RESEARCH ARTICLE

Functional gene array–based analysis of microbial communities in heavy metals-contaminated lake sediments

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Abstract

Lake DePue (IL, USA) has been contaminated for > 80 years by an adjacent Zn-smelting facility. Previous work indicated that sulfate reduction increased and biomass declined as pore-water metal concentrations increased, while 16S rRNA gene profiles remained relatively stable. To better understand this phenomenon, the sediment microbial community structure and functional potential were investigated using a functional gene microarray (GeoChip) targeting > 10 000 functional genes. Nonmetric multidimensional scaling and clustering analyses showed that the overall community structure was similar across all sites based on the relative abundance of all detected genes, but some individual gene categories did show differences. A subset of sulfate reduction genes (*dsr*) and the most relevant metal resistance genes were more abundant than other categories and were highly correlated with metal contamination. The most significant correlations were between pore-water metal concentrations and *dsr*, with Zn, Cd, and Mn as the most predictive for the presence of *dsr*. These results suggest that metal contamination influences sediment microbial community structure and function by increasing the abundance of relevant metal-resistant and sulfate-reducing populations. These populations therefore appear to contribute significantly to the resistance and stability of the microbial communities throughout the gradient of metal contamination in Lake DePue.

Introduction

Environmental contamination is a widespread legacy of past industrial activities (Konstantinidis *et al.*, 2003; Becker *et al.*, 2006; Joynt *et al.*, 2006; Zhang *et al.*, 2006; Wang *et al.*, 2007), and sediments are often the final repository for these pollutants, especially metals (Giusti, 2001). However, information is lacking about the consequences of metal pollution on sediment biogeochemistry. Previous studies of microbial communities at contaminated sites have focused on culturable microorganisms (Reyes *et al.*, 1999; Konstantinidis *et al.*, 2003), phospholipid fatty acid analysis (Kandeler *et al.*, 2000; Abaye *et al.*, 2005; Hinojosa *et al.*, 2005; Åkerblom *et al.*, 2007), genetic fingerprinting (Frey *et al.*, 2006; Li *et al.*, 2006; Linton *et al.*, 2007; Lazzaro *et al.*, 2008), and PCR-based cloning and sequencing of 16S rRNA genes (Joynt *et al.*, 2006; Lazzaro *et al.*, 2008), but no comprehensive study of a metal-contaminated site has yet integrated measurements of metals concentration and speciation with alterations in community structure, diversity, and specific functional genes. In this study, a comprehensive functional gene microarray (GeoChip 2.0) (He *et al.*, 2007) was used to assess potential impacts of metal contamination on the structure and functional potential of sediment microbial communities.

In general, environmental disturbances, such as heavy metal contamination, are thought to exert a selective pressure that induces compositional change within the affected microbial community (Junca & Pieper, 2004; Anderson

et al., 2009). Wang *et al.* (2007) reported a negative correlation between enzymatic activities and metal concentrations for microbial communities near a Cu smelter. The community structure, as determined by 16S rRNA gene–based DGGE, was also affected with clear differences observed between high- and low-contaminant-level communities (Wang *et al.*, 2007). Becker *et al.* (2006) found a high variability in the metabolic potential of microbial communities within *c.* 30 cm distance of Pb- and Cr-contaminated soil samples. The coefficient of variation for both metabolic potential and metal concentration of the contaminated site was three to ten times higher than for background samples (Becker *et al.*, 2006). These studies indicate that metals contamination generally has a negative impact on microbial community structure and function.

The experimental site, Lake DePue (Bureau County, IL, 41°19′N, 89°18′W), a small backwater lake on the Illinois River, has been contaminated for almost 80 years by waste from an adjacent zinc-smelting facility (http://www.epa.state.il.us/community-relations/fact-sheets/new-jersey-zinc/index.html). Metal contaminants originate at the northeast corner of the lake, near a man-made creek that drains the grounds surrounding the facility. Metal levels in sediments were substantially higher than documented at other contaminated sites, with Zn concentrations as high as 99 000 mg kg^{-1} (Gough *et al.*, 2008a). Previous studies of the lake sediments revealed that while metal contamination levels vary significantly in different areas of the lake, other environmental parameters such as nutrient concentrations (e.g. organic carbon and nitrogen) are generally constant (Gough *et al.*, 2008a), except that total organic carbon was higher in sediments with the highest metal contamination levels. Additionally, significant correlations between higher pore-water Zn and As concentrations with reduced microbial biomass (Gough *et al.*, 2008a) and with higher sulfate reduction rates (Gough *et al.*, 2008b) suggested that metal contamination likely has impacted microbial abundance and activity. Community structure inferences using terminal restriction fragment analysis of the 16S rRNA gene revealed some differences in the archaeal community associated with metal contamination levels; however, no significant associations were indicated for bacterial diversity, richness, or community composition (Gough & Stahl, 2011).

Here, we present results further examining the bacterial communities focusing on functional genes using GeoChip. The main hypothesis tested in this study was that metal contamination level has a significant effect on the diversity and structure of functional gene groups. The objectives for this study were to (1) characterize the functional gene diversity of the metal-contaminated site, (2) identify key functional groups important to survival at this site, and (3) identify key environmental factors shaping the sediment microbial communities.

Materials and methods

Site description and sampling

To allow direct comparison with previous results, archived sediment samples collected in September 2000 were used. Samples were collected representing two depth intervals (0–1 and 1–2 cm) for triplicate sediment cores (e.g. S1–1, S1–2, and S1–3) from five sites in the lake and stored at –80 °C (Gough *et al.*, 2008a) (Fig. 1). These sediments represent a range of metal contamination levels from 3100 to 21 000 mg kg^{-1} total Zn and 0.30 to 3.8 μM pore-water Zn. Concentrations of additional metals are in Table 1. More detailed information on the contaminants is documented in Gough *et al.* (2008a).

Samples representing the two depth intervals for a single core were pooled to produce sufficient DNA for analysis. Previously reported analytical results (metal concentrations and environmental variables) were averaged for the pooled sample depths to allow direct comparison.

DNA extraction, amplification, and labeling

Due to excessive divalent metal ion contamination (Gough *et al.*, 2008a), which causes premature DNA precipitation (Kejnovsky & Kypr, 1997), sediment samples were prewashed with 40 mM EDTA (pH 7.2) as previously described (Gough & Stahl, 2011). DNA was extracted using a freeze-thaw method (Zhou *et al.*, 1996) using PIPES as the extraction buffer and purified using a Wizard® PCR Preps DNA Purification System (Promega, Madison, WI) following the manufacturer's instructions. This method was tested on several different soil types and for most soils resulted in *c.* 70% or greater lysis efficiency and had a crude DNA yield within the expected range (Zhou *et al.*, 1996). In addition, this extraction method provides high molecular weight DNA, which is important in the subsequent amplification step. DNA was checked for quality using a NanoDrop™ 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE) and for quantity using Quant-It™ PicoGreen® (Invitrogen, Carlsbad, CA).

Whole-community genome amplification was performed on *c.* 100 ng DNA using the TempliPhi amplification kit (Amersham Biosciences, Piscataway, NJ) in a buffer modified to increase amplification efficiency and reduce representational bias (Wu *et al.*, 2006). To control for experimental error associated with amplification and hybridization, each individual sample was amplified in triplicate as technical replicates. Minimal bias for pure culture or community DNA was observed when at least 1 ng DNA was used, and bias for 10 ng DNA was 0.021–0.161, where 0 indicates no bias and 1 indicates total bias (Wu *et al.*, 2006). Amplifications were

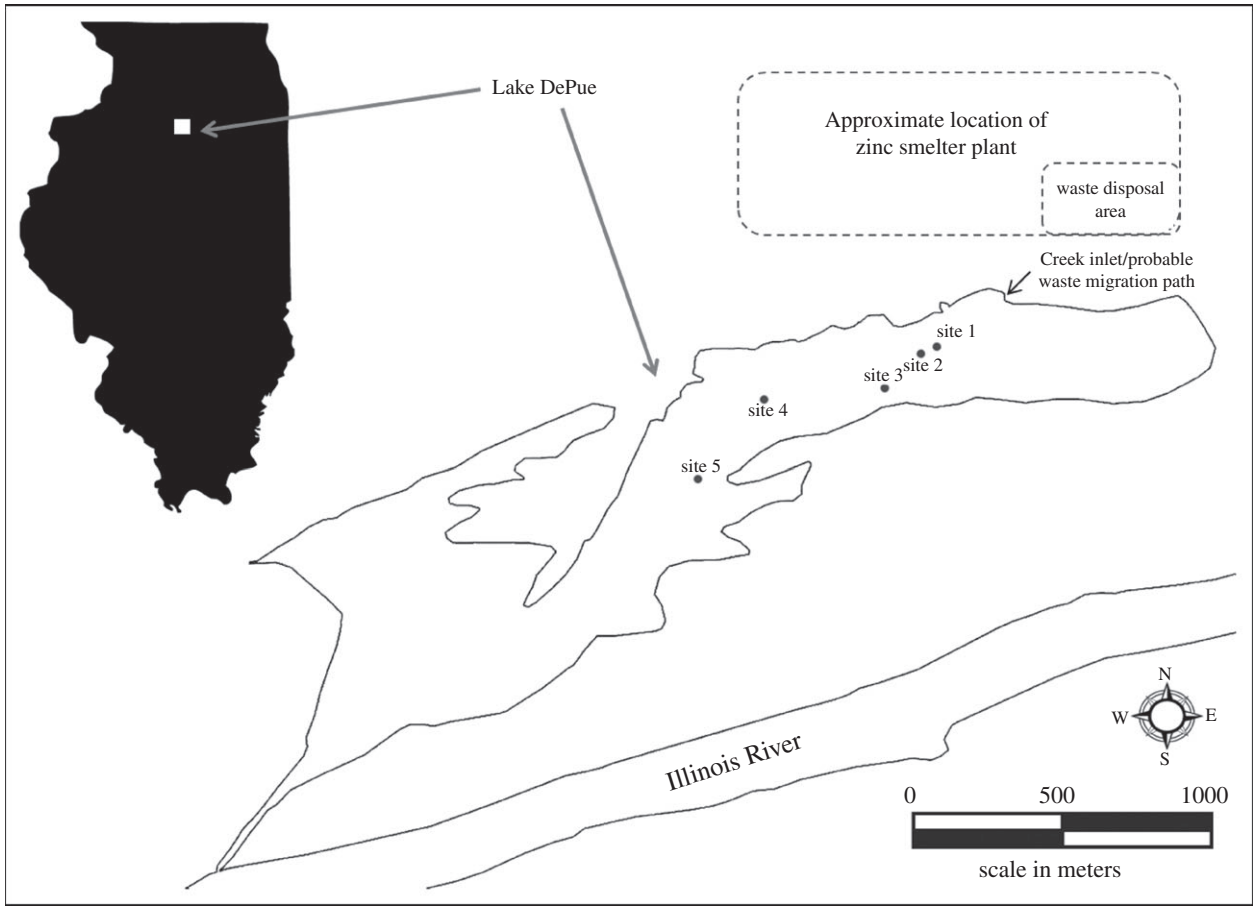

Fig. 1. Map of Lake DePue. Sampling sites are indicated.

incubated at 30 °C for 3 h and then denatured by boiling for 5 min at 99.9 °C in the presence of random octamers (15 μg) and then fluorescently labeled by incubating at 37 °C for 3 h in a reaction solution containing 250 μM dNTP (125 μM dTTP) (USB, Cleveland, OH), 1 mM Cy5 dUTP (Amersham Pharmacia Biotech, Piscataway, NJ), and 40 U of Klenow fragment (Invitrogen).

GeoChip hybridization and data preprocessing

Labeled DNA was purified with a QIAquick PCR purification kit (Qiagen, Valencia, CA), dried, and resuspended in 130 μL hybridization solution [50% formamide, 3 × SSC (saline sodium citrate) buffer, 0.3% SDS, 0.7 μg μL^{-1} herring sperm DNA, and 0.85 mM DTT]. The fluorescently labeled DNA was hybridized with GeoChip 2.0 (He *et al.*, 2007) on an HS 4800™ Pro Hybridization Station (TECAN US, Durham, NC) in triplicate at 42 °C for 10 h. Hybridized slides were scanned on a ScanArray 5000® Microarray Analysis System (PerkinElmer, Wellesley, MA) at a resolution of 10 μm. The laser power and photomultiplier tube (PMT) gain were adjusted for each slide to obtain maximum dynamic range while saturating positive control spots.

Scanned images were quantified using ImaGene® version 6.0 (BioDiscovery, Inc., Los Angeles, CA) and processed in the Microarray Data Manager (MGM) system on the Institute for Environmental Genomics (IEG) website (http://ieg.ou.edu/microarray). Probes were considered positive if the signal-to-noise ratio (SNR) was ≥ 2.0, and the probes were detected in at least two of the three technical replicates. Outliers were removed based on Grubbs' test of outlier at $\alpha = 0.01$ (Grubbs, 1969). The signal intensities of each remaining probe were then averaged across the three technical replicates to provide one dataset per sample.

GeoChip data processing and statistical analysis

Hierarchical cluster analysis was carried out using CLUSTER and visualized in TREEVIEW. All other analyses were performed in R version 2.6.1 with the packages vegan version 1.11–3, ecodist version 1.1.3, qvalue version 1.1,

Table 1. Chemical summary of Lake DePue sediments (September 2000)*

	S1	S2	S3	S4	S5
pH	7.8	7.9	7.7	7.6	7.8
DOC (mM)	1.77	1.80	1.65	1.25	1.36
TOC (mg kg^{-1})	53 000	57 000	43 000	47 000	40 000
Sulfate (mM)†	3.8	2.1	ND	ND	0.75
Total metal concentrations					
Cd (mg kg^{-1})	89	91	37	24	14
Cu (mg kg^{-1})	801	434	237	177	101
Mn (mg kg^{-1})	963	834	777	829	764
Pb (mg kg^{-1})	260	288	162	119	71
Zn (mg kg^{-1})	21 383	13 862	5963	4255	3104
Pore-water metal concentrations					
As (μM)	1.80	0.553	0.054	0.083	0.092
Cd (μM)	0.015	0.009	0.007	0.006	0.005
Cu (μM)	0.203	0.091	0.047	0.243	0.053
Cr (μM)	0.168	0.059	0.061	0.077	0.096
Mn (μM)	24.4	19.8	28.9	33.3	26.9
Pb (μM)	0.019	0.015	0.005	0.004	0.006
Zn (μM)	3.83	1.21	0.348	0.298	0.564

ND, not measured.

*Summarized from Gough *et al.* (2008a).

†Sulfate concentrations from Gough *et al.* (2008b); values are the average of measurements from 0–1 and 1–2 cm.

and others. Nonmetric multidimensional scaling (NMDS) was used for unconstrained ordination, and results were quantitatively evaluated with analysis of similarity (ANOSIM) (Clarke & Ainsworth, 1993).

Canonical correspondence analysis (CCA), Mantel test, and variation partitioning analysis (VPA) were used to evaluate two-factor relationships. Three categories of environmental variables were considered as independent variables during data analysis: basic sediment characteristics (five variables), individual pore-water metal concentration (eight variables), and total metal concentration (six variables). Environmental variables were first standardized by Z transformation to resolve differences in scale and then prepared in three categories for further analyses (Sokal & Rohlf, 1995).

A detailed description of all statistical analyses performed is in Data S1. The microarray data presented are available at http://ieg.ou.edu/4download/.

Results

Functional gene diversity

For clarity, here and throughout the text, the term sample will be used to indicate individual soil samples (i.e. individual cores), and site will be used to indicate the aggregate data (i.e. average of replicates) from each site. A total of 2034 gene variants [*c.* 250–600 per site (Table 2)], representing 18.6% of the gene sequences on the GeoChip 2.0, were detected. These gene numbers are similar to those detected in other studies using this same array [504–656 gene variants in soils from strawberry fields (Reeve *et al.*, 2010), 177–1076 in uncontaminated soils near oil fields (Liang *et al.*, 2011), *c.* 500–750 in uncontaminated soil (He *et al.*, 2010)]. The number of gene variants detected and the α-diversity of functional gene variants were highly variable within (between biological replicates) and across sites (i.e. sites 1–5) (Table 2), but no significant differences in diversity indices were observed, suggesting that contaminant level did not affect the overall functional gene diversity. Approximately 15–30% of genes detected in one sample were shared with another sample, regardless of whether the samples were from the same site or not. About 10–20% of all gene variants detected were unique to a specific

Table 2. Functional gene diversity, similarity, and evenness

	S1	S2	S3	S4	S5
S1	17.3 ± 4.9*	23.8 ± 2.9	20.4 ± 5.0	23.1 ± 5.6	24.6 ± 2.4
S2†		21.3 ± 0.5	19.5 ± 6.6	24.8 ± 8.2	24.6 ± 1.4
S3			9.9 ± 3.5	20.8 ± 7.0	25.1 ± 3.2
S4				14.0 ± 6.9	24.1 ± 2.7
S5					10.7 ± 4.0
S	403 ± 162	587 ± 15	249 ± 167	471 ± 295	324 ± 30
H′	5.55 ± 0.41	5.95 ± 0.06	4.94 ± 0.61	5.51 ± 0.93	5.43 ± 0.09
J′	0.92 ± 0.02	0.93 ± 0.01	0.92 ± 0.00	0.93 ± 0.02	0.94 ± 0.01
-ln(*D*)	5.12 ± 0.44	5.56 ± 0.14	4.51 ± 0.58	5.15 ± 0.83	5.10 ± 0.11
$E_{(1/D)}$	0.41 ± 0.06	0.44 ± 0.07	0.42 ± 0.03	0.46 ± 0.07	0.51 ± 0.03

S = Number of genes detected, *H′* = Shannon–Weaver diversity index, *J′* = Shannon–Weaver evenness index, -ln(*D*) = Pielou-Kemp transformed Simpson index, $E_{(1/D)}$ = Simpson evenness index.

*Numbers in shading indicate the percentage of genes detected at that site (*S*) that are unique to that site. Numbers without shading indicate the percentage of genes detected at that site that were detected at the second site.

†S2 only had two replicates.

site. The highest percentage of unique gene variants was detected in sites with higher metal contamination levels (S1 and S2).

Sediment microbial community structure

To assess differences in the overall structure of sediment microbial communities at this site, hierarchical clustering analysis of all detected gene variants was performed (Fig. 2). Communities from S1 and S2 grouped together, while S3–S5 formed a second group. A total of 12 clusters were detected based on the presence and abundance of gene variants across sites (tree to the left). Gene variants in clusters 2, 3, 4, 8, 9, 10, and 12 were observed across multiple sites, while those in clusters 1, 5, 6, 7, and 11 were detected primarily in one or two sites (S4; S1 and S2; S1 and S2; S2; and S3 and S5, respectively). At least one gene from each major category was present in all clusters. Cluster 11, with genes largely found in S3 and S5, had the highest percentage of genes coding for the dissimilatory sulfite reductase (*dsr*). Clusters 2, 4, 10, and 12, containing genes detected across all sites, had the highest percentage of metal resistance genes (17% or greater). Cluster 6, which was mostly comprised of genes from S1 and S2, had higher percentages of genes for organic contaminant degradation (41%); cluster 4 also had a high percentage of organic contaminant degradation genes. Cluster 9 had the lowest percentage of metal resistance genes (*c.* 8%). Groups 5, 6, and 8 had general trends of decreasing gene abundance in the less contaminated sites (S3, S4, and S5).

Next, ordination plots of all gene variants detected by GeoChip were used to visualize differences between samples and sites. Nonmetric multidimensional scaling (NMDS) did not show consistent clustering of samples by location (Supporting Information, Fig. S1). Samples from S4 and S5 (the least contaminated sites) clustered together near the center of the ordination plots, whereas samples from more contaminated sites (S1–S3) were variably scattered further from the center.

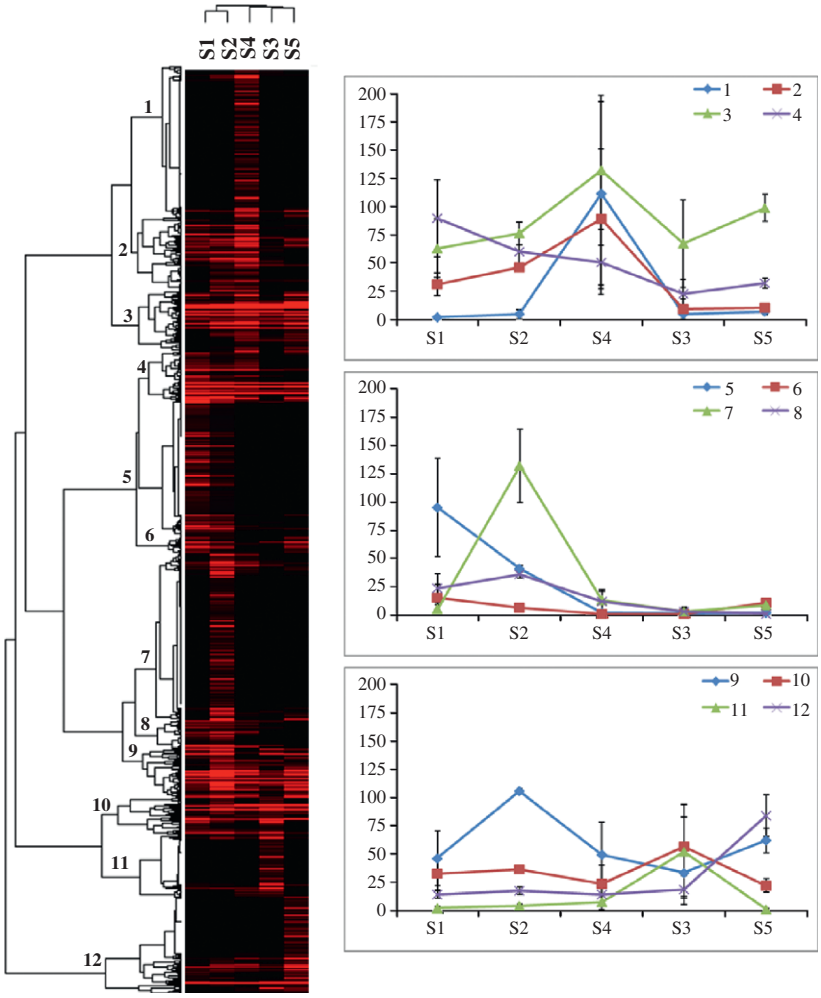

Fig. 2. Hierarchical cluster analysis of all genes detected at all Lake DePue sites. Signal intensities were averaged for samples from the same site (i.e. S1–1, S1–2, and S1–3). Higher intensity red color indicates higher signal intensities. Graphs to right indicate average signal intensities of each cluster. Error bars represent standard deviation of biological triplicates. Clusters were determined based on clustering of genes and are indicated by numbers on the left side of the tree.

Relative abundance of functional gene groups

The relative abundance of all functional gene categories was similar across all samples and sites (Fig. 3; Fig. S2). About 30–35% of the detected gene probes were for genes involved in organic contaminant degradation; another 20–25%, in N cycling; 3–6%, in methane oxidation/generation; about 20%, in metal resistance; 5–8%, in sulfate reduction (*dsr*); 2–5%, in C fixation; and 10–15%, in C degradation. Sites 2 and 3 had slightly higher abundance of sulfate reduction gene variants (*dsr*, 7.1–8.2%) compared with the other sites (5.0–5.7%). Methane generation gene variants were in lower abundance at sites 3 and 4 (0.2–0.3% vs. 0.9–1.4%).

GeoChip 2.0 has a total of 646 dissimilatory sulfate reductase gene variants (*dsr*AB), and the overall detection (22.0%) was higher than the percentage detection of all gene probes (20.5%) and two other functional gene categories [13.4% metal resistance and 15.8% carbon cycling, implying a higher proportion of sulfate-reducing bacteria (SRB)]. While metal resistance genes account for 20.5% of all probes on the GeoChip, this category accounted for only 13.4% of all positive gene probes.

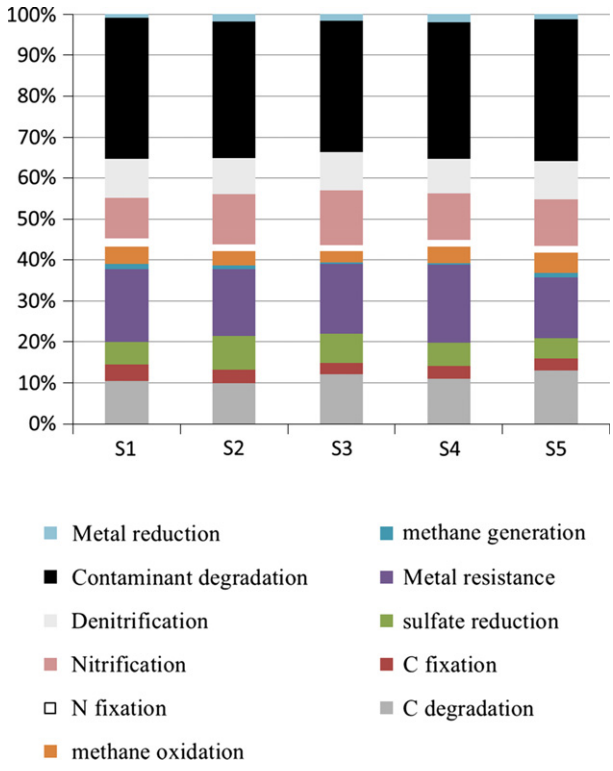

Fig. 3. Relative richness of all functional gene groups detected. The total number of genes detected at each site was used to calculate the relative richness of each gene group. Values were averaged for samples from the same site. Numbers along *x*-axis are the site names. Fig. S2 shows the relative richness for each individual sample.

Detailed analysis of key functional genes

Individual gene categories were examined in more detail using hierarchical clustering. Those coding for the dissimilatory sulfite reductase (*dsr*) were examined because many SRB can also reduce various metals (Fude *et al.*, 1994; White *et al.*, 1998), and previous studies of Lake DePue identified a positive correlation between sulfate reduction rate and pore-water metal concentrations (Gough *et al.*, 2008a, b). The distribution of different *dsr* gene variants did not cluster based on contaminant level (total or pore water), as S1 was most similar to S5, while the greatest number of gene variants/highest abundance was in S2 and S4 (Fig. 4). Most of the *dsr* gene variants detected were from uncultured SRB or clone sequences from environmental samples although there were five gene variants detected from *Desulfotomaculum* and *Desulfovibrio* spp. (in red font). The *dsr* gene variants affiliated with *Desulfomicrobium norvegicum* (NCBI gene number from protein database, gi 14090293) and *Desulfotomaculum kuznetsovii* (gi 14276806) (underlined) were detected at all sites.

Metal resistance in bacteria is largely conferred by transporters because it is energetically unfavorable for many metals to be reduced and/or reduction does not reduce toxicity (Nies, 2003). Most of the metal resistance genes covered by the GeoChip are transporters (Cd, Co, Cr, Cu, Ni, Pb, and Zn) although arsenate reductase, mercuric reductase, and organomercurial lyase are also covered. A number of such genes detected (251 gene probes from all sites) confer resistance to a variety of metals, including Hg, As, Ni, Cd, Te, Zn, and Cu, and clustered by metal contamination level as S3, S4, and S5 were closely clustered (Fig. 5). Several gene variants were found in higher abundance at the more contaminated sites (S1 and S2; in red font). Two metal resistance gene variants were observed in high abundances at all sites, with one (gi 6689526) derived from *Xanthomonas campestris* for Hg resistance and the other (gi 1749680) from *Schizosaccharomyces pombe* for Zn and Cd resistance (underlined). These encompass many of the major metal contaminants of Lake DePue, which include Zn, Cd, Cu, Pb, and As, and previous examination of Lake DePue sediments did detect Hg (Cahill & Bogner, 2002). In addition, cytochrome genes can contribute to resistance of some metals through their reduction, including U(VI), Fe(II), and Mn(IV) (Lovley & Phillips, 1994; Beliaev *et al.*, 2001). Cytochrome gene variants did cluster based on metal contaminant level with S1 and S2 clustering separately from the other sites (Fig. 6). Twenty-six cytochrome gene variants were detected across all sites with the greatest number and abundance detected in S2 and S4. Most of the gene variants were from *Geobacter*

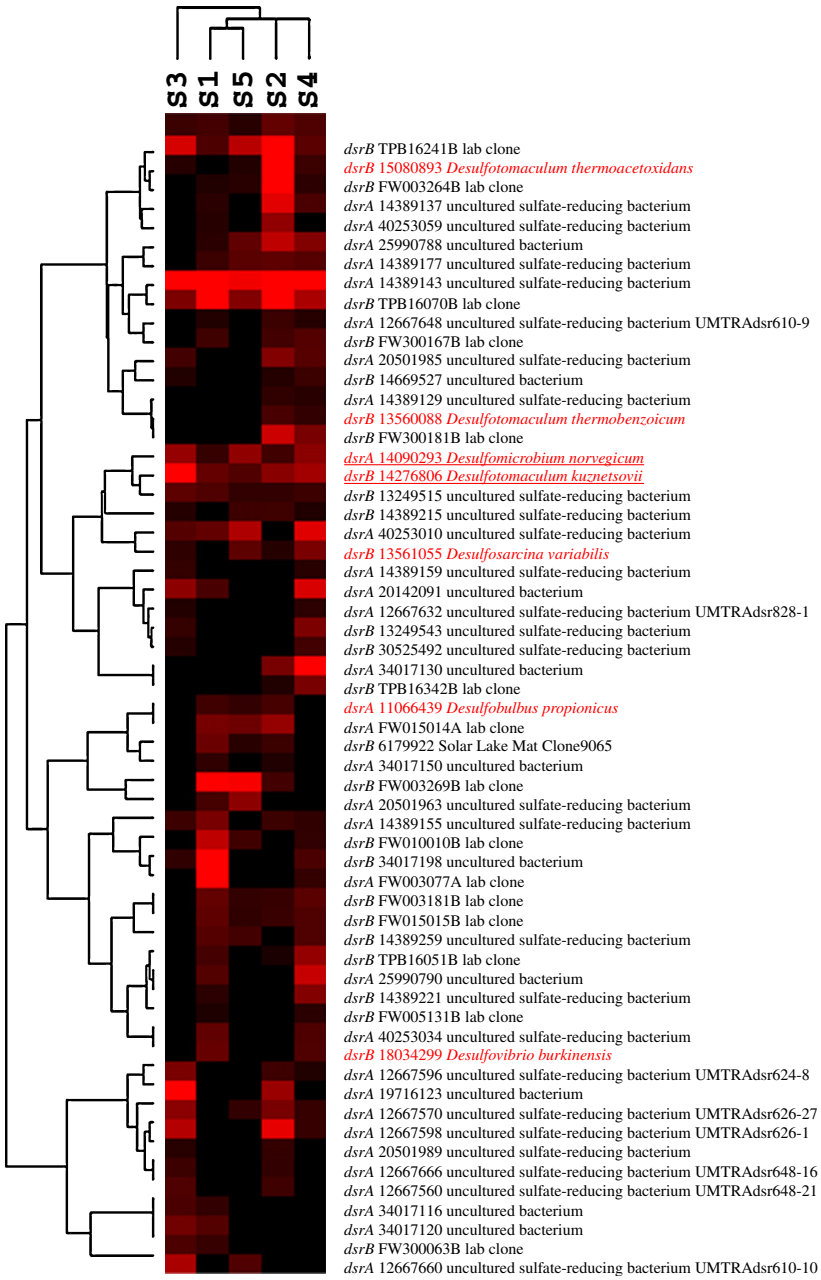

Fig. 4. Hierarchical cluster analysis of dissimilatory sulfite reduction genes. Values were averaged for samples from the same site. Numbers along *x*-axis are the sample names. Brighter red coloring indicates higher signal intensities.

sulfurreducens (in red font). A cytochrome gene similar to that found in *Rhodopseudomonas palustris* (gi 39933283) (underlined) was detected at all sites. Both metal resistance and cytochrome (involved in metal reduction) gene variants clustered by contamination level (Figs 5 and 6).

Previous work in Lake DePue indicated that organic carbon accumulation was associated with metal contamination (Gough *et al.*, 2008a). Because accumulation could derive from a combination of decreased organic matter mineralization and increased carbon fixation, carbon degradation and fixation genes were examined in greater detail (data not shown). The relative abundance of the carbon degradation and carbon fixation gene variants detected indicated that there were more carbon fixation gene variants present, suggesting that this functional group would have provided a greater contribution to organic carbon accumulation (C degradation: 17.0%; C fixation: 19.0%). Genes for polygalacturonase, mannanase, cellulase, chitinase, and laccase were detected in all sites. Site S3 had the fewest number of carbon degradation gene variants (43), followed by site S5 (65), while sites S1, S2, and S4 had similar numbers (73–86). About half of the carbon degradation gene variants detected are involved in recalcitrant compound degradation (e.g.

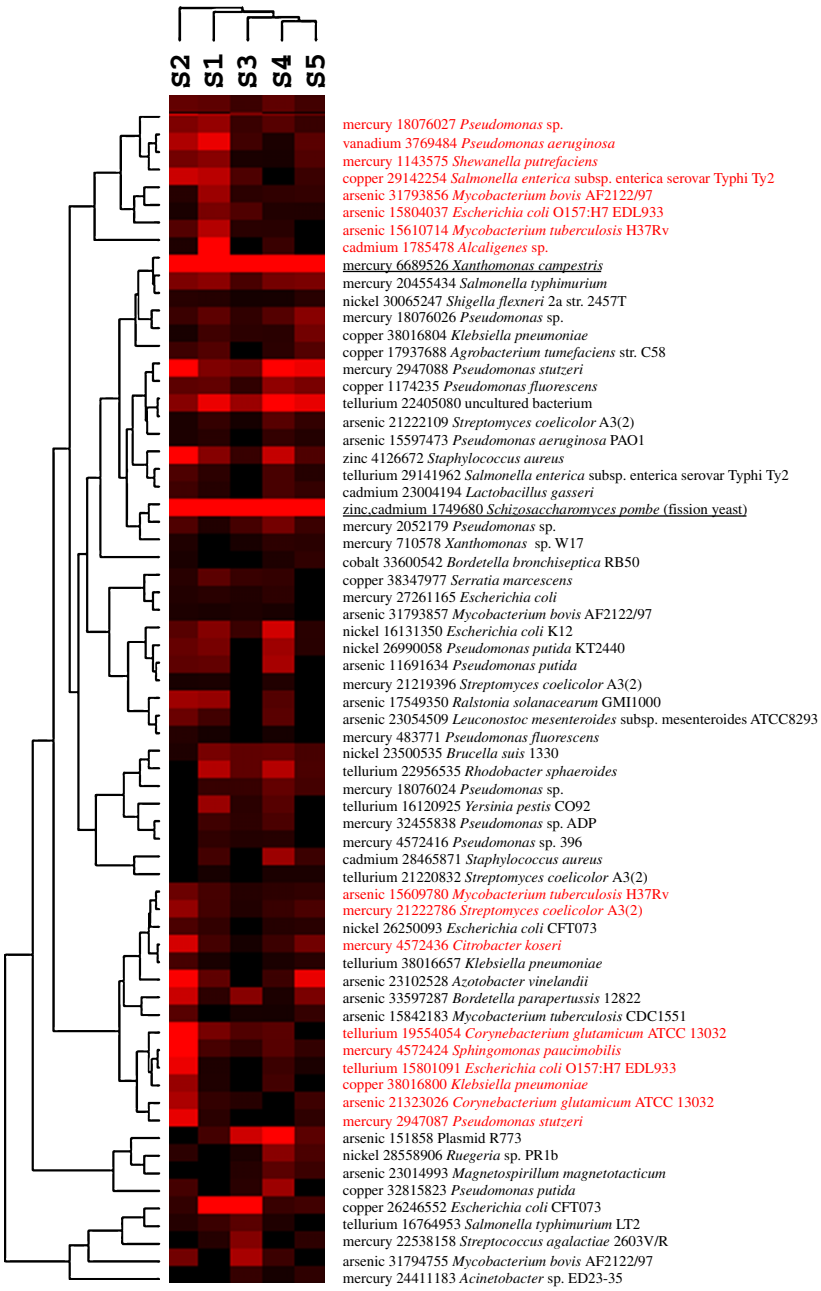

Fig. 5. Hierarchical cluster analysis of metal resistance genes. Values were averaged for samples from the same site. Numbers along *x*-axis are the site names. Brighter red coloring indicates higher signal intensities.

lignin). Clustering based on C fixation gene variants grouped sites by contaminant level. The greatest number/abundance of C fixation gene variants was detected in S1 and S2. Most of the detected gene variants were from uncultured bacteria.

Relationship between sediment microbial community and environment

Environmental variables for this site were divided into three categories: lake sediment characteristics, pore-water metals, and total metals. The lake sediment characteristics included general sediment properties (pH, water content, etc.), pore-water metals were metal concentrations measured within the pore water, and total metals were the metal concentrations measured in the bulk sediment. Among the three groups of environmental variables available, pore-water metals were significantly correlated with functional structure as determined by the Mantel test. Thus, all subsequent analyses were focused on pore-water metal concentrations. A subset of pore-water metals were chosen by sequentially removing variables with the

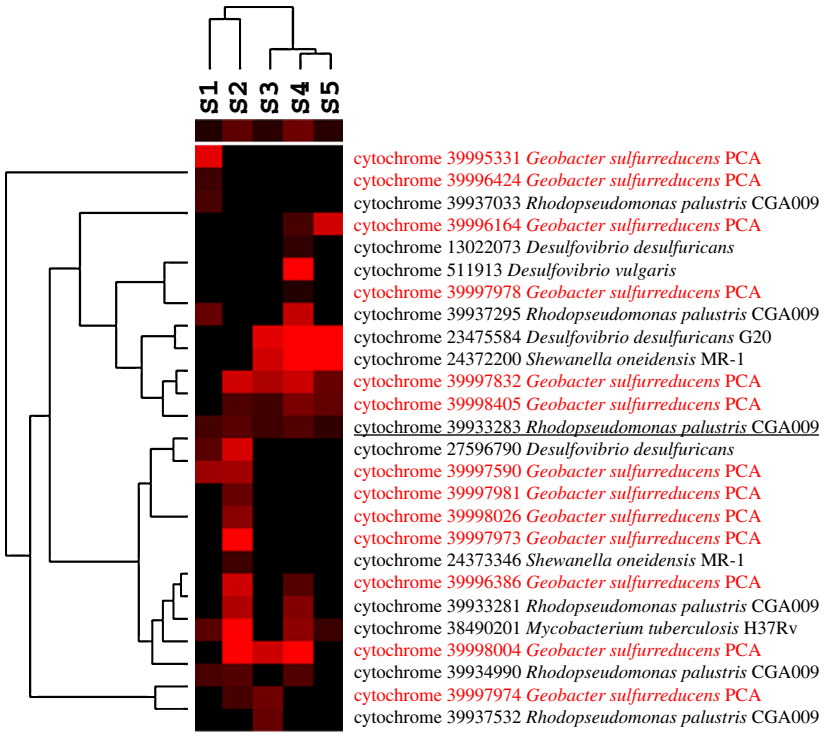

Fig. 6. Hierarchical cluster analysis of cytochrome genes. Values were averaged for samples from the same site. Numbers along *x*-axis are the site names. Brighter red coloring indicates higher signal intensities.

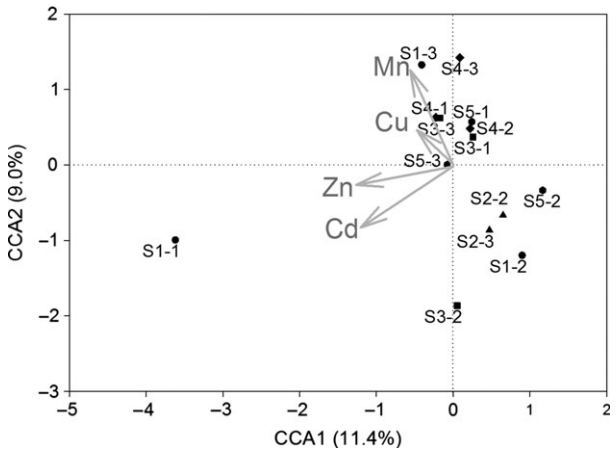

Fig. 7. Canonical correspondence analysis for all Lake DePue samples. Analysis was carried out using all functional genes detected (symbols) and environmental variables (arrows). Environmental variables were chosen by automatic selection significance calculated from individual CCA results and variance inflation factors calculated during CCA. Numbers indicate sample name.

highest variance inflation factors (VIF) from the canonical correspondence analysis (CCA) until all remaining variable VIFs were < 10. The specified model with four pore-water metals (Mn, Cu, Zn, and Cd) using all gene probes detected by GeoChip was not significant, although the *P*-value was < 0.1 ($P = 0.075$) and explained 20.4% of the variation observed in the microbial community structure (Fig. 7). All pore-water metals showed a negative correlation with the first axis. Mn and Cu showed a positive correlation with the second axis, while Zn and Cd showed a negative correlation.

The results of CCA and partial CCA were used to partition the variations of microbial community structure between pore-water metals and geographic distance (Borcard *et al.*, 1992). Pore-water metals accounted for 35.2% of the variation ($P = 0.08$), while geographic distance only accounted for 14.75% of the variation. There was only a small portion of variation (0.27%) shared by pore-water metals and distance, indicating a relative independence between the pore-water metals and distance. Almost half (49.79%) of the variation was unexplained. Similar results were obtained when metal resistance, sulfate reduction (*dsr*), or carbon cycling gene variants were used (Fig. S3).

Results of the Mantel and partial Mantel (distance controlled) tests for selected pore-water metals using all gene probes detected by GeoChip ($r_M = 0.302$; $P = 0.108$; $r_M = 0.332$; $P = 0.088$, respectively) indicated some marginal shared contribution of pore-water metals and distances among samples. Notably, detected *dsr* genes were more significantly correlated with pore-water metals than with genes for other functional groups (partial Mantel, $r_M = 0.365$, $P = 0.036$).

Functional gene categories (as listed in Fig. 3) were then analyzed. No significant associations were revealed by the Mantel test or CCA. Mantel test suggested a relationship between *dsr* gene variants and pore-water metals

($r_M = 0.151$, $P = 0.146$). Based on multiple regression analysis, the functional gene subgroup for Cd resistance was significantly associated with both pore-water As ($P = 0.005$) and Zn ($P = 0.004$), even after Bonferroni correction and *p*FDR. In fact, the Cd resistance subgroup was the most significantly regressed with all pore-water metals except Mn, which was most significantly regressed with Cr resistance.

Next, individual genes were examined. Multiple regression analysis on each functional gene identified significant models between functional gene variants and certain pore-water metals (Supporting Information, Table S1). The functional gene variants significantly correlated with the concentration of different pore-water metals were similar among As, Cd, Cr, Zn, and Pb, while Cu and Mn had distinct gene composition (Tables S2–S5). The majority of correlations were with Cu, although a few were with Zn, Cr, and Mn. All metal resistance gene models were positive (i.e. positive relationship between signal intensity and metal concentration), and about half were negative for other functional gene groups. The normalized signal intensity, which represents abundance, of these selected functional gene variants was then tested by correlation analysis with pore-water metal concentration. With few exceptions, they were significantly correlated with the corresponding pore-water metals. These findings suggest that pore-water metals other than Cu might have a greater influence on the distribution of gene variants highly correlated with metals. A more detailed description of individual gene correlations is in Data S1.

Discussion

Impacts of metal contamination on microbial communities

Previous studies at Lake DePue suggested that reduced biomass correlated with metal contamination levels (Gough *et al.*, 2008a), while bacterial diversity and similarity remained high among microbial communities at the different sampling sites transecting the gradient of metal contamination (Gough *et al.*, 2008a; Gough & Stahl, 2011). In these previous studies, microbial communities were characterized using 16S rRNA gene–based terminal restriction fragment length polymorphism analysis (Gough *et al.*, 2008a; Gough & Stahl, 2011). In the present study, the microbial communities were characterized using a functional gene array (GeoChip), thus expanding on these previous observations by providing a greater level of resolution (species vs. strains) (He *et al.*, 2007). About 85% of the detected gene variants were detected in at least two sites, which is not unexpected as similar communities would be expected prior to contamination. In addition, transport of microorganisms could be expected for both planktonic forms and on sediment particles due to water flow and other disturbance of the sediment. Although, as in the earlier studies at Lake DePue, the overall community structure and functional gene diversity determined by GeoChip were similar across all sites regardless of contamination level (Fig. 3, Figs S1 and S2, Table 2), sulfate reduction (*dsr*) and metal resistance gene variants did show a strong correlation with pore-water metals (Tables S1–S4). The multivariate ordination analysis also indicated a strong influence of metal contamination level on the microbial community structure in that samples with lower contamination levels (S4 & 5) were tightly clustered, while highly contaminated samples were scattered across the ordination space (Fig. S1).

Controlling factors of microbial communities at metal-contaminated sites

While bulk sediment metal concentrations are often used in metal contamination studies, primarily due to their higher values (Gillan *et al.*, 2005; Cordova-Kreylos *et al.*, 2006), the use of the bulk concentration may provide an inaccurate picture of metal bioavailability. The pore-water fraction of metals are the most biologically available portion because they are more chemically labile and are thus a more relevant measure for evaluating metal toxicity to microorganisms (Loureiro *et al.*, 2005), although other factors (e.g. pH, organic content, and metal speciation) will also affect bioavailability (Giller *et al.*, 1998). There can be orders of magnitude differences between the bulk and pore-water metal concentrations (Sowder *et al.*, 2003; Gough *et al.*, 2008a). Thus, metal toxicity may not occur at concentrations found in the pore water even if the site is highly contaminated (Van Nostrand *et al.*, 2007). In a study of radionuclide-contaminated pond sediments, although much higher concentrations of U were present in the bulk sediments compared with Ni, higher levels of Ni were present in the pore-water fraction, suggesting that the Ni would be more bioavailable (Sowder *et al.*, 2003), and subsequent studies, demonstrating uptake of Ni by on-site plants (Punshon *et al.*, 2003) and isolation of Ni-tolerant bacteria (Van Nostrand *et al.*, 2007), support the increased bioavailability of Ni at this site.

A previous study of Lake DePue identified pore-water metals as the most significant independent variable correlated with microbial biomass (Gough *et al.*, 2008a). In the current study, pore-water metals were identified as the most significant environmental factor affecting the functional structure and potential of the microbial communities. Pore-water metal concentration had a greater influence on the microbial community than even spatial differences. Pore-water metals account for *c.* 35% of the

variation observed in the community structure, while spatial differences accounted for < 15%. Although bulk Zn concentrations were very high (3100–21 400 mg kg^{-1}) in the Lake DePue sediments and decreased steadily with distance from the site of input (Gough *et al.*, 2008a), the corresponding pore-water concentrations were much less (3.83–0.298 μM) and were more similar, with S3–S5 all having *c.* 0.3–0.6 μM Zn in the pore water (Gough *et al.*, 2008a). Sequential metal extraction, which extracts metals in a stepwise manner from most labile to least, was also carried out on these soils (Gough *et al.*, 2008a). The exchangeable and carbonate-bound/acid-soluble fractions are generally considered potentially bioavailable (Wong *et al.*, 2002). The Zn concentrations in these two fractions were much higher than in the pore-water measurements (*c.* 4000 ppm in site 1, *c.* 1700 ppm in site 2, *c.* 250 ppm in site 3, *c.* 70 ppm in site 4, and *c.* 30 ppm in site 5 vs. 0.25, 0.08, 0.0003, 0.0003, and 0.0005 ppm, respectively) (Gough *et al.*, 2008a). For sensitive strains, the toxic effects of Zn are expected to start at *c.* 1 μM (0.06 ppm) and have a minimum inhibitory concentration of 1 mM (65.4 ppm) (Nies, 2000).

While metal resistance gene probes account for *c.* 16% of the probes on GeoChip 2.0, only *c.* 12–14% of the gene probes that were positive in the Lake DePue samples were related to metal resistance. Because this is a metal-contaminated site, we would have expected an enrichment of metal resistance gene variants. This could be the result of several reasons. Most genes represented on the GeoChip 2.0 are transporters as this is the most commonly observed mechanism of metal resistance in bacteria (Nies, 2003). However, other mechanisms include sequestration (within/on the cell or using a secreted substance), down-regulation of nutrient metal influx transporters (Hausinger, 1993), or reduction. Most of these mechanisms would not have a corresponding gene or would not be specific for metal resistance. For example, excretion of sulfide by sulfate-reducing bacteria would function to sequester many heavy metals but not be considered a function specific to metal resistance. The appropriate metal resistance genes may not be covered on the GeoChip, or some of the metal resistance genes or gene variants may not be relevant to this site. Mn and Pb were abundant in Lake DePue sediment, so we would expect to find populations resistant to these metals, but there were no probes for genes involved in resistance to Mn and only a very few for Pb. A Mn efflux transporter was recently described (Rosch *et al.*, 2009), although at the time of GeoChip 2.0 development, this transporter was unknown. While transporters for Pb resistance have been known, few sequences are available in public databases.

While many metals are present in Lake DePue sediment at fairly high concentrations, the amount actually available to the microorganisms may be much lower, thus providing little to no selection pressure for resistant strains (discussed above). When only a subset of resistance gene variants corresponding to metals found in higher concentration in the lake sediment (Zn, As, Cu, and Cr) were examined, the percent of positive probes increased to 19.7%, suggesting that relevant metal-resistant populations did increase and were affected by the metal contamination. In this study, metal resistance gene variants from the lesser contaminated sites (S3-S5) with similar pore-water Zn concentrations clustered together (Fig. 4). In addition, cytochrome gene variants, which may be involved in metal reduction, clustered into two groups: one of the higher contaminated sites S1 and S2 and another of the three lesser contaminated sites (Fig. 6). Cd resistance gene variants were the most significantly regressed of all metal resistance genes with all of the pore-water metals except Mn. Cd resistance was significantly associated with both pore-water As ($P = 0.005$) and Zn ($P = 0.004$). Several metal resistance systems are known to confer resistance to more than one metal. For example, the *czc* operon confers resistance to Cd, Zn, and Co (Nies, 2003). Additionally, all metal resistance gene variants showed a positive relationship between signal intensity and pore-water metal concentration, and the majority were significantly correlated with their corresponding pore-water metal (Tables S2–S5). These results taken together strongly suggest that metal-resistant microorganisms provide a significant contribution to the stability and resistance of the sediment microbial community at this metals-contaminated site.

While beyond the scope of this work, sites with high levels of metals contamination are ideal for the isolation of novel metal-resistant microorganisms because metal tolerance or resistance is an adaptive response to excessive metal exposure (Stoppel & Schlegel, 1995). For example, *Cupriavidus metallidurans* CH34, one of the most highly metal-resistant strains identified to date (Mergeay *et al.*, 2003), was initially isolated from a decantation tank at a zinc factory (Mergeay *et al.*, 1978). Obligate anaerobic bacteria from Lake DePue sediments have been isolated and shown to grow in the presence of 10 mM Zn (Webb *et al.*, 2001).

In addition to Zn-smelting, waste was generated from paint pigment, sulfuric acid, and fertilizer production as well as other industrial activities (http://www.epa.state.il.us/community-relations/fact-sheets/new-jersey-zinc/index.html). There was also some release of organic contaminants, including solvents (xylene and toluene), gasoline, and diesel fuel (ATSDR; http://www.atsdr.cdc.gov/hac/pha/pha.asp?docid=534&pg=2). These contaminants could influence the microbial community structure, and genes for the degradation of benzene, atrazine, biphenyl, catechol, naphthalene, MTBE, phenol, and other chemicals were detected (data not shown). However, organic contaminants are not the primary

contaminant of concern at this site, and no concentration information was available, so further analysis was beyond the scope of this work.

Sulfate-reducing bacteria

Sulfate concentrations are often low in lakes, and most sulfate reduction occurs in the first 10 cm of the sediment (Holmer & Storkholm, 2001 and references therein). Sulfate reduction has been measured in oligotrophic, mesotrophic, and eutrophic lake sediments, with generally higher rates observed in eutrophic systems (Sass *et al.*, 1997; Holmer & Storkholm, 2001 and references therein; Gough *et al.*, 2008b). SRB have been detected in Lake DePue sediments, with similar numbers detected at both sites 1 (most contaminated) and 5 (least contaminated) (Gough *et al.*, 2008b). Higher sulfate-reducing rates and sulfate concentrations were measured in samples from sites 1 and 2, where metal concentrations were highest in this freshwater lake (Gough *et al.*, 2008b; Table 1). Sulfate concentrations in Lake DePue are as high as or higher than what would be expected in an uncontaminated lake (< 1 mM in eutrophic lakes and ≤ 2 mM in sulfate enriched lakes) (Holmer & Storkholm, 2001).

Sulfate-reducing bacteria (SRB) are known to reduce various heavy metals such as As, Cr, Cu, Fe, Mn, and others (Fude *et al.*, 1994; Scala *et al.*, 2006; Stone *et al.*, 2006). This reduction can decrease metal availability by precipitation or by making metals less toxic by altering their speciation. In addition, SRB also produce metal sulfides, which reduce metal availability and toxicity (Fortin *et al.*, 1994). The increased sulfate-reducing rates at sites with higher metal concentrations (Gough *et al.*, 2008b) support a functional significance in the lake. The increased production of H_2S may serve as a mechanism to reduce metal toxicity by binding the metals and reducing availability. Sitte *et al.* (2013) found increased metal resistance in SRB enrichment cultures derived from metals-contaminated creek sediment due to the formation of metal sulfides, which likely provided a less toxic environment for the microbial community members. A large proportion (47–70%) of the Zn concentrations in the sediment was in the form of ZnS (Gough *et al.*, 2008b). While the relative abundance of detected *dsr* gene variants was similar among all samples (Fig. 2), the data suggest that the *dsr*-containing population was greatly influenced by metal concentration. The *dsr* gene was most significantly correlated with pore-water metals ($P = 0.036$), and regression analysis suggested a relationship between *dsr* and pore-water metals ($P = 0.146$). In addition, several *dsr* gene variants from specific SRB showed significant correlations with pore-water metals (Tables S1 and S4). A higher proportion of *dsr* gene probes were detected (20.3% of the *dsr*A/B probes on the array) than other gene categories (18.6% of all gene probes on the array), indicating an enrichment of SRB compared with other microbial populations. Finally, results of CCA indicated that Pb, Zn, and Cd were important in shaping the SRB community. All these results together implicate SRB as significant contributors to the resistance and stability of sediment microbial communities at this long-term metals-contaminated site.

Several SRB were shown to have strong correlations with pore-water metals suggesting these species in particular may play important roles in reducing metal toxicity or otherwise helping to stabilize the microbial community at this contaminated site. These include *dsrA* and/or *dsrB* from *Desulfotomaculum putei* (Zn), *Desulfovibrio longus* (Cu), *Desulfobacterium oleovorans* (Cu) and *Desulfotomaculum kuznetsovii* (Cu), *Desulfovibrio piger* (Pb, Cu), and cytochrome c from *Desulfovibrio vulgaris* (Cu). A *Desulfotomaculum* strain similar to *D. putei* (95%) was isolated from landfill leachate in Japan and was able to completely precipitate up to 3 mM Cd^{2+} or 2 mM Cu^{2+} as CdS and CuS (Mori *et al.*, 2000), indicating a fairly high level of metal tolerance. *Desulfovibrio longus* was originally isolated from production fluid of an oil-producing well, although the number of electron donors it is able to use is limited (Magot *et al.*, 1992). 16S rRNA gene sequences most closely related to *D. kuznetsovii* were detected at a Cu-Pb-Zn mine in Toyoha, Japan (Nakagawa *et al.*, 2002). *Desulfovibrio vulgaris* is known to reduce chromate enzymatically via a cytochrome c (Lovley & Phillips, 1994). These previous studies are consistent with the functional significance of SRB in metal-contaminated sites, reducing metal toxicity by precipitation or reduction.

Community ecology of contaminated site microbial systems

Community stability in ecology has been framed in terms of resistance to disturbance (community structure little altered) or resilience (community structure returns to its original state following short term disturbance), and these factors have also been shown to be associated with biodiversity (Tilman, 1996). After a stress event, there is often an initial reduction in biomass due to the stress, but then, the biomass begins to stabilize as tolerant species gain dominance (Schindler, 1990; Gough & Stahl, 2011). Microbial communities adapt to the stress event, such as metals contamination, by either selective growth or introduction of metal-resistant microorganisms (Turpeinen *et al.*, 2004). Studies have shown that stable and functionally comparable populations have developed in response to historical metals contamination (Yin *et al.*, 2000; Franklin & Mills, 2006; Yannarell *et al.*, 2007).

Our data are most consistent with a dominant role of resistance in the evolution of stable, and functionally comparable, communities as indicated by increased abundance of relevant metal-resistant populations and SRB capable of metal reduction or precipitation. While diversity and overall community structure and functional potential were not associated with the level of metal contamination, there was significant enrichment of SRB and metal resistance gene variants, which indicate the importance of these populations to maintaining the community structure and overall diversity. Although sulfate-reducing bacteria are not generally thought to be of great functional importance in mesotrophic or eutrophic freshwater systems, this study points to their significance in reducing metals toxicity through immobilization or altered speciation and therefore functioning as significant contributors to the resistance and stability of sediment microbiota under long-term metals contamination.

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Authors' contribution

S.K. and J.V.N. contributed equally to this work.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Non-metric multidimensional scaling for sediment samples. Analysis was done using all of the functional genes detected from all samples. Each site is labeled with a different symbol.
Fig. S2. Relative richness of all functional gene groups detected in each sample. The total number of genes detected at each site was used to calculate the relative richness of each gene group. Numbers along x-axis are the site and sample names.
Fig. S3. Variance partitioning of environmental variables analyzed by CCA. The diagram represents the relative effect of each variable upon the entire functional community (A), metal resistance genes (B), and sulfate cycling genes (C). The ovals represent the effects of individual variables by partitioning out the effects of the other variables. Variables used in CCA were used for the VPA.
Data S1. Experimental procedures.
Table S1. List of important genes selected by multivariate multiple regression analysis with pore water metal concentration.
Table S2. List of functional genes of MRRG group significantly correlated with corresponding pore water metal concentrations.
Table S3. List of functional genes of MRRG group significantly correlated with each pore water metal concentration.
Table S4. List of functional genes of SRG group significantly correlated with each pore water metal concentration.
Table S5. List of functional genes of CCG group significantly correlated with each pore water metal concentration.