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Hyporheic Microbial Community Development Is a Sensitive Indicator of Metal Contamination

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Accurate natural resource damage assessment necessitates monitoring organisms or communities that respond most sensitively to contaminants. Observational studies have demonstrated a correlation between fluvial heavy metal deposition and hyporheic microbial community structure. To establish a causal relationship between sediment metal content and the structure of colonizing bacterial communities, we performed a controlled field experiment. River sediments of 1.75−2.36 mm in diameter with five different contaminant concentrations were collected from an environmental metal contamination gradient. Sediments were sterilized and then recolonized by incubation in the hyporheic zone of an uncontaminated river (i.e., a common garden experiment was performed). A significant correlation between hyporheic microbial community structure and heavy metal contamination ($R^2 = 0.81$) was observed. The abundance of two phylogenetic groups was highly correlated with the level of heavy metal contamination (Group I, $R^2 = 0.96$; Group III, $R^2 = 0.96$, most closely affiliated with the α- and γ-proteobacteria, respectively). Microbial community structural responses were detected at metal concentrations an order of magnitude lower than those previously reported to impact benthic macroinvertebrate communities. We conclude that hyporheic microbial communities could offer the most sensitive method for assessing natural resource damage in lotic ecosystems in response to fluvial heavy metal deposition.

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Introduction

The contamination of aquatic environments with heavy metals is widespread and can result in natural resource damage (NRD) (*1*−*5*). NRD is defined as the change in biotic properties of an ecosystem in response to the introduction of an anthropogenic contaminant (*1*). Current methods for determining the presence and extent of aquatic resource damage due to heavy metal contamination include (1) determining the metal content of benthic macroinvertebrate tissues (*2*), (2) testing chronic and acute metal toxicity to invertebrates (*3*), (3) comparing fish communities in contaminated and reference streams (*4*), and (4) monitoring changes in aquatic algal community structure after exposure to heavy metals (*5*). We have proposed that hyporheic microbial communities are the first components of an aquatic food web to be affected by and respond to fluvial heavy metal deposition (*6*−*8*). Therefore, these communities may offer a more sensitive option for assessing metal-induced aquatic resource damage. This study examines how fluvial deposition of heavy metals affects the establishment of hyporheic microbial communities, determines levels of contamination at which effects can be measured, and evaluates the use of microbial communities for establishing NRD.

Lotic ecosystems are dynamic combinations of abiotic and biotic factors (*9*) with the stream channel and riparian zone representing the best-studied components (*10*). However, the hyporheic zone, the region of saturated sediments beneath the channel of a stream, has become recognized as the third major component of lotic ecosystems and one where physiochemical gradients play important roles in contaminant transport (*11*). Indeed, hyporheic microbial communities may constitute the majority of ecosystem biomass and activity (*12*−*14*), accounting for 76−96% of the ecosystem respiration (*15*), regulating nutrient cycling (*16*), and feeding grazing aquatic insects (*17, 18*). Therefore, although little is known about the relationship between microbial community structure and ecosystem functioning in the hyporheic zone, it is reasonable to expect that disruption of community structure (i.e., species richness, composition of species present, and relative abundances of those species (*19*)) could alter ecosystem functioning.

This study was designed to determine how fluvial deposition of heavy metals influences hyporheic microbial community structure development. Prior observational studies (*8, 20*) exploring this question were unable to control for potentially confounding environmental factors between streams such as organic matter quality and quantity, dissolved inorganic nutrient levels, temperature, consumer pressure, etc. This field experiment was designed to control for these factors. For example, the metal treatments were from environmental sources, so we sterilized them to remove potential competition between communities living on the sediments and the newly colonizing communities that we were interested in comparing. Other environmental factors were controlled by performing the experiment in a single stream along a reach with relatively homogeneous hydrogeomorphological properties (i.e., a common garden). Grain size has an inverse relationship with microbial biomass (*21*) and, in combination with slope, influences nutrient delivery rates (*22, 23*). To control for this, we used the same sediment size fraction for all metal treatment levels and planted sediment columns in an evenly sloped reach. Previous work demonstrated that there were no significant differences in the concentrations of NO_3^- and PO_4^{3-} along this stream reach (*20*). Therefore, our experimental design left the fluvially

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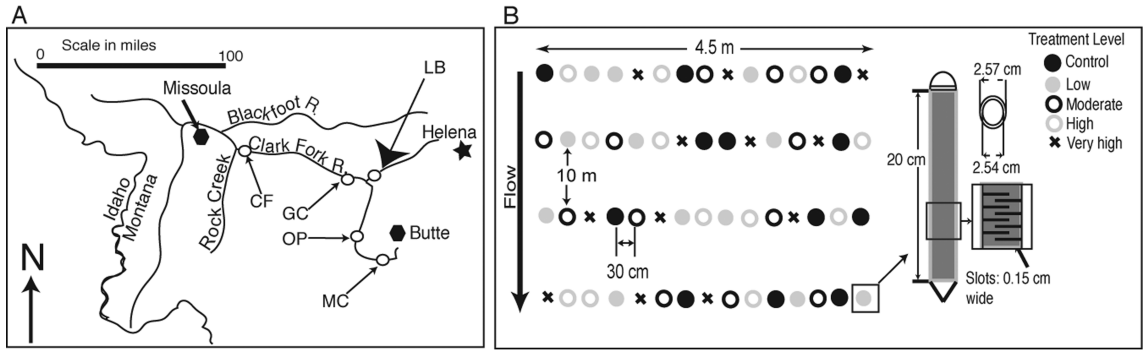

FIGURE 1. (A) Map of field site and locations from which sediments were gathered for use as metal treatments. (LB): Little Blackfoot River, site of common garden experiment and source of control sediments. (MC): Silverbow Creek at Miles Crossing, source of sediments for very high metal treatments. (OP): Silverbow Creek at Opportunity Ponds, source of sediments for high metal treatment. (GC): Clark Fork River at Gold Creek, source of sediments for moderate metal treatment. (CF): Clark Fork River at Rock Creek, source of sediments for low metal treatment. (B) Schematic of sediment columns and how they were arranged in the field experimental site (Little Blackfoot River). Location of treatment replicates within each experimental replicate is indicated.

TABLE 1. Concentration of Heavy Metals, Contamination Index (CI) values, and Carbon Associated with Sediments[a]

sediment collection site	treatement level	As (μg/g)	Cd (μg/g)	Cu (μg/g)	Pb (μg/g)	Zn (μg/g)	CI	Carbon (μg/g)
MC	very high	44.8 ± 4.4	0.9 ± 0.1	220 ± 14.2	111.4 ± 13.9	412 ± 45.8	1.81 ± 0.02	7.24 ± 2.3
OP	high	40.9 ± 2.3	0.8 ± 0.1	209.5 ± 12.3	68.1 ± 6.6	363 ± 29.3	1.74 ± 0.01	5.83 ± 0.22
GC	moderate	7.3 ± 0.3	0.1 ± 0.0	69.4 ± 4.0	16.0 ± 0.9	160.7 ± 5.7	1.12 ± 0.08	6.14 ± 0.47
CF	low	4.5 ± 0.1	0.2 ± 0.0	29.7 ± 1.0	9.8 ± 0.6	108.8 ± 4.0	1.01 ± 0.01	4.71 ± 0.39
LB	control	7.8 ± 0.4	bdl[b]	4.9 ± 0.2	9.4 ± 0.2	29.6 ± 0.6	0.49 ± 0.07	4.56 ± 0.47

[a] Sediments were collected from five different locations along a fluvial heavy metal contamination gradient and employed as our experimental treatments. See Figure 1 for definition of sediment collection sites. [b] bdl is below detection limit (< 0.005 μg/g).

deposited metals on the implanted sediments as the main factor controlling hyporheic microbial community structure.

Materials and Methods

Experimental Design. A controlled, replicated ($n = 4$, Figure 1) field experiment testing the effects of fluvially deposited heavy metals on hyporheic microbial community structure was conducted. Medium-grained sand fraction river sediments were collected by hand-sieving bulk sediment (0−20 cm depth) with stacked 2.36 and 1.7 mm stainless steel sieves from the streambed at the control location (The Little Blackfoot River) and from four different locations within the Clark Fork River. These sediments were employed as five levels of an environmentally relevant metal treatment (very high, high, moderate, low, and control) (Figure 1). It is important to note that because of historic mining activities in the drainage, metal concentrations in the Little Blackfoot are 3 to 5 times higher than might be expected in a truly pristine site (*8*). However, the scope and extent of the mining activity in western Montana makes finding a true "control site" impossible. Sediment collection locations were as follows: Little Blackfoot River (LB, control treatment) third-order stream, 46 31′11″ N, 112 47′33″ W, elevation 1324 m; Silverbow Creek at Miles Crossing (MC, very high metal treatment), a headwater tributary of the Clark Fork River, third-order stream, 46°06′28″ N, 112°48′17″ W, elevation 1497 m; Silverbow Creek at Opportunity Ponds (OP, high metal treatment), third-order stream, 46°06′28″ N, 112°48′17″ W, elevation 1497 m; Clark Fork River at Gold Creek (GC, moderate metal treatment), fourth-order stream, 46°35′26″ N, 112°55′40″ W, elevation 1271 m; and the Clark Fork River at Rock Creek (CF, low metal treatment), fourth-order stream, 46°49′34″ N, 113°48′48″ W, 1011 m. Heavy metal content of the sieved sediments is presented in Table 1. Additional detail describing the characterization of the sediment metal content is available in the Supporting Information. Sediments were gathered in one 12 h period (post spring runoff), placed in sterile containers, and transported to the laboratory on ice. After sterilization (3× for 1 h on 3 occasions) approximately 100 g of sediment representing each metal level was packed into separate sterile and acid-washed PVC slotted columns (Figure 1B). Packed columns were stored at 4 °C until placed in the test stream (<24 h). Sets of columns representing each treatment were buried in the hyporheic zone of the Little Blackfoot River (5−25 cm) at the heads of four sequential riffles. To bury the columns, we hammered a solid steel rod of the same diameter as the sediment columns approximately 30 cm into the hyporheic zone; sediment columns were driven by hand to ∼30 cm as the steel rod was removed and then covered by cobbles. Placing the columns at the heads of riffles ensured that the pore water at these locations would be dominated by the influx of surface water, thus reducing variability between experimental replicates due to potential groundwater influx. After four months the columns were removed from the streambed, rinsed on site in streamwater, transferred to sterile sampling bags (Fisher Scientific Company, Hanover Park, IL), and stored on dry ice until returned to the laboratory. At the laboratory, sediments were lyophilized using a Freezemobile 24 (Amoco Productions Co., Tulsa, OK) and then stored at −70 °C prior to analysis.

DNA Extraction. One gram samples of lyophilized sediment were extracted on the basis of the method of Yu and Mohn (*24*) with modification as described previously (*20*).

Community Analysis. *Denaturing Gradient Gel Electrophoresis and Gel Pattern Analysis.* PCR amplification for denaturing gradient gel electrophoresis (DGGE) analysis was performed using the generally conserved 16S rRNA gene primer pair 536 fc and 907r as described previously (*20*). Microbial community composition was determined by DGGE using the BioRad D Gene System (BioRad Laboratories, Hercules, CA) and a linear gradient of denaturants ranging from 25% to 60% urea:formamide in a 6% acrylamide gel.

Electrophoresis run conditions and standards for comparative analyses were as described previously (*20*). DGGE images were analyzed with GelCompar v.4.0 software (Applied Maths, Kortrijk, Belgium). All band patterns were normalized to the positional markers in each gel, thereby eliminating variation between individual gels. A similarity index comparing all samples was calculated on the basis of the Dice coefficient (Supporting Information) and used as the input for a nonmetric dimensional scaling (NMDS) analysis of community composition. Phospholipids [phospholipid fatty acid analysis (PLFA)] were purified and analyzed according the method of White and Ringelberg (*25*) with slight modifications as described previously (*8, 25*) (see the Supporting Information for more detail).

Real-Time Quantitative PCR. A suite of group-specific primers corresponding to three major phylogenetic groups (Groups I, II, and III; most closely related to the α, β, and γ-proteobacteria, respectively) were used to monitor group-level abundance as previously described (*20*). Real-time quantitative PCR (qPCR) reactions were performed using a Bio-Rad iCycler (BioRad Laboratories) and a SYBRGreen I detection method as described previously (*20*). Typically, standard curves for qPCR reactions were linear across 5 orders of magnitude (10^7-10^2 copies). Samples that fell above or below this linear range were diluted or concentrated, respectively, to bring the target copy number into the linear range of detection. All qPCR values are expressed as Log 16S rRNA gene copy number g^{-1} (dry wt) of sediment.

Geochemical Analyses. Total recoverable metal content and total carbon of sediments were analyzed at the end of the experiment by inductively coupled plasma mass spectrometry (ICP-MS) (see the Supporting Information for complete methods).

Statistical Analysis. Within each experiment (i.e., each set of columns placed at the head of a riffle), all four treatment levels were replicated three times and were analyzed separately by DGGE, PLFA, and qPCR. The relationship between the microbial community response variables and heavy metal treatments was assessed using NMDS, principle components analysis (PCA), multivariate and univariate analysis of variance, and linear regression. When applied to DGGE data, NMDS analysis produces sets of coordinates that can be used to graphically represent relative differences in community composition between treatments and replicates. Additional detail on the application and interpretation of NMDS to molecular microbial community analysis is provided in the Supporting Information. Analysis of variance and posthoc multiple comparison tests (Tukey−Kramer) determined which levels of metal amendment resulted in significant changes in biotic response variables ($p < 0.05$). Linear regression modeled microbial community responses in relation to heavy metal treatments. Statistical tests were performed with NCSS 2001 software (NCSS, Kaysville, UT) and SPSS software v. Ten (SPSS Inc.) for DNA and PLFA data, respectively (see Supporting Information for more detail). qPCR data were log transformed to meet assumptions of ANOVA and MANOVA.

Results and Discussion

Metal Treatments and Contamination Index (CI). Metal treatments consisted of sediments gathered from locations within the Clark Fork River drainage. This river was contaminated by the introduction of mine waste from large-scale mining around Butte, MT (*26*). Consequently, a gradient of elevated sediment heavy metal loads extends ~500 km downstream from the source, with sediment metal concentrations decreasing logarithmically downstream (*27*). Sediment metal concentrations used for the experimental treatments are presented in Table 1. By using sediments from this gradient of contamination as the source for metal treatment materials, we encompassed all of the potential differences in form and bioavailability of fluvially deposited heavy metals.

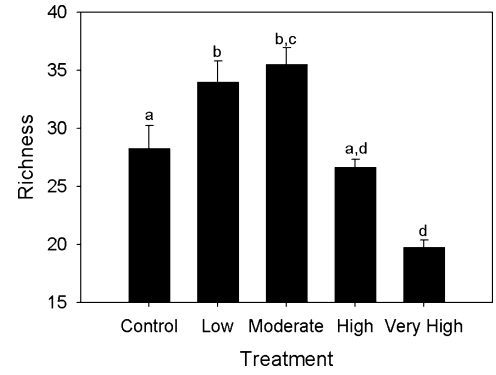

FIGURE 2. Bacterial species richness (based on the number of detectable DGGE bands) associated with each treatment level. Treatment levels that share a letter are not significantly different from one another ($p > 0.05$).

Rather than attempting to determine individual metal effects, we characterized treatment levels by converting concentrations of sediment-associated heavy metals to contamination index (CI) values (*28*) to which community responses were related (Table 1). Mineral assemblages of fluvially deposited mine waste are very complex, consisting of irregular mixtures of metal oxides, iron oxyhydroxides, and metal ions and complexes sorbed to oxyhydroxide-coated surfaces and chelated with organic matter (*26, 28*). These fluvial metal deposits consist of a variety of covarying heavy metals, making the determination of individual metal effects difficult (*29*). By employing CI, we avoid problematic and potentially erroneous attribution of contamination effects to individual metal contaminants. Similar applications of a contamination index have been used to evaluate the response of benthic macroinvertebrate assemblages to a suite of heavy metal contaminants (*30*).

Natural sediment surfaces and those coated with Fe and Mn oxyhydroxides (enhanced as a result of mining contamination) can adsorb organic matter (*31*). No significant difference in carbon between treatments was detected (range = 3.78−11.71 μg C g^{-1}; $F_{\text{organic carbon}} = 1.024$, and $p = 0.441$) (Table 1), indicating that microbial responses to the applied treatments were due to metals and not the ability of sediments to adsorb organic matter. However, because carbon quality and quantity can affect microbial community structure (*32, 33*), percent carbon was analyzed separately from CI.

Community Composition. Fluvial deposition of heavy metals affected hyporheic microbial community richness. DGGE pattern analysis was used to assess differences in microbial community composition between metal treatments. Species richness as measured by DGGE band number differed between treatments ($F_{\text{richness}} = 6.68$ and $p = 0.01$), with the highest richness values in the low and moderate metal treatment levels (mean band numbers = 35 and 34, respectively), while significantly lower richness values were obtained for the control sediment and the high and very high metal treatments (Figure 2). If elevated metal levels are viewed as a disturbance, then this pattern of species richness is in agreement with the intermediate disturbance hypothesis (*34*). Our results suggest, that during initial community establishment, species richness increases to moderate levels of contamination and is only reduced at relatively high levels. This pattern of species richness may also be due to a combination of physical factors and toxic metal effects. Fe and Mn oxyhydroxide coatings could facilitate colonization and increase richness by acting as sorption sites for bacterial cells where toxic components are not prevalent (*35, 36*). However, metal toxicity may prevent species from becoming

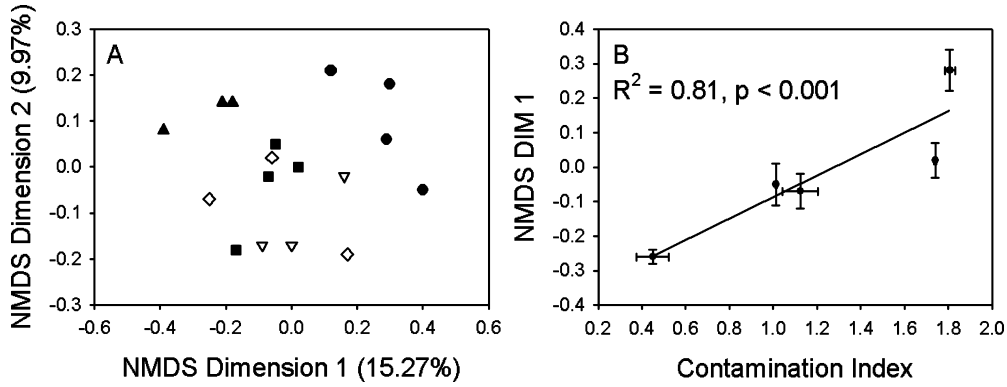

FIGURE 3. (A) NMDS plot of the first and second NMDS dimensions relating DGGE patterns of microbial community compositions between treatments. Symbols representing communities present in each treatment level are as follows: ▲ control; ◇ low; ■ moderate; ▽ high, and ● very high. Percentages given in axes labels are the % variation in community composition explained by the respective NMDS dimensions. (B) Linear regression of the contamination index versus dimension 1 of the NMDS analysis. Points and error bars plotted represent the mean ± standard error of each variable. The strength of the linear relationship is indicated by the regression coefficient (R^2) on the plot.

TABLE 2. Correlation Coefficients and Significance Values for Linear Regressions of Microbial Community Response Variables versus Contamination Index, Percent Carbon, and Total Multiple Regression Model[a]

	richness		community composition		Group I		Group II		Group III	
independent variable	R^2	*p* value	R^2	*p* value	R^2	*p* value	R^2	*p* value	R^2	*p* value
CI	0.04	0.81	0.86	<0.01	0.96	0.02	0.83	0.09	0.96	0.02
%C	0.17	0.59	0.45	0.03	0.70	0.16	0.20	0.55	0.69	0.17
total model	0.01	0.57	0.87	<0.01	0.98	0.07	0.77	0.28	0.96	0.11

[a] CI and %C are used as predictor variables. In the total model row, R^2 = adjusted R^2.

established where heavy metal ions make up a larger component of the coatings in highly contaminated areas (*37*−*39*). Because richness does not exhibit a linear relationship with metal contamination (Figure 2), this community-level response may not be a good predictor of aquatic resource damage. This interpretation is contrary to previous studies suggesting that physiological stress caused by metal toxicity leads to selection of less diverse communities comprised of metal resistant populations (*40*−*42*).

The composition of hyporheic microbial communities was directly related to CI. A similarity matrix of DGGE banding patterns comparing communities between treatment levels was constructed and analyzed by nonmetric dimensional scaling (NMDS) (Figure 3). NMDS indicated that community composition in controls and very high treatments were different from communities present in other treatment levels (Figure 3A). While NMDS alone did not separate communities inhabiting the low, moderate, and high metal treatments, the first dimension of the NMDS analysis of community composition plotted against the CI indicated a predictable community response ($R^2 = 0.81$ and $p < 0.001$, Figure 3B). Similarly, total benthic invertebrate species richness is also a relatively insensitive measure of heavy metal contamination (*30, 43*). Rather, the specific composition of benthic invertebrate assemblages is a more reliable indicator of heavy metal effects in aquatic ecosystems (*30, 43, 44*). A similar effect appears to exist in hyporheic microbial communities.

We employed PLFA as a second and independent measure of community structure for assessing relationships to CI. Similar to our approach with the DGGE data, PLFA patterns were analyzed by an ordination method (principle components analysis; PCA). The first principle component (PC-1) explained 35% of the variation in the PLFA data and also showed a strong correlation between CI and community structure ($R^2 = 0.98$ and $p = 0.002$). Fatty acids thought to be synthesized primarily by actinomycetes and the ratio of fungal to bacterial biomarkers were significantly correlated with the CI ($R^2 = 0.86$, $p = 0.032$ and $R^2 = 0.86$, $p = 0.30$, respectively). Individual fatty acids that were positively correlated ($p < 0.05$) with the index were 16:1ω5c, 16:1ω7c, i15:0, 16:0, i15:1, i17:0, 14:0, and i16:0. The indicated shorter chain monoenoic and branched fatty acids are typical constituents of bacterial membranes. The indicated fatty acids have been hypothesized to represent gram positive abundance (see ref *29* and references therein). Steric acid (18:0), 10me16:0, 18:2ω6, and 18:1ω9 were negatively correlated with the CI ($p < 0.05$). The indicated fatty acids are suggested to be of actinomycete and fungal origin, respectively (*29*). Differences in carbon associated with metal treatments did not influence this relationship (Table 2).

Others have observed similar relationships between microbial community structure and heavy metal contamination in soils (*29, 41*). However, the strength of the relationship between metal contamination and hyporheic microbial community composition reported here appears to be stronger than that observed in soils (*29, 41*). These high correlation values imply that heavy metals associated with sediment surfaces in the oxic shallow hyporheic zone are bioavailable. This contradicts the prediction that at the alkaline pH (7.9−8.3) observed in the sediment source sites, heavy metals will be in either oxide forms or chelated to surface-associated iron oxyhydroxide coatings, both assumed to be relatively unavailable forms (*45*) (i.e., oxide forms or sorbed to surface-associated iron oxyhydroxide coatings). Previous work in the Clark Fork River demonstrated that diel variations in stream pH, attributed to ecosystem respiration, can result in release of sorbed metal ions from oxyhydroxide surfaces (*46*). Further, biofilms on sediment surfaces create spatial variability in pH and redox, two parameters that affect the bioavailability of heavy metals (*47*). The adsorption, establishment, and growth of bacterial communities on sediments may alter bioavailability of heavy metals and selection for metal tolerant organisms. The strong correlation between community composition and CI leads us to hy-

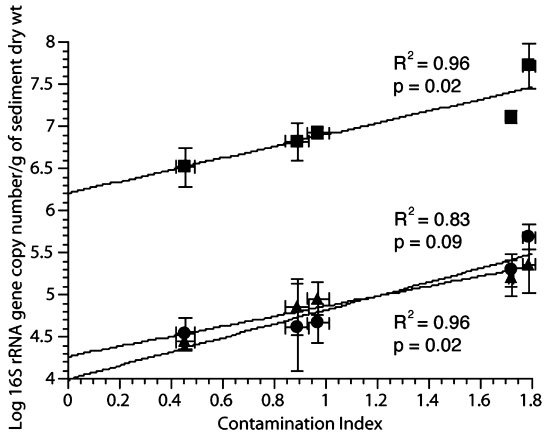

FIGURE 4. Means and standard errors of log transformed abundance of Group I (■), Group II (●), and Group III (▲) versus the contamination index.

pothesize that the association of these communities with contaminated sediment surfaces may explain the sensitivity of microbial communities.

Phylogenetic Group Abundance as Determined by qPCR. Phylogenetic Groups I, II, and III, most closely affiliated with the α, β, and γ-proteobacteria, respectively (*20*), were previously identified as the most abundant groups associated with hyporheic sediments (*20*). Relative abundance of these groups in this study was in agreement with previous observations describing their distribution in the hyporheic zone of pristine streams (*20*). For example, Group I was significantly more abundant than Groups II or III in all samples ($F_{\text{Groups I,II,III abundance}} = 40.4$ and $p < 0.001$). Abundance of Groups II and III was not significantly different regardless of metal treatment level ($F_{\text{groups II,III abundance}} = 0.635$ and $p = 0.431$). Conversely, linear modeling of phylogenetic group abundance revealed strong significant ($p < 0.05$) positive linear correlations between CI and the abundance of Groups I and III (Table 2 and Figure 4). Group II abundance displayed a similar though not significant trend (Table 2 and Figure 4). These results indicate that the absolute abundance of each group was affected more than the relative distributions of the three groups. Significant effects of percent carbon on group-level abundance and CI were not detected (Table 2).

Although elevated heavy metals may promote the establishment of Groups I and III, other factors likely influence this relationship as the community develops. Previous studies of intact hyporheic microbial communities along a contamination gradient indicated that Group II can be selected for in heavy metal contaminated environments, while Groups I and III are selected against (*8, 20*), and that these relationships vary seasonally (*8*). Species within Groups I and III may be more metal tolerant and thus able to colonize metal contaminated substrates. However, it appears that as a hyporheic microbial community continues to develop in the presence of fluvial heavy metal contamination Group II organisms eventually replace Groups I and III as dominant members of the community. Elevated relative abundance of α-proteobacteria along with decreases in gram positive and other gram negative lineages has been noted in soils amended with metal-rich sewage sludge (*42*). Also, Roane and Pepper, identified γ-proteobacteria that are resistant to Cd, Cu, and Zn, three of the metals included in our index (*37, 48*). Members of these phylogenetic groups present in the hyporheic zone may be resistant to the metal treatments we applied and thus able to colonize the contaminated sediments. Furthermore, the proteobacterial groups we measured are common and numerically abundant members of aquatic heterotrophic microbial communities (*8, 20*). Therefore factors affecting their abundance could alter carbon cycling rates and secondary productivity in situ. Ongoing work by our group is examining these aspects of the hyporheic biotic response to chronic heavy metal contamination.

The data presented herein suggest that the richness, composition, and abundance of select phylogenetic groups within the hyporheic microbial community are affected by fluvially deposited heavy metal contamination. We detected changes in hyporheic microbial community structure at very low levels of metal contamination, approaching an order of magnitude lower than that at which benthic macroinvertebrates exhibit a measurable response. On average, sediment metal concentrations equivalent to 84 μg/g As, 100 μg/g Cu, 36 μg/g Pb, or 355 μg/g Zn are required to cause a shift in benthic macroinvertebrate community structure (*2, 30, 43, 44, 49*). These contamination levels are almost an order of magnitude higher than the low metal treatment (4.5 μg/g As, 30 μg/g Cu, 9.8 μg/g Pb, and 110 μg/g Zn) used in this experiment. Thus, the composition of hyporheic microbial communities and abundance of Groups I and III are potentially more sensitive indicators of heavy metal contamination in streams than are benthic macroinvertebrates.

Acknowledgments

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Supporting Information Available

Detailed descriptions of experimental design and methodologies employed. This material is available free of charge via the Internet at http://pubs.acs.org.

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