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Title:

Down-stream spatial distribution of antibiotic resistance along metal contaminated stream reaches

Running Head:

Spatial patterns of aminoglycoside resistance in sediments

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Abstract

Sediment bacteria samples were collected from three streams in South Carolina, two contaminated with multiple metals (Four Mile Creek and Castor Creek), one uncontaminated (Meyers Branch), and another metal contaminated stream (Lampert Creek) in northern Washington State. Growth plates inoculated with Four Mile Creek sample extracts show bacteria colony growth after incubation on plates containing either one of two aminoglycosides (kanamycin or streptomycin), tetracycline or chloramphenocol. This study analyzes the spatial pattern of antibiotic resistance in culturable sediment bacteria in all four streams that may be due to metal contamination. We summarize the two aminoglycoside resistance measures and the 10 metals concentrations by Principal Components Analysis. Respectively, 63% and 58% of the variability was explained in the 1st principal component of each variable set. We used the respective multivariate summary metrics (i.e. 1st principal component scores) as input measures for exploring the spatial correlation between antibiotic resistance and metal concentration for each stream reach sampled. Results show a significant and negative correlation between metals scores versus aminoglycoside resistance scores and suggest that selection for metal tolerance among sediment bacteria may influence selection for antibiotic resistance differently than previously supposed.. In addition, we borrow a method from geostatistics (variography) wherein a spatial cross-correlation analysis shows that decreasing metal concentrations scores are associated with increasing aminoglycoside resistance scores as the separation distance between sediment samples decreases, but for contaminated streams only. Since these results were counter to our initial expectation and to other experimental evidence for water column bacteria, we

suspect our field results are influenced by metal bioavailability in the sediments and by a contaminant promoted interaction or "cocktail effect" from complex combinations of pollution mediated selection agents.

Introduction

All living organisms are spatially dispersed in the environment. Typical spatial distribution patterns include uniform, random, or aggregated and are conferred by a multitude of environmental (biotic and abiotic) factor combinations. Microorganisms are no exception. However many microbiologists still adhere to the "everything is everywhere, for which the environment selects" mindset. This means that representatives of all groups of microorganisms can be found everywhere but that the environment favors certain groups over others. Those not favored remain rare and not easily detected until such times when evolutionary preadaptations among them are favored due to environmental change. However certain species of bacteria may be more "plastic" or facultative than others, because of various strains or ecotypes and thus the species composition of the bacterial community may not differ substantially along the environmental gradients although some strains may be predominant along the gradient because of differential selection.

McArthur and Tuckfield [1] argued that the distribution of bacteria and or their genes should follow predictable patterns in stream ecosystems. Freshwater streams are particularly suitable for studying spatial relationships of microbial traits because of the one-way transport vector for stream inputs, whether natural or anthropogenic. Specific genes or combinations of genes that are adaptive under one set of environmental conditions may not be so under a different set. Since stream ecosystems often change dramatically in physicochemical attributes, often over fairly short geographic distances, the distribution of bacteria and bacterial genes are predicted to change similarly and certain genes may be adaptive or maintained over longer reaches of stream than other genes [1]. This ecological perspective fits the microbiological perspective as well if evolutionary adaptation is occurring both among bacterial strains and bacterial species.

McArthur and Tuckfield [2] sequentially sampled stream sediment bacteria from a historically metal contaminated stream to assess the relationship of a bacterial trait, e.g., prevalence of antibiotic resistance, with downstream distance. They discovered similar spatial relationships in the prevalence of both streptomycin and kanamycin resistance among stream sediment bacteria with downstream distance in a contaminated stream. The highest levels of antibiotic resistance were reached near the confluence of a stream with historical Cd and Hg contamination. No matching spatial patterns among sediment bacteria were detected in an uncontaminated stream although the prevalence of antibiotic resistance was substantial. They also showed that the prevalence of streptomycin resistance was positively correlated with Hg sediment concentration within a historically metal contaminated tributary. However, they did not estimate the maximum stream distance over which these antibiotic resistance traits were maintained, nor did they conduct a metals analysis on the corresponding stream sediment samples.

Bacteria in aquatic environments with known heavy metal contamination often demonstrate resistance to antibiotics as well to heavy metals [3-14]. The common explanation for such phenomena is horizontal gene transfer via the plasmid mediated exchange of genetic material among bacteria [15]. It is not uncommon to find genetic elements for antibiotic and metal resistance on the same the same plasmid. In fact, there are well known integrons that contain both [16]. However, no prior field study has examined the spatial relationship or correlation between either of these phenotypic traits and the increased selection pressure from a known point source of metal contamination in the same aquatic environments. Geostatistical variograms are often used to identify patterns of spatial relationships for a variety of ecological variables in lakes [17], streams [18-21], groundwater [22, 23] and among birds, barnacles, arthropods, shrubs and trees [24-30], with equal utility for exploring the same patterns among microbes in sediment samples as well.

The purpose of this study was to demonstrate the applicability of using geostatistical methods to describe the spatial distribution of bacterial phenotypic traits within streams. We sought to determine through field collections whether phenotypic traits were spatially correlated. Specifically, we sought to determine whether or not anthropogenically (metal) contaminated streams would show a spatial downstream pattern in antibiotic resistance among culturable sediment as a function of metal concentration, which pattern should not exist within an uncontaminated stream.

Materials and Methods

Study area and streams

The Savannah River Site (SRS) is an 802 km^2 manufacturing facility bordering Georgia and South Carolina that has contributed to the production of nuclear defense materials for approximately 50 years. The actual Savannah River is the western site boundary for SRS and receives discharge from several contaminated and uncontaminated streams. We collected 67 sediment samples along a 13 km reach of Four Mile Creek (Fig. 1) during the summer of 1998 and 62 samples along a 12 km reach of Meyers Branch in the summer of 1999 (see [2]). In addition 22 sediment samples along a 2 km reach of Castor Creek in 1999 and 14 sediment samples along a 1.5 km reach of Lampert Creek that same year. The first three streams are part of the SRS watershed.

Four Mile Creek (FMC) is a third order upper coastal plain stream draining a 5,894 ha watershed. Annual stream temperatures range from 9.0 to 25 ºC and pH from 5.10 to 8.10, (median = 6.09). FMC received thermal effluent (>50 ºC) from reactor operation until 1987 at flows 10 times higher than ambient (40 m^{-3} to over 400 m^{-3}). These flows caused major geomorphological changes within the stream essentially scouring the channel of all organic matter and in-stream structure. All riparian vegetation was killed. In addition, several chemical seepage basins were established near the headwater reaches of the stream and used continuously for over 30 years. These basins received chemical effluent composed of tritium, nitrate, organic solvents, and various metals. Although the basins have been capped, leachate continues to seep into the stream. The stream has been undergoing natural recovery since cessation of thermal inputs in 1987.

Castor Creek (CC) is a third order upper coastal plain stream that flows into FMC with an environmental profile similar to FMC. The catchment for CC drains runoff and effluent from the SRS C-Reactor facility.

Meyers Branch (MB) is a third order, relatively pristine blackwater stream on the SRS, set-aside for ecological research. MB drains an approximately 5,085 ha watershed. It originates in the sand hills of the upper coastal plain and has an extensive riparian floodplain. Annual stream temperature ranges between 0.1 and 25.5 °C and pH ranges from 5.8 to 8.3 with a median of 6.9. This stream has for the past 50 years had no known metal contamination by anthropogenic input and is more remote to human activity at SRS than FMC or CC.

Lampert Creek (LC) is a fourth order montane stream in Ferry Co., Washington immediately south of the Canadian border on the eastern slope of the Kettle mountain range (in the Belcher Mine drainage, Section 8, Township 37N, Range 34E, Willamette Meridian, WA) whose catchment feeds into Curlew Creek and Curlew lake and eventually empties into the Sanpoil River which leads to the Columbia River. LC receives runoff from an abandoned precious metals mine immediately adjacent to the midreachs of that stream and is therefore contaminated with elevated concentrations of several heavy metals.

Sediment sampling and laboratory analysis

Sediment samples were collected from all four streams during summer months – June 1998 (FMC), and May, June and August 1999 (MB, CC, and LC, respectively). Sampling locations were established 200m (+/- 1.5m) apart within the main stream channel of both FMC and MB using Geographic Information System (GIS) technology. Points were identified using interpolation of coordinates from the UTM North American Datum 1927 projections. The Global Positioning System (GPS) coordinates were generated for all sampling points in these two streams and portable GPS equipment used to locate them.

Sampling locations in both CC and LC streams were not obtained by GPS telemetry but were located approximately 100m apart. Twenty two locations in the former were paced upstream from the confluence of FMC for nearly 2 km. Fourteen locations in the latter were obtained by first locating the source of the mine drainage and following the stream bed to its confluence with a continuously flowing stream and collecting samples for approximately 1.5 km downstream thereafter. Each sampling location regardless of stream also possessed linear coordinates as well, a number from 1 to N (N = # of sampling locations) as the northing coordinate and a constant value of 1 for the easting coordinate. Linear coordinates were used in spatial statistics methods (see below).

At each stream sampling location, one 10 cm x 2.5 cm diameter core was taken from near-bank bottom sediments, placed on ice in a sterile bag, and immediately transported to the laboratory. At each sampling location the side of the stream to sample was chosen randomly.

For FMC, a direct plating method ~~as~~ was used wherein 10mL sterile saline (0.85% sodium chloride) was added to approximately 5g wet weight of sediment and sonicated for five minutes in a Bransonic Water Bath sonicator to detach bacteria. Next, 250 μL of the resulting slurry was spread onto each of five plates: control, kanamycin, streptomycin, tetracycline and chloramphenicol. Control plates consisted of half strength nutrient broth agar with 100 μg ml^{-1} of cycloheximide added to control fungal growth. The remaining plates were identical to the control plates except the addition of 100 μg ml^{-1} of kanamycin, streptomycin, tetracycline, or chloramphenicol. This concentration was used for each antibiotic since the minimum inhibitory concentrations for these bacteria were unknown. Each set of five plates was inoculated from a single sediment sample. Remaining sediments were dried (60 °C) and weighed. Colony counts were made after 6 d incubation at room temperature (~20 °C). Each count was adjusted for sample volume by adding 1 to the count and dividing by the corresponding sediment dry weight.

For all other streams, samples were processed similarly with the following exceptions that a) only the control plates were inoculated and only the aminoglycosides (kanamycin and streptomycin) were used as experimental treatments and b) serial dilutions (0, 10^{-1}, 10^{-2}) were made. 250 µl of liquid were drawn from each of the three dilutions and spread on control plates. Bacteria colonies were counted on the control plates after 6 days incubation at 20 ^{o}C. The plate with the "best" colony growth (~50-100 colonies/plate) was selected as the control plate. These control plates were then used as a replica plating source (Bel-Art Products, Pequannock, NJ) for two other plates each containing 100 µg ml^{-1} of either streptomycin or kanamycin. Bacteria colonies were counted on the replica plates after 3-4 days incubation at 25 ^{o}C.

Concentrations for 10 metals (ppm) (Hg in ppb) were obtained from all sediment samples by ICP (Inductively Coupled Plasma) methods from services provided by the inorganic chemistry laboratories of the University of Georgia. Metal concentration measurements reported below the laboratory detection limit (DL) were replaced by DL/2 prior to data analysis, a typical environmental data analysis practice [31].

Statistical methods

A common log transformation of bacteria colony counts was performed as a variance stabilizing method and to preserve the statistical assumption of normality prior to statistical modeling. A Sharpiro-Wilk W-test [32] was then performed to ensure that the transformed data adequately fit a normal distribution and justify parametric statistical methods. Specifically, the prevalence of antibiotic resistance (*pAR*) was defined as the logarithm of the ratio of the adjusted antibiotic resistant bacteria colony count to the adjusted control colony count, for each antibiotic and sample. An errors-in-variables (i.e. orthogonal) linear regression [33] was performed to quantify the relationship between the *pAR* for kanamycin and streptomycin as well as between the principal component scores (see below) for aminoglycoside resistance and the metals principal component scores. An analysis of variance (ANOVA) was performed on the *pAR* data among streams. Stream means were compared using the method of linear contrasts [34].

All pairwise correlations between each measure of aminoglycoside antibiotic resistance and each of the 10 metals concentrations were calculated by a parametric statistical (Pearson's r) correlation method [35] on the common logarithms of these measures since colony counts often crossed more than one order of magnitude.

Each sediment sample produced a multivariate response vector, with two measures of antibiotic resistance and 10 measures of metal concentration. Principal Component (PC) Analysis [36] was used as a dimensionality reduction technique in order to obtain a summarized univariate measure of both antibiotic resistance and metal concentration. Data from all streams were combined and PCs generated based on the correlation matrix, as opposed to the variance-covariance matrix. This was done to preserve the relative contribution (i.e. eigen vector coefficients) of each univariate response measure to the information contained in each antibiotic resistance or metal PC. Since PCs were calculated from the correlation matrix, the number of dimensions summarized in a PC is represented by the corresponding eigen value. The PC score is the linear combination of measurement values for all variables in the PC weighted by the corresponding eigen vector coefficients.

The metals data were also subjected to a Discriminant Function (DF) analysis [36]. DFs allowed the classification of individual stream samples based on all metals data. DFs also indicate the relative contribution of each metal to the discrimination among streams.

All univariate and multivariate data analyses were performed with the JMP® 5.21 statistical computing software from SAS Institute Inc. Box-and-whisker plots were produced by S-PLUS 6.1 and JMP® 5.21.

Geostatistical methods [37] were used to estimate the amount of spatial correlation for measures of antibiotic resistance and metal concentration within streams by means of the well known variogram, given by

$$\gamma(\mathbf{h}) = \frac{1}{2N(\mathbf{h})} \sum_{i=1}^{N(\mathbf{h})} \left[\mathrm{z}(\mathbf{x_i}) - \mathrm{z}(\mathbf{x_i} + \mathbf{h}) \right]^2 \qquad (1)$$

where $N(\mathbf{h})$ is the number of pairs of stream sample locations with a between-location or spatial separation distance $\mathbf{h}$, $z(\mathbf{x_i})$ and $z(\mathbf{x_i} + \mathbf{h})$ is the response measures (such as *iAR*) for the ith easting (x_e) and northing (x_n) coordinates for sampling location $\mathbf{x_i} = (x_e, x_n)_\mathbf{i}$ within the stream and its paired location +$\mathbf{h}$ distance units away, respectively. Variogram ($\gamma(\mathbf{h})$) and cross-variogram ($\gamma_{12}(\mathbf{h})$) model fits were performed using the 1st PC scores (log scale) for metals and for aminoglycosides as response measures. The emboldened letter **h** indicates a separation vector; that is, a Euclidean distance between sampling locations in 2-space, or a linear distance along the stream channel. The variogram range (r_v) parameter is defined as the minimum **h** between sampling locations such that the correlation between measurements for each successive measurement pair is effectively zero, and the standardized variogram $\gamma_s(\mathbf{h})$ approaches 1.0 [38]. Since $\gamma(\mathbf{h})$ increases with **h**, the estimate of r_v is obtained by plotting $\gamma(\mathbf{h})$ versus **h**. When **h** is sufficiently large, $\gamma(\mathbf{h})$ is asymptotic and the value of the *pAR* measurement at $\mathbf{x_i}$ is no longer a useful predictor of the *pAR* value at $\mathbf{x_i + h}$. Thus, the variation among all *pAR* measurement differences at paired sampling locations $\geq r_v$ units apart is strictly random. A geostatistical variogram can be modeled to estimate r_v for a given spatial response measure such as the *pAR* or for metal concentration. Standardized variograms and cross-variograms were fit to *pAR* PC scores and to metals concentration (logarithms) PC scores using a spherical model [38] to obtain estimates of r_v for each stream. Cross-variograms demonstrate the degree of spatial correlation between different response measures for the same pair of sampling locations, in this case the *pAR* PC scores and the metals concentrations PC scores, as **h** increases between sampling locations. All model fits were obtained by the VARIOWIN software [38].

Results

The prevalence of antibiotic resistance (*pAR*) among antibiotics and streams are presented as a series of box plots in Figure 1. The *pAR* for each of the four antibiotics used in the initial treatment of FMC sediment bacteria (Fig. 1A) show markedly higher resistance to the two aminoglycosides, kanamycin and streptomycin, compared to chloramphenicol and tetracycline. Table 1 shows the strongly positive and statistically significant ($p<.05$) pairwise correlations among these four *pAR* measures. The magnitude of the antibiotic resistance response was much smaller, i.e. the bacterial sensitivity was much higher for tetracycline and chloramphenicol. In a majority of samples, there were few or zero bacteria resistant to the latter two antibiotics. Thus, because of the substantial loss of information and paucity of data, only the responses of the two aminoglycosides were used in subsequent spatial pattern analyses for a bacterial trait.

Metals and antibiotic resistance correlations

A correlation analysis between the *pAR* for streptomycin vs kanamycin without regard to stream indicates a positive ($r = 0.25$) relationship ($p < 0.05$). The 1st PC between these two *pAR* measures explains 63% of the *pAR* variability among all stream samples or 1.25 of these 2 dimensions. In addition, both kanamycin and streptomycin contribute equally to this PC. ANOVA results of the 1st PC scores among streams indicate no significant differences among streams. The largest *pAR* PC score mean occurred in CC but no pairwise linear contrast with any other stream mean was statistically significant.

Metal concentration results are presented in Figure 2. Three metals (Al, Fe, and Mn) show consistently higher concentrations compared to the other seven in all four streams. The pattern of relative magnitude in median (geometric mean) metal concentration among all four streams was largely the same for 8 of 10 metals. The exceptions are Hg and Cu. There are much lower concentrations of Hg in MB (the uncontaminated stream) relative to the other three streams. Over 70% of the Hg measurements in MB were < 10^{-4} ppm. The highest median Hg concentration (approx. 20 ppb) occurred in CC relative the other streams. In addition, the median Cu concentration was higher in LC than FMC.

All pairwise correlations among metal concentrations on the log scale, regardless of stream, were positive and all but two (i.e., Cr vs. Cd, and Cr vs Hg) of the 45 pairs were significant ($p < 0.05$) (Table 2). Pairwise correlations between an individual metal and each of the two *pAR* measures were all negative. For 7 of 10 metals (Al, Cd, Cu, Fe, Mn, Pb, and Zn), there is strong evidence ($p < 0.05$) of negative correlation with streptomycin resistance, while there is only weak evidence ($p < 0.10$) of negative correlation for 3 of 10 metals (Al, Mn, and Zn) with kanamycin resistance.

The 1st PC calculated for all metals explains 58% of the total variability among N=165 sediment sample measurements. In descending order, Zn, Fe, Al and Cd had the largest relative contribution to this PC (coefficient values not shown). The eigen value for the 1st PC was 5.8 and indicates that this PC represents nearly 6 of the 10 metal dimensions in one summary measure. ANOVA results using the 1st PC scores for metals concentrations among streams shows a statistically significant ($p < .05$) stream effect. Linear contrasts among stream means show that MB PC scores are significantly ($p < .01$) lower than either FMC or LC.

Orthogonal regression of *pAR* PC scores vs. metal PC scores for each of the four streams (Fig. 3) shows a statistically significant ($p < 0.05$) and negative correlation between these summary measures for FMC and LC ($r = -0.33$, $r = -0.61$, respectively) but not for CC or MB.

Figure 4 is a scatter-plot of the 1st and 2nd DF scores for the *pAR* measures (Fig. 4A) and metal concentrations (Fig. 4B) among N = 165 sediment samples. The distinctness of each stream is shown by bounding each set of scores by a 50% normal contour around the stream centroid. The DF analysis of the *pAR* measurements shows that 93% of the variability among these data can be explained by the 1st DF. Greatest discrimination occurred between Lampert Creek and the other three streams along the 1st DF axis. The three SRS streams are relatively indistinguishable. For metals, the 1st DF accounted for 53%, while the 2nd DF accounted for an additional 31% of the variation among metals. Copper contributed most to discrimination along the 1st DF axis whereas Mn and Fe contributed most to discrimination in the 2nd DF. The uncontaminated stream (MB) separates distinctly from the contaminated streams along the 1st DF axis whereas the latter separate along the 2nd DF axis. In addition, only 19 of the 165 sediment samples were misclassified to a stream identity based on their metals data, and 10 of these 19 were between CC and FMC where the former is a tributary of and flows into the latter.

Spatial Pattern Analysis

Each contaminated stream (FMC, CC, and LC) shows a comparatively similar spatial pattern in that as the concentration of metals decreases with downstream distance, the *pAR* among sediment microbes appears to increase. These *spatial patterns* are clearly manifested by plotting the difference between the metal and antibiotic resistance PC scores vs. downstream distance (Fig. 5). In all streams, the difference pattern can be fit by a 2nd order polynomial that largely supports a negative relationship between metals concentration and antibiotic resistance, but suggests that this relationship may be stream reach dependent. However this curvilinearity is statistically significant ($p < .05$) in only CC, FMC, and MB. The relationship between metals concentration and antibiotic resistance is strictly linear and negative ($p <.05$) for LC.

Spatial Statistics Analysis

There is also strong evidence for a *spatial correlation* and *cross-correlation* among PC scores for metals and antibiotics (Fig. 6) that decreases with increasing separation distance (**h**) between sampling locations for the three contaminated streams (CC, FMC, and LC). This decrease in spatial correlation is manifest by the increase in $\gamma_S(\mathbf{h})$ (y axis) to its maximum value at which point the spatial correlation is effectively zero (upper panels of Figure 6). For these comparisons the estimated range parameter r_v (the intersection of vertical dotted line and **h** axis, Fig. 6) is much larger than the distance between sampling locations (100 to 200 m). At each of these streams, r_v is approximately the same distance for both antibiotic resistance and metals concentrations. For example, in FMC $r_v \cong 6500$m for antibiotic resistance and $r_v \cong 7300$m for metal concentrations and indicates that these variables are spatially correlated for up to approximately 7 km of stream channel length between sample measurements. In CC and LC, there is spatial correlation over a much smaller distance from ~500m in LC to >800m in CC. However, MB shows only a spatial correlation for the metals PC scores and not *pAR* PC scores (Fig 6D). Finally the $\gamma_{S_{12}}(\mathbf{h})$ between the *pAR* and metals PC scores indicate negative spatial correlation which decreases with increasing separation distance (lower panels of Fig. 6A-C). No model fit could be obtained to the MB cross-variogram data (lower panel of Fig. 6 D).

Discussion

The distribution of bacteria in stream ecosystems should be strongly influenced by the major unidirectional flow of water. Given the time most freshwater lotic systems have been in existence we might expect a uniform geographic distribution of bacteria and or their genes for various traits along the stream continuum [39]. Depending on the stream, somewhere between 10^4 and 10^6 bacteria per ml of water are continually imported into lower reaches. Point sources of contamination alter the natural variation in stream environmental conditions and thus the selection pressures. In this study we examined the spatial distribution of a specific class of antibiotic resistances among sediment bacteria along four streams in relationship to heavy metal contamination in those sediments. The evidence suggests that there is a downstream spatial pattern in the prevalence of resistance to two aminoglycosides that is a function of metal concentration in three streams with known metal contamination, whereas such a pattern does not exist for the uncontaminated stream. One criticism of these observations is that the concentration of the antibiotics themselves was not measured. It could be that the contaminated streams actually had higher concentrations of the antibiotics as selection agents for higher bacterial resistance. FMC does have known seep line inputs from previously unlined seepage basins upstream and CC is part of a catchment drainage system to the C-reactor area both with potential and unknown anthropogenic inputs in their entirety. However, LC which shows a similar spatial pattern (Fig. 6) to the other two metal contaminated streams is a high elevation montane stream and mining drainage catchment, defunct for more than two decades and unlikely to have had substantial or continuous aminoglycoside inputs from human activity. Nonetheless, measuring antibiotic concentrations in the sediments is requisite to future research.

For each sample collected in this study 12 variables were measured that included resistance to two antibiotics and the sediment concentrations of 10 heavy metals. Because of the large number of measurements, we sought a method to reduce the dimensionality of the per sample response vector by the use of PC Analysis. The PCs therefore are not phenotypic traits, but as summary measures that behave as do the traits, correlative relationships can be explored between them and other summary statistics that would otherwise require multiple analyses of the individual variables or traits, i.e., each metal concentration and aminoglycoside resistance, separately. We found that ~60% of the total variation among metal concentrations can be explained by the 1st PC which, therefore, serves as a summary variable in 1 dimension from among 10. All pairwise correlations among metals concentrations were positive and with only three exceptions were also statistically significant. This means that large PC scores are indicative of large metal concentrations and vice versa. Likewise, our evidence suggests that *pAR* PC scores can be viewed as a 1 dimensional summary measure of antibiotic resistance traits. Larger PC scores in this instance indicate a greater prevalence of aminoglycoside resistance.

The strong negative relationship between the 1st PC scores for metals and the 1st PC scores for antibiotic resistance for FMC and LC (Fig. 3) is interesting because it is counter to results from other recent studies [40-43]. Our data indicate that as heavy metal concentrations increase the prevalence of antibiotic resistance decreases. Although these two streams are from geographically different regions of the country (western montane and southeastern blackwater streams), they demonstrate a similar spatial pattern (Fig. 6).

Discriminant function analysis of the antibiotic resistance data and the metals data (Fig. 4) indicates that the streams can be reliably classified based on these variables. For example the patterns of aminoglycoside resistance in LC are distinctly different from that found in the southeastern streams (Fig. 4A). This difference was primarily due to higher levels of kanamycin resistance in LC and higher levels of streptomycin resistance in FMC and CC. While all streams had measurable levels of the ten metals, the uncontaminated stream (MB), can be discriminated from the three contaminated streams (Fig. 4B). We would expect from this evidence and the ANOVA results on PC scores that selection pressure for metal toxicity resistance traits is lower in ~~this stream~~MB and perhaps insufficient to produce spatial patterns or relationships with other associated and indirectly linked bacterial traits.

The most important finding of this study is that spatial cross-correlations, like the statistical correlations, between measures of antibiotic resistance and metal concentration are negative in contaminated streams as indicated by the cross-variogarm $\gamma_{S_{12}}(\mathbf{h})$ model fits in the lower panels of Figure 6A, B and C. Regarding the interpretation of cross-variogram models, Journel and Huijbregts [44] state

> "A cross-semi-variogram [i.e. cross-variogram] $\gamma_{k'k}(h)$ can take on negative values, whereas a direct semi-variogram [$\gamma(h)$] is always positive. A negative value of the cross-semi-variogram indicates that a positive increase in one of the variables (k') corresponds, on average, to a decrease in the other (k)."

Note that $\gamma_{S_{12}}(\mathbf{h})$ values with increasing **h** (Fig. 6A, B and C) are all negative. Note also the stronger negative spatial correlation between the antibiotic resistance and metal PC scores when sampling locations are nearer each other than at larger separation distances **h** $< r_v$.

A puzzling study outcome is the evidence in MB for strong spatial correlation for metals, but the absence of such for antibiotic resistance (Fig. 6D). There are just as many metal species in MB as the other streams and ~~T~~the only metal which is substantially lower in MB than all other streams was Hg (Fig. 4). Nonetheless, a strong spatial pattern and correlation persists for metal concentrations in MB for up to 9000 m (Fig. 6D). As an ancillary observation, recent research [45-47] has demonstrated the importance of metals bioavailability on bacterial toxicity. Bioavailability of various metals can be influenced by the amount of organic matter. We found that the average ash free dry weight (measure of organic matter content) of all sediment samples was 6.8 mg/kg in FMC and 13.9 mg/kg in MB, a statistically significant difference ($p < 0.001$). Perhaps the bioavailability for some metals may be substantially reduced due to the chelating or binding capacity of larger amounts of organic matter in the uncontaminated stream.

Spatial semivariance and correlation are dependent on the sampling scale, i.e. the separation distance **h** between sampling locations. We have shown relatively large range parameter estimates for metals correlation ($r_{v_met} \cong 7{,}800$m) and antibiotic resistance PC scores ($r_{v_abr} \cong 6{,}300$m) in FMC compared to both CC and LC ($r_{v_met} \cong 650$m $r_{v_abr} \cong 460$m, and $r_{v_met} \cong 820$m $r_{v_abr} \cong 880$m, respectively). Hubberten et al. [48] also reported large variogram ranges up to 55 km as well as considerably large nugget variances in a study of spatial variation among diatom assemblages in a large Siberian lake. Note also that range parameter estimates between FMC or MB and CC or LC differ by nearly an order of magnitude (Fig. 6). FMC and MB were sampled every 200m for nearly 13km each whereas CC and LC were sampled every 100m for 2.2km and 1.4km each. This indicates that either the biological processes in CC are dissimilar from FMC based on stream length, or that spatial patterns can be detected at more than one scale along the total length of stream. Stream processes will certainly dominate at the ecological scale of downstream transport and deposition [39]. The contaminant deposition process as well as water flow rate and water chemistry within the stream are likely therefore to govern the spatial distribution of bacteria.

Although previous studies have investigated the density of bacteria in transport [48], no attempts have been made to determine the spatial distribution of transported bacterial traits. We have shown spatial correlation among samples taken at either 100m or 200m intervals. Decreasing the sampling intervals to say 10m between sampling locations may reveal a smoother elevation in the fitted variogram from nugget to sill, but we predict that the scale of spatial correlation should not change.

Our samples, though spatially numerous, represent only a single snapshot in time. Should we expect to find similar spatial trends and correlations if we were to sample these sites on some temporal basis? Dent and Grimm [19] have shown a spatial as well as a temporal pattern in the variogram range parameter for nutrient concentration in a desert stream. In our study, spatial correlation and range parameter estimates are likely to change temporally only when there is substantial sediment turnover and transport. If, for example, a flood event were to occur simultaneously in both FMC and MB we would predict a measurable temporal progression toward and an eventual return to a variogram range value near to the one attained prior to the flood event in FMC due to continued contaminant inputs from the upstream seepage basin. MB on the other hand should demonstrate no such temporal progression or return capacity. Meteorological flood events do occur on the SRS watershed but perhaps more important is the past history of scouring in CC due to nuclear reactor secondary cooling water releases into this stream. Although it has been nearly 2 decades since such occurrences, metal contamination has been washed downstream to low lying wetland areas near its confluence with FMC. In fact, a post hoc linear contrast of metals PC score means shows that CC and MB are not statistically different while both are each significantly ($p < .05$) smaller that either LC or

FMC, the two most metal contaminated streams.

Another potential explanation for our counter-predictive results may be called the "cocktail effect". In addition to the multiple high concentrations of metals in FMC, this stream receives other input from a seepage basin near its headwaters on the SRS. These inputs include polycyclic aromatic and chlorinated hydrocarbons known also to be associated with antibiotic resistance traits among microorganisms [49]. One at a time, a specific metal or a hydrocarbon may indirectly select for increased antibiotic resistance. The interaction among two or more of these challenge agents introduces a complexity of selection that may produce nonadditive, interfering, and unexpected results. It should be noted that the "cocktail effect" hypothesis does not necessarily require pollutant mixtures of both metals and hydrocarbons. This is because the CC and LC streams both show the same spatial pattern as FMC. The former has no record of having received polycyclic aromatic and chlorinated hydrocarbon pollutants while the latter is unlikely to have had such.

In summary, we have found intriguing evidence for recurrent spatial pattern and negative correlation between metal contamination and resistance to two antibiotics among sediment bacteria in certain streams. It is intriguing because it is counter to our prediction from recently published evidence on water column bacteria where levels of exposure to heavy metals is positively correlated with resistance to multiple antibiotics [40-42]. It is highly unlikely that the obvious and statistically significant downstream pattern in antibiotic resistance and metal concentration *in all four study streams* as well as the statistically significant product moment correlations between these same two variables are due to random mutations among exposed microbes. It is plausible that resistance to these antibiotics is related to the environmental exposure. We initially used three distinct classes of antibiotics in our monitoring of FMC sediments with similar and positively correlated results suggesting that environmental exposure is related to distributional patterns of unrelated bacterial traits. Correlations between metal concentrations and antibiotic resistance are generally accepted even though few studies have collected sufficient samples to determine spatial patterns. Our data is indicative of the complex interactions between bacteria, their natural environs, and anthropogenic inputs that affect a multitude of outcomes over various temporal and spatial scales. We have provided evidence that a seemingly unrelated phenotypic trait in stream sediment bacteria, is likely a spatially correlated response to anthropogenic inputs.

In summary, we present a statistical perspective for interpreting multivariate response measures on unknown bacteria collected as a function of their environment. It is a study that is retrospective not prospective, observational not experimental. It is a field study not a lab study and as such, the standard precaution against concluding causality from correlation should be invoked. However, our findings elucidate intriguing spatial patterns consistent with prior research linking the occurrence of antibiotic resistance with elevated metal concentrations in the environment, although the directionality of that relationship in this case is unexpectedly inverted. These findings simply beg the question regarding which microorganisms, plasmids, and genes are at issue and which are the province of controlled and future laboratory experimentation. Such experiments should confirm the suggested linkage between metal concentrations (or PC scores) and the prevalence of antibiotic resistance by first showing that elevated metals concentrations (or PC scores) are correlated with an increased prevalence of metal resistance among bacteria in contaminated microcosms, but not in uncontaminated microcosms or in those where metals bioavailability has been compromised. This will also confirm the utility of the PC scores method for quantifying the relationship between these two variables. PC scores are used here as measure of explanation, not prediction.

Finally, we note a recent call for more research on the distribution of bacteria at the biogeographic scale [50]. We would argue however, that small scale and local ecological investigation of microbes will provide important and prerequisite knowledge for large scale insight.

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Figure Legends

Figure 1. Box-and-whisker plots of (A) the prevalence of antibiotic resistance (*pAR*) of antibiotic resistant bacteria colony count divided by the control colony count, for each of the four antibiotics used in the initial experimental treatment of Four Mile Creek stream sediment bacteria, and (B) the *pAR* data for each of the four study streams and for the two aminoglycosides only for comparison. The center line and point within each box plot indicates the average value. All points outside the upper whiskers indicate nonparmetric outliers beyond the 75th percentile + 1.5*(inner quartile range). Each horizontal dashed line runs through the arithmetic average *pAR* value of each aminoglycosides for Meyers Branch to allow comparison to the averages of the other three streams.

Figure 2. Box-and-whisker plots of all stream sediment sample concentration measurements for each of the 10 metals analyzed per sample among all four study streams (CC, FMC, LC, and MB) which as a set, are gradient shaded in grayscale for comparison among metals. The center line and point within each box indicate the median value. All points outside the upper whiskers indicate nonparametric outliers beyond the 75th percentile + 1.5*(inner quartile range).

Figure 3. Scatter-plot of the 1st principal component (PC) scores for antibiotic resistance vs the 1st PC scores for metals. Plot symbols and gray scale colors distinguish each set of study stream data. Also shown is the parametric statistical correlation (r) between these two variables within each stream. **Bold** orthogonal regression lines indicate the statistically significant ($p<.05$) relationships among streams.

Figure 4. Scatter-plot of the 1st and 2nd Disriminant function scores for the two incidences of antibiotic resistance measured, kanamycin and streptomycin (A), and the 10 different metals analyzed (B) per sediment sample for each study stream. Plot symbols distinguish each set of study stream data. Encapsulating circles indicate 50% normal contours around the group (stream) centroid.

Figure 5. Scatter-plots and statistical model fits of the differences between the 1st principal component (PC) scores for metals concentration and aminoglycoside resistance (difference=former-latter) versus downstream distance in each of the four study streams Four Mile Creek (FMC), Castor Creek (CC), Lampert Creek (LC) and Meyers Branch (MB).

Figure 6. Geostatistical variograms (upper two panels) and cross variograms (lower panel) of the 1st principal component scores for both antibiotic resistance and metals concentration and in each of the four study streams Four Mile Creek (A), Lampert Creek (B), Castor Creek (C), and Meyers Branch (D). Vertical dotted lines from the fitted curve to the ordinate axis indicate estimates of the variogram range (r_v) parameter. All direct or cross-variograms are standardized. Negative values of the cross variograms indicate negative spatial correlation between metal concentration and the prevalence of antibiotic resistance.

Table 1. Statistically significant ($p<0.05$) and positive pairwise correlations between the incidences of antibiotic resistance defined as the common logarithms of the ratio of antibiotic resistance colony counts to the total (control) colony counts among four antibiotics – kanamycin (KRatio), streptomycin (SRatio), tetracycline (TRatio), and chloramphenicol (CRatio) – for sediment bacteria obtained from Four Mile Creek in 1998. Note that each of the $\log_{10}$ *X*Ratios presented in this table is equivalent to an *pAR* measurement for that corresponding antibiotic (*X*) as defined in this paper.

	$\log_{10}$ KRatio	$\log_{10}$ SRatio	$\log_{10}$ TRatio	$\log_{10}$ CRatio
$\log_{10}$ KRatio	1.0000	0.4975	0.5524	0.5494
$\log_{10}$ SRatio		1.0000	0.4798	0.4789
$\log_{10}$ TRatio			1.0000	0.6688
$\log_{10}$ CRatio				1.0000

Table 2. Statistically significant ($p < .05$) and positive pairwise correlations among the common log transformed concentrations of 10 metals measured from the same sediment samples corresponding to bacterial colony plating experiments for antibiotic resistance. Only two of the 45 correlations were not significant, viz., Cd vs Cr, and Hg vs Cr. Hg was measured on the ppb scale.

	$\log_{10}$ Al	$\log_{10}$ Cd	$\log_{10}$ Cr	$\log_{10}$ Cu	$\log_{10}$ Fe	$\log_{10}$ Hg	$\log_{10}$ Mn	$\log_{10}$ Ni	$\log_{10}$ Pb	$\log_{10}$ Zn
$\log_{10}$ Al	1.0000	0.6567	0.2950	0.7308	0.7058	0.6314	0.6159	0.5515	0.6086	0.7278
$\log_{10}$ Cd		1.0000	0.1185	0.6406	0.7599	0.5986	0.6227	0.6264	0.3766	0.8240
$\log_{10}$ Cr			1.0000	0.2434	0.2343	0.1327	0.3275	0.2685	0.3147	0.2518
$\log_{10}$ Cu				1.0000	0.7072	0.6385	0.3943	0.4536	0.4708	0.6500
$\log_{10}$ Fe					1.0000	0.5131	0.7580	0.5866	0.4528	0.8490
$\log_{10}$ Hg						1.0000	0.2463	0.2950	0.3144	0.5035
$\log_{10}$ Mn							1.0000	0.6083	0.3435	0.8444
$\log_{10}$ Ni								1.0000	0.3862	0.6566
$\log_{10}$ Pb									1.0000	0.4100
$\log_{10}$ Zn										1.0000