressively with depth. Correlation analysis supported this conclusion: when data from all four boreholes were considered, the r values for both log AODC·$gdw^{-1}$ vs depth ($r = -0.21$) and log CFU· $gdw^{-1}$ vs depth ($r = -0.05$) were below the threshold for statistical significance ($r < -0.25$, for a negative relationship). Moreover, correlation between our index of potential for heterotrophic growth, log CFU/log AODC, and depth was very close to zero ($r = -0.01$). Although fungi and protozoa were present in lower numbers than bacteria, their population densities also showed no decreasing trends with depth (log fungi vs depth, $r = -0.20$; and log protozoa vs depth, $r = -0.26$ (the threshold value for significance here was $r = - 0.30$ because the sample size was smaller than for other groups)).

The variability within each vertical profile was substantial. AODC varied over 2 orders of magnitude and CFU over 7 orders of magnitude (Fig. 1). Some of this variability might be attributed to variations of environmental factors between geological formations. Therefore, we compared the mean densities of bacteria (AODC and CFU), fungi and protozoa within each geological formation (Table 1). These results suggested that some formations (e.g., the Congaree) are more productive biologically (i.e., have higher mean population densities horizontally over the four sample sites) than others (e.g., Tobacco Road). Attractive as this suggestion may be, our t-tests did not support it; no pair of mean values for individual formations differed at a significance level < 0.05. This lack of statistical support for the data in Table 1 was surprising; it may be explained by the small number of samples (3-17) obtained from each formation. Alternatively, facies changes within a geological formation, which represent changes in depositional environment over time or space, could cause substantial variability in environmental factors within a formation and, thus, have led to the variation we observed in microbial densities.

The horizontal distribution of microbes was examined by comparing the mean values for biomass estimates in the vertical profiles of each borehole (Table 2). It was found that bacterial (AODC and CFU), fungal and protozoan population densities all decreased along the transect between the P28-P29 cluster and C10 (Table 2). T-tests indicated that for bacteria, the differences among the profile means for P28, P29 and P24, which were separated by 3-10 km, were not statistically significant, while the mean for C10, which was 20-30 km downdip from the other boreholes, was significantly lower than the others. C10 was a much deeper borehole than the others (530 m vs <300 m), but depth did not explain its overall lower biomass; the bacterial densities in C10 samples from above 300 m depth were as low as or, in some cases, lower than those in samples from deeper than 300 m (Fig 1). For fungi, the boreholes fell into two groups: P28 and P29 contained higher fungal biomass overall than P24 and C10. Within these two groups, the borehole mean values for fungal biomass were not significantly different. The values for protozoan biomass also were lowest in the C10 borehole. Protozoan densities differed significantly in each borehole, except for the P29-P24 pair, whose means could not be distinguished statistically.

## Correlations between bacterial abundance and environmental factors

As anticipated from previous work (6, 17), both total (AODC$\cdot$ gdw$^{-1}$) and viable (CFU$\cdot$ gdw$^{-1}$) bacterial densities correlated with several sediment texture variables (negatively with % silt and % clay (C), and positively with % sand (S), S/C, and log S/C) at a significance level of <0.05 (Table 3). In addition, significant relationships were found between both log AODC and log CFU and hydraulic conductivity (K). However, the magnitude of r for these last relationships was strongly influenced by a few samples with low K values; only 7 of 42 K values reported in the Deep Probe study fell between $10^{-8}$ and $10^{-4}$, whereas 35 were between $10^{-3}$ and $10^{-1}$. Log K and the sediment texture variables were highly interrelated ($r > 0.6$ or $< -0.7$).

Neither AODC nor CFU correlated at a statistically significant level with the dissolved inorganic nitrogen (DIN) concentration of pore water (Table 3), an indicator of nitrogen availability. Furthermore, despite the dominance of subsurface microbial communities by aerobic heterotrophic bacterial populations, bacterial biomass estimates did not correlate significantly with dissolved organic carbon (DOC) concentration. For CFU, however, the weakness of the relationship to DOC may have been caused by a small group of clay-rich samples from which few, if any, bacteria were cultured. When only sandy samples (sand ≥ 70%) were included in the analysis, a significant negative relationship ($r = -0.48$) was obtained for CFU vs DOC. Here it is interesting to note that C10, which had a significantly lower bacterial biomass than the other boreholes, also had the greatest mean DOC concentration, 8 $\text{mg l}^{-1}$, compared with means of 2, 5, and 3 $\text{mg}\cdot\text{l}^{-1}$ in boreholes P29, P28, and P24 (all these means were significantly different at a level of 0.05).

Analysis of the combined data set for the four boreholes showed no correlations between bacterial biomass (AODC or CFU) and pH (Table 3); but significant relationships were found when data were examined borehole by borehole. Pore water pH values correlated positively with AODC in boreholes P28 and P29, and with CFU in P29, P24 and C10. Lack of correlation for the overall data set may be explained by the range of pH values represented. Neither very high ambient pH nor very low ambient pH would favor prolific microbial growth on the neutral pH plate-count medium; an optimum environmental pH would be expected to occur nearer to neutrality. The lack of correlation might also be explained by nonlinearity of the relationship between microbial biomass and pH. For non-linear relationships, the correlation analysis we employed is not appropriate.

In borehole P29, AODC and CFU correlated significantly (and negatively) with the pore water concentrations of several metals: Al, Cd, Cu, Fe cations ($Fe^{+2} + Fe^{+3}$), Ni and Zn. Fewer of such relationships were found in P28 (AODC correlated negatively with Al and Zn, and CFU correlated only with Zn), and no such correlations were found for P24 and C10. Strong relationships between biomass and metals in P29 ($-9.0 < r < -7.0$), biased the significance of relationships within the overall data set; AODC correlated with Zn, and CFU with Al, Ni, Cu and Zn (Table 3).

## Correlations between microeucaryote abundance and environmental factors

Relationships between fungal density and environmental factors changed from borehole to borehole. Furthermore, the combined data set for all boreholes yielded a different set of relationships than was found for the individual boreholes. For borehole P28, none of the measured environmental factors was be related to fungal biomass. At P29, CFU, K, % sand, S/C, pH, and the concentrations of DIN, Al, Ni, and Cd all correlated positively with biomass, while Fe cations and % clay correlated negatively. For borehole P24, there was a negative correlation with Zn, and for C10, a positive correlation with Fe cations. The overall data set indicated positive relationships between fungi and all three of the other biological parameters (AODC, CFU, and protozoa), negative relationships with DOC and pH, and a positive relationship with DIN concentration (Table 3). It showed no relationship between fungal density and either the sediment texture variables or K.

Protozoan density correlated strongly with AODC and CFU ($r > 0.7$) at the two up-dip sites (P28 and P29), but not at the lower sites (P24 and C10). No relationship between protozoan abundance and pore water chemistry was indicated for boreholes P28 and P29, whereas at P24 and C10 significant correlations were found between protozoan biomass and the concentrations of Fe cations (negative) and Ni , respectively. Protozoan density correlated with K only in borehole P29. It correlated with sediment texture variables both in this borehole and in P28. Using the entire data set (Table 3), we found significant relationships between protozoa and each of the following: AODC, CFU, fungi, sediment texture variables (% sand, % clay (negative), and log S/C), Cu and Cd (negative).

## DISCUSSION

This study provides statistical support for the now common observation that below the root zone and down to several hundred meters, microbial abundance does not diminish progressively with depth (2, 14, 17). However, this observation should not be interpreted to imply that microbial biomass is uniform within subsurface sediments. To the contrary, the microbial distribution and abundance data set examined in this work showed considerable spatial variability. Over vertical profiles, bacterial, fungal and protozoan densities fluctuated over at least 2 orders of magnitude, and boreholes 3-30 km apart had significantly different profile means for microbial population densities (Table 2).

We hypothesize that underlying the observed spatial variability in microbial abundance are physical and chemical factors that control growth and that reflect environmental heterogeneity. Our long term objective is to identify the factors controlling growth. This would enable us to predict the influence of environmental factors on the function of subsurface ecosystems.

Several Deep Probe investigators (2, 5, 9, 11, 17) have already reported relationships between bacterial biomass or activity, and sediment clay content or S/C ratio. We confirmed these relationships, and also found that other sediment texture variables (% S, log S/C) correlated with abundance of microorganisms (Table 3). Sediment texture may simultaneously affect many other environmental factors important to microbes: hydraulic conductivity (K), pore size and water availability, to name a few. Thus it would be desirable to examine the influence of these sediment-texture related factors independently.

In this work, we were able to examine the influence of K. Our statistical analyses showed that bacterial density (both AODC and CFU) correlated with K at a level of statistical significance similar to that of the density-sediment texture relationship (Table 3). In addition, K and the sediment texture variables were all highly interrelated. Thus, it appears the impact of sediment texture on distribution and abundance of microbial biomass may be linked to its affect on K. In turn, the influence of K is likely to be linked to its influence on nutrient and electron acceptor supply rates.

The productivity of most ecosystems is nutrient limited, usually by nitrogen (e.g., in forests and coastal oceans) or phosphorus (e.g., in freshwater). Unequivocal evidence for nutrient limitation usually is achieved through experimentation. However, exploratory statistical analyses such as those presented in this paper can provide some clues about the regulatory roles of nutrients. In the Deep Probe sediments, neither C nor N appeared to be growth limiting, as indicated by lack of strong positive correlations between microbial abundance and the DOC and DIN concentrations of pore water. However, these estimators of C and N availability neglect nutrients associated with particles (either adsorbed on sediments or temporarily stored in microbes), and they do not adequately reflect nutrient levels in throughflowing water. DOC and DIN values can be taken as measures of standing stock, but they are really residual quantities which reflect an instantaneous balance between pore water nutrient sources (mineralization, desorption, inflow, etc.) and sinks (microbial uptake, adsorption, outflow, etc).

Nevertheless, nutrient concentrations can provide valuable information about relationships between environmental supplies (S) and microbial demand (D). When S>>D, the dissolved concentration of a nutrient is controlled by abiotic processes (i.e., microbial uptake would be a small flux relative to other fluxes). This seems to have been the case for nitrogen in the Deep Probe studies: neither the AODC nor the CFU of bacteria correlated with pore water DIN. Furthermore, many sediment samples showed relatively high pore water DIN concentrations ($mg \cdot L^{1}$) when compared to the concentrations in N-limited pelagic ecosystems, typically µg·L$^{1}$ (10, 18)). Drilling fluid intrusion could not be blamed for the high concentrations in pore waters. In most instances, the DIN concentration in drilling fluids was below the detection limit.

Microbial processes become important in controlling the dissolved concentrations of nutrients as the magnitude of S approaches that of D (see Levine (13) for details). Provided that S > D, an inverse relationship between biological density and nutrient pool size should develop. Such a relationship was found for CFU and DOC concentration in the Deep Probe samples. Drilling fluid intrusion could not have produced this negative relationship since both microbial densities and DOC concentration were greater in drilling fluid than in most of the sediments.

For a growth-limiting nutrient, S = D at steady state. Equality is maintained by microbial populations, whose demands rise and fall with S. The dissolved pools of limiting nutrients are as fully depleted as is physiologically possible at all microbial densities. None of the nutrients which we examined in the Deep Probe data set showed the characteristics of a limiting nutrient. However, we did not attempt to correlate bacterial density with phosphorus concentration, for the pragmatic reason that Total P was undetectable in almost every pore water sample (thus there was no spread in scatter plots). Although low pore water P concentrations might be attributable in part, or wholly, to abiotic processes such as phosphate precipitation and adsorption of P onto sediment particles, it is also possible that they reflect exhaustion of P resources by microorganisms. Evidence supporting the latter hypothesis was presented by Phelps and White (15), who found that phosphate additions to subsurface sediments consistently stimulated $^{14}$C-acetate incorporation into lipids, whereas nitrogen and sulfur amendments only occasionally had this effect.

The exploratory statistical analysis reported in this paper sets the stage for experimental work to test hypotheses concerning nutrient limitations on microbial abundance in oligotrophic subsurface sediments. Such experimental work should also lead to a better understanding of the physical and chemical factors affecting microbial activity in these sediments.

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## FIGURE CAPTIONS

Fig. 1. Vertical profiles for AODC and CFU densities within four boreholes at the Savannah River site, SC.

# VERTICAL DISTRIBUTION OF BACTERIA

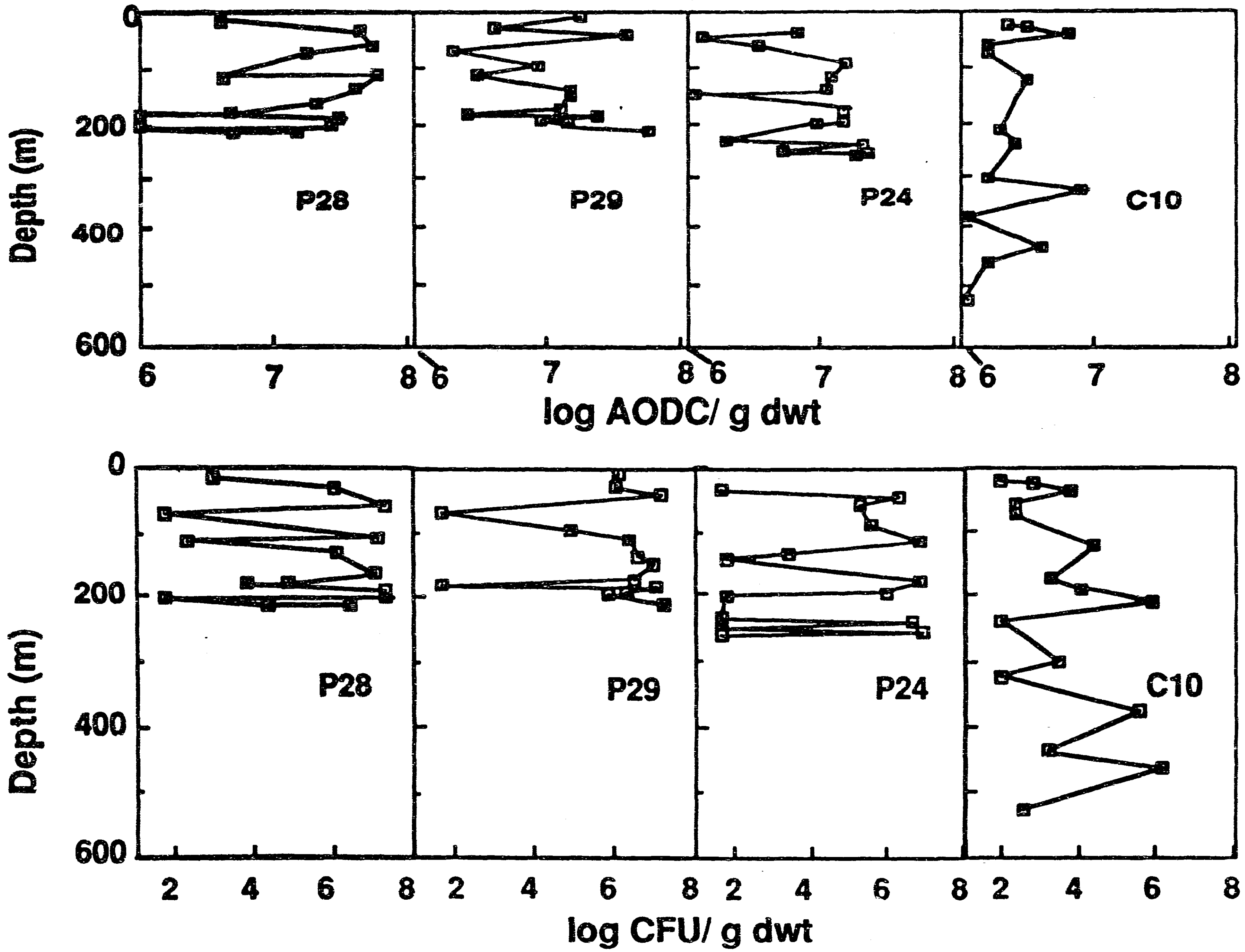

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Table 1. Mean microbial densities for different geological formations. Only formations from which 3 or more samples were obtained are included here.

| | Logarithm of numbers per gram dry weight | | | |
|---|---|---|---|---|
| **Formation** | **AODC** | **CFU** | **Fungi** | **Protozoa** |
| Tobacco Road | 6.62 | 2.57 | -0.03 | -0.70 |
| Dry Branch | 6.54 | 4.44 | 0.32 | -0.11 |
| Griffins Landing | 6.80 | 4.56 | 0.81 | 0.39 |
| Congaree | 7.51 | 5.81 | 0.52 | 1.11 |
| Ellenton | 6.90 | 3.55 | 0.27 | -0.05 |
| Peedee | 6.81 | 4.80 | 0.30 | 0.23 |
| Black Creek | 6.97 | 4.87 | 0.40 | 0.12 |
| Middendorf | 6.89 | 4.86 | 0.61 | 0.61 |

Table 2. Vertical profile means for microbial biomass within the four boreholes at the Savannah River Site.

| | Logarithm of numbers per gram dry weight | | | |
|---|---|---|---|---|
| **Borehole** | **AODC** | **CFU** | **Fungi** | **Protozoa** |
| P28 | 7.07 | 7.02 | 6.87 | 6.40 |
| P29 | 7.02 | 5.73 | 4.29 | 3.51 |
| P24 | 6.87 | 0.72 | 0.26 | 0.03 |
| C10 | 6.40 | 0.40 | 0.31 | -0.69 |

TABLE 3. Correlation coefficients (r) for relationships between microbial density in SRS sediments and geochemical and physical factors. Data from all four bore holes are combined. Statistical significance ($p \leq 0.05$, *) was indicated for bacteria ($N = 60$) at $r > 0.25$ or $< -0.25$, for fungi ($N = 56$) at $r > 0.26$ or $< -0.26$, and for protozoa ($N = 44$) at $r > 0.30$ or $< -0.30$. The r for individual boreholes are available from the authors at request.

| | Log AODC | Log CFU | Log Fungi | Log Protozoa |
|---|---|---|---|---|
| **Physical Variables** | | | | |
| Sediment Texture | | | | |
| International Units | | | | |
| % Sand (S) | 0.57* | 0.59* | 0.14 | 0.39* |
| % Silt | -0.45* | -0.52* | -0.24 | -0.36 |
| % Clay (C) | -0.56* | -0.57* | -0.09 | -0.36* |
| S/C | 0.58* | 0.53* | 0.17 | 0.44* |
| log S/C | 0.58* | 0.58* | 0.12 | 0.39* |
| American Units | | | | |
| % Sand (S) | 0.50* | 0.62* | 0.11 | 0.37* |
| % Silt | -0.28* | -0.49* | -0.12 | -0.24 |
| % Clay (C) | -0.56* | -0.57* | -0.09 | -0.36* |
| S/C | 0.56* | 0.52* | 0.15 | 0.43* |
| log S/C | 0.54* | 0.59* | 0.11 | 0.38* |
| Log K | 0.54* | 0.53* | 0.07 | 0.18 |
| **Chemical Variables** | | | | |
| DOC | -0.10 | -0.23 | -0.29* | -0.16 |
| DIN | 0.12 | 0.23 | 0.33* | 0.11 |
| pH | -0.03 | 0.17 | -0.40* | -0.28 |
| Metallic Cations | | | | |
| log Al | -0.23 | -0.28* | -0.11 | -0.06 |
| log Cd | -0.18 | -0.24 | -0.13 | -0.50* |
| log Cu | -0.11 | -0.26* | 0.21 | 0.54* |
| log $Fe^{+2} + Fe^{+3}$ | 0.03 | -0.19 | 0.05 | 0.17 |
| log Ni | -0.12 | -0.32* | -0.11 | -0.13 |
| log Zn | -0.27* | -0.39* | -0.01 | 0.06 |

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