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Microbial community signature in Lake Coeur d'Alene: Association of environmental variables and toxic heavy metal phases

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Abstract

The water and sediments of Lake Coeur d'Alene in northern Idaho (USA) have been impacted by decades of mining operations within the Coeur dâ€™Alene mining district. Using a multivariate statistical approach, correlations were explored between the microbial community (via 16S rDNA microarray) in sediment cores and operationally defined heavy metal phases (via continuous sequential extractions). Candidate phyla NC10, OP8 and LD1PA were only detected in metal contaminated cores and diversity doubled among Natronoanaerobium in metal contaminated cores compared to the uncontaminated control site. This may suggest some increased fitness of these phyla in contaminated sediments. In contrast, diversity within the phyla Aquificae, Coprothermobacteria, and Synergistes was at least double in the uncontaminated control site. In linear models composed of two geochemical variables from the presumed sulfate reducing lineages detected in this study, orders Desulfobacterales, Desulfuromonadales, Desulfotomaculum, and Syntrophobacterales were highly correlated with Pb (positive influence) and Zn (negative influence) in the operationally defined residual fraction, and most taxa within orders from Desulfovibrionales. Bdellovibrionales highly correlated with Pb in the exchangeable/carbonate (negative influence) and oxyhydroxide (positive influence) phases. Diversity within families from metal reducing bacterial lineages Shewanellaceae, Geobacteraceae, and Rhodocyclaceae showed high correlation with Pb in the exchangeable/carbonate (negative influence) and oxyhydroxide (positive influence) phases. To our knowledge, this is the first time these techniques have been used in combination to describe a contaminated system. Resulting correlations suggest the diversity of the microbial community was influenced primarily by partitioning of heavy metals into exchangeable Pb over other Pb phases and, to a lesser extent, residual Pb to residual Zn phase partitioning.

1. Introduction

The Coeur d'Alene (CDA) Mining district has had 90 mines in operation producing Pb, Zn, Ag, and Sb (Balistrieri et al., 2003; Horowitz et al., 1992, 1995; Tonkin et al., 2002). Over seven million tons of Pb, three million tons of Zn, and 34 thousand tons of Ag have been mined from the CDA Mining district, which stretches from Coeur d'Alene, Idaho to Superior, Montana (Leach et al., 1985).

The history of this district as well as the type of ore deposits and mineralogy has been summarized by Leach and others (Fleck et al., 2002; Leach et al., 1985; Mauk and White, 2004; Panneerselvam et al., 2006; Rosenberg and Larson, 2000). Briefly, the mineralogy of the CDA Mining District consists primarily of quartz [SiO2] and siderite [FeCO3] veins containing deposits of galena [PbS], sphalerite [ZnS], and tetrahedrite [$Cu_{12}Sb_4S_{13}$] (Leach et al., 1985). Pyrite [FeS_2], chalcopyrite [$CuFeS_2$], and pyrrhotite [Fe_xS x = 0.8,1] are also locally abundant (Leach et al., 1985). Fe minerals including siderite, magnetite, pyrite, pyrrhotite, goethite, hematite, and ferrihydrite have been reported in the sediments of Lake Coeur d'Alene (LCDA) and the mining district (Cummings et al., 2000; Farrand and Harsanyi, 1997; Toevs et al., 2006).

Horowitz et al. (1995) reported the Coeur d'Alene River (CDAR) and adjacent lake sediments showed the greatest level of contamination with heavy metals. Batch sequential extractions indicate that heavy metals in the delta region of LCDA appear to be associated with an operationally defined sulfidic phase (Harrington et al., 1998b), while those elsewhere in LCDA appear to be

predominantly associated with the more mobile hydroxides (Horowitz et al., 1995; Woods and Beckwith, 1997), though there is some controversy on this point (Horowitz et al., 1999). Haus et al. (2008) employed sequential extractions in combination with high resolution mass spectrometry and electron microscopy to study seasonal influences on arsenic speciation in the lateral lakes surrounding the CDAR. They found that stable water column height was a better predictor for partitioning of arsenic to less mobile phases when compared to a seasonally fluctuating water column. Additionally, studies of mixing and neutralization of acidic, metal contaminated water with uncontaminated surface waters (Balistrieri et al., 1999; Paulson and Balistrieri, 1999) and surface complexation of metals onto oxyhydroxides (Balistrieri et al., 2003; Tonkin et al., 2002) have been performed to predict metal fate and transport in this contaminated system. Sengör et al. (2007) developed models that incorporated microbial influences with surface complexation and chemical species changes during neutralization.

Concerns over repartitioning of extracted elements during selective chemical extractions have given rise to continuous sequential extraction (CSE) techniques. CSE are thought to limit transient metals redistribution during chemical extractions due to continuous removal of extracted elements when compared to equilibrium batch processes (Miro et al., 2005; Shiowatana et al., 2001; Wisotzky and Cremer, 2003). While a variety different analytical methods have been employed to study metal contamination and heavy metal phase association in and around LCDA, CSE techniques have not been employed at this site. This study utilizes CSE to measure heavy metal phase association while limiting repartitioning of metals during extraction processes.

The microbial ecology of LCDA has been studied through culture dependent (Cummings et al., 1999, 2000; Harrington et al., 1998a; Niggemyer et al., 2001; Sass et al., 2009) and independent techniques (Cummings et al., 2003; Ramamoorthy et al., 2009; Rastogi et al., 2009). Novel organisms have been cultivated from LCDA, including *Ferribacterium limneticum* (Cummings et al., 1999), *Desulfovibrio idahonensis* (Sass et al., 2009), *Arthrobacter* sp. (Moberly et al., 2010), and *Geobacter* sp. (Cummings et al., 2000). Using taxa specific primers for real-time polymerase chain reaction (rt-PCR) and denaturing gradient gel electrophoresis (DGGE), Cummings et al. (2003) reported that *Geobacteraceae* were abundant and diverse in LCDA across metal contaminated and uncontaminated sites sampled. Similarly, Ramamoorthy et al. (2009) used most probable numbers and quantitative PCR of the a-adenosine 5′-phosphosulfate reductase to estimate sulfate reducing bacteria (SRB) populations in LCDA, and reported non-culture based estimates of SRB populations were higher in contaminated sites than pristine sites. Utilizing clone libraries of *rpoB* and 16S rRNA genes, Rastogi (Rastogi et al., 2011, 2009) found β-*Proteobacteria* and *Crenarchaeota* to be the dominant bacterial and archaeal communities in the CDAR, respectively. However with advancements in microbial community analysis tools, larger scale, community level analyses have yet to be reported in the LCDA system.

In the past, molecular approaches to characterize diversity and abundance of microbial community have relied on amplification of a portion of the 16S rRNA gene, followed by cloning and sequencing of the amplified product. The number of clones needed to describe a majority of the community taxa can be large (Brodie et al., 2006) and this method is relatively expensive. Gene-based microarrays allow for rapid characterization of community assemblages by hybridizing amplified sample DNA onto short sequences of known probes bonded to the microarray surface; producing a florescent signal when bound. Microarray based techniques can capture diversity not generally observed by cloning methods (Bohorquez et al., 2012; Brodie et al., 2006) but only detect DNA with similar nucleotide composition to the designed probes. Advanced sequencing techniques, also known as next generation sequencing, have become the "gold standard" for microbial community analysis and through rapid advancements in recent years continue to decrease cost while providing increased information. Microarray based community analysis was employed in this study to characterize microbial community composition and was compared to clone libraries for limited samples.

Microbes are known to catalyze reactions that alter their environment resulting in detoxification (e.g. precipitation, reduction, (de)methylation, production of metal-binding proteins, cell surface complexation) and adaptation (e.g. horizontal gene transfer of metal resistant genes, mutation) (Lloyd and Lovley, 2001). Additionally, metal toxicity has been shown to depend on chemical speciation and geochemical factors, such as surface area for adsorption and redox active phases (e.g. hematite, goethite, and ferrihydrite) (Balistrieri et al., 2015; Gadd, 1992; Hoang and Tong, 2015; Meyer et al., 2015, 2006; Moberly et al., 2010; Morton et al., 2000; Sani et al., 2003; Tipping and Lofts, 2015; Van Genderen et al., 2015). To better understand the influence of toxic metals on microbial communities, simultaneous characterization of both heavy metal speciation and microbial community is important. Effects of anthropogenic heavy metal contamination on microbial community structure have been studied in other systems (Ancion et al., 2013; Feris et al., 2003, 2004a, 2004b; Gillan et al., 2005; Gough and Stahl, 2011), but are lacking in the LCDA system. In this study, we examine correlations between operationally defined heavy metal phases and microbial community diversity at the phylum and family levels using linear models and GGE biplots (Yan et al., 2001, 2007), with inferences to metabolic function.

2. Methods

2.1. Sediment sampling

Core samples were collected in July 2008 from the LCDA delta region reported to be most contaminated with heavy metals (Horowitz et al., 1992). As a comparison site with similar geochemistry, samples were also collected from the relatively uncontaminated St. Joe River delta, also in LCDA. Core sleeves were disinfected with ethanol prior to sampling. Sealed sediment cores were flash frozen immediately after removal from the lake bed by submersion in a mixture of dry ice and ethanol. Cores were transported on dry ice to Montana State University and frozen (−25 °C) for approximately two days until sectioning. Samples were sectioned into sub-cores while frozen according to visible stratification and divided for geochemical and molecular analyses. Samples for molecular analyses were taken with sterile spatula from the inner diameter of the core to avoid contamination from both sample handling and spatial contamination from organisms transported between different levels of the core while sampling. Samples destined for geochemical analysis were sealed in serum bottles under nitrogen and frozen (−25 °C) until analysis. See Tables 1 and 2 for further information on core and subcore samples.

2.2. Microbial community characterization

Genomic DNA was extracted from 1.0 g of sediment from each stratified sub-core using a PowerSoil DNA extraction kit (Molecular Biosciences, Carlsbad, CA). Extracted DNA was divided between a 16S rDNA microarray (PhyloChip) analysis developed by Lawrence Berkley National Lab (Brodie et al., 2006) and, as a comparative tool, clone libraries to provide another means of microbial community comparison that includes relative abundance. DNA for PhyloChip analysis was prepared as previously described by Brodie et al. (2006). An equal mass of DNA amplicons were added to each

Table 1
Sample site characterization from July 2008.

Parameter	Coeur d'Alene river delta			St. Joe river delta
	CDAR-1	CDAR-2	CDAR-3	STJOE-5
pH	6.04	6.00	6.02	5.97
Water depth (cm)	150	90	170	100
Dissolved oxygen (mg/L)	8.7	8.6	8.9	9.2
Water temperature (°C)	19.4	19.8	20.1	18.7
GPS coordinates (Lat)	N 47° 27.612′	N 47° 27.594′	N 47° 27.517′	N 47° 21.972′
GPS coordinates (Long)	W 116° 47.878′	W 116° 47.877′	W 116° 47.834′	W 116° 44.336′
Total average Fe (mg/kg)	86,089	125,156	94,577	11,822
Total average Mn (mg/kg)	9638	12,198	9897	173
Total average Zn (mg/kg)	6029	5224	2199	169
Total average Pb (mg/kg)	3422	3180	1514	29

Table 2
Heavy metal phase association, depth from sediment water interface, and OTU presence from samples taken July 2008.

Parameter	Coeur d'Alene river delta							St. Joe river delta		
	CDAR-1A	CDAR-1B	CDAR-1C	CDAR-2A	CDAR-2B	CDAR-3A	CDAR-3B	STJOE-5A	STJOE-5B	STJOE-5C
Core depth(cm)	0–7	7–31	31–52	0–27	27–45	0–21	21–45	0–30	30–41.5	41.5–60
OTU presence (#)	18	909	235	1048	365	73	124	583	146	737
Metal (mg/kg dry wt.)										
Mn-exchangeable/CO_3	1700	2892	2082	1038	2452	1028	1500	10	27	NQ
Mn-(Oxy)hydroxides	5700	4169	7722	6783	9162	5963	7324	151	99	172
Mn-sulfidic/organic	1057	274	2177	1106	3078	1495	1709	NQ	ND	ND
Mn-residual	330	536	275	157	620	466	308	9	15	26
Fe-exchangeable/CO_3	7801	14,314	9584	5910	13,067	5386	6563	ND	ND	NQ
Fe-(Oxy)hydroxides	52,774	31,733	69,502	65,205	90,848	54,600	75,062	5142	5603	6920
Fe-sulfidic/organic	9233	2548	21,651	13,509	34,454	11,885	17,930	ND	ND	1582
Fe-residual	17,421	13,724	7981	8251	19,068	10,146	7581	1967	3609	10,530
Zn-exchangeable/CO_3	1091	1179	625	1080	1388	564	684	NQ	173	NQ
Zn-(Oxy)hydroxides	650	596	171	741	305	146	140	NQ	NQ	ND
Zn-sulfidic/organic	4616	3575	4307	4662	2038	1329	1162	NQ	ND	NQ
Zn-residual	558	560	158	82	151	121	252	ND	89	ND
Pb-exchangeable/CO_3	2789	1588	3625	1744	3515	906	1674	3	3	2
Pb-(Oxy)hydroxides	273	303	331	286	304	111	120	10	12	8
Pb-sulfidic/organic	177	157	797	155	318	71	112	17	12	16
Pb-residual	50	156	21	14	23	17	16	<1	1	1

OTU: Operational Taxonomic Unit; ND: Not Detected; NQ: Detected But Not Quantifiable; Theoretical Detection Limits-3σ (mg/kg dry wt.) Mn(8), Fe(500), Zn(80), Pb(0.2).

microarray. Gene-based microarrays can be calibrated by the addition of a known quantity of indicator DNA prior to hybridization to obtain a relative abundance response (relative to the indicator loading), however this is not trivial due to competition for binding sites, hybridization potential of indicator DNA with environmental DNA, and differences in binding affinities of DNA fragments from the indicator and environmental DNA and this procedure was not done for these samples. Clone libraries were produced using TOPO-TA kits (Invitrogen, Carlsbad, CA). Approximately 300 clones were picked and sequenced from site CDAR-1A. Protocols for clone library, PCR amplification, sequencing, and molecular analysis were performed as previously described (Field et al., 2010).

PhyloChip results were analyzed as previously described by Brodie et al. (2006), using a positive fraction (pf) cutoff of 0.92. Operational taxonomic unit (OTU) detections were combined at each of the taxonomic levels. Out of 63 demarcated phyla and 445 families represented on the PhyloChip, 40 phyla and 246 unique families were detected in core samples. Nineteen of the 246 families were eliminated from further analysis either due to single OTU detections or uniform detections across all samples. Due to low diversity measured in the sample, duplicate PhyloChips were performed on the site 1A sample from the contaminated LCDA and compared to clone libraries. Both techniques supported repeatable, low OTU diversity results in this sample.

2.3. Statistical tools

The relationship between microbial community structure and geochemical variables were first explored by evaluating all possible one and two geochemical parameter multiple linear regression models to each microbial community detection (as OTUs), and choosing models which fit best. In the simplest case, a single environmental variable can be used to predict the response of a phylum via linear regression (Supplemental Fig. 2). For a two component model (two environmental variables predicting a single phylum response), the data is fit in three dimensional space (Supplemental Fig. 3). Restriction of these exploratory analyses to these simple models was necessitated by the limited number of samples collected. Models which were statistically significant at a 5% false discovery rate were selected as candidate models. Candidate models were grouped based on R^2 value with the highest numerical value considered the best model for that microbial community representative (phylum or family level). Models with nearly identical p-values were also included in analyses. The square root of the R^2 for each linear regression model is Pearson's multiple correlation coefficient r, which varies between −1 and 1. For two variables, values of r close to either −1 or 1 indicate a strong linear relationship between the variables. The software R (R Core Team, 2013) was used to fit these regression models. Greater detail on the formation of these linear models is included in supplemental materials.

Relationships in higher dimensional data can be difficult to visually elucidate without first presenting the data in a manner that can capture variability in responses and parameters in two- or three-dimensional space, where most human minds operate. Dimensional reduction algorithms, such as principle coordinate analysis, canonical correspondence analysis, or principle component analysis, are ordination methods that condense multidimensional data onto lower dimensional space so that greatest variability described in the data is captured in the newly formed lower dimensional orthogonal axes. Ordination methods are exploratory analyses that seek patterns in multivariate data (Austin, 1985). Biplot analyses, which includes GGE biplots proposed by Yan et al. (2001; 2007), are another method for visually interpreting different types of multivariate data and are complementary to ordination methods.

GGE biplots have been used for multivariate analyses of environmental datasets in plant ecology (Yan, 2002; Yan et al., 2001). The axes in GGE biplots are similar to those used in principle component analysis, and are calculated by taking a singular value decomposition of the correlation matrix between the environmental factors and microbial responses (Yan et al., 2001, 2007). These correlations were further investigated using GGE biplots (Yan et al., 2001), which is a graphical representation of the correlations between the environmental variables and the microbial responses. A workflow diagram presenting how environmental (geochemical) and ecology (microbial community) data were carried through these analyses (Supplemental Fig. 1) is included in supplementary materials.

2.4. Geochemical characterization

Sub-cores from each site were analyzed using continuous sequential extractions (CSE) designed to identify operationally defined phase associations of heavy metals in these sediments. The CSE techniques used here follow Shiowatana et al. (2001), but employed the revised Community of Bureau of Reference (BCR) extraction methods, as outlined by Mossop and Davidson (2003) and van Hullebusch (2005). The benefit of CSE is that transient metals are less likely to redistribute during flow extractions than in equilibrium batch processes (Miro et al., 2005; Shiowatana et al., 2001; Wisotzky and Cremer, 2003). Metals selected for analysis were Fe, Mn, Zn, and Pb. Fe and Mn were selected for their ability to adsorb heavy metals, compete with other heavy metals for reactive sulfides in anaerobic conditions, and for their potential as a terminal electron acceptor for metal reducing bacteria. Zn and Pb were selected for their abundance in LCDA and toxicity to microorganisms. Sediment samples were thawed, weighed, and loaded into the extraction vessel under anaerobic conditions. Anaerobic conditions were maintained during extraction via sparging ultrapure nitrogen into the solvent phases and extractant chamber. The extractions proceeded as follows: 0.1 g of solids was initially extracted with 0.11 M acetic acid (pH 2.85) to remove exchangeable and water/weak acid soluble phases (e.g. carbonates). This was followed by digestion using 0.5 M hydroxylamine hydrochloride (pH 1.5) to remove Fe and Mn (hydr)oxides phases. The solids were then washed in water (adjusted to pH 2 with nitric acid) to reduce the reactivity for the next step of the extraction. A 30% hydrogen peroxide and 1 M ammonium acetate solution (pH 2) was then used to extract elements associated with organic and sulfidic phases. The residual solids from these sequential extractions were dried and digested in *aqua regia* (3 HCl:1 HNO_3) under batch conditions to remove remaining metals. As a comparative tool for extraction efficiency, a parallel sample of 0.1 g was directly digested using *aqua regia* digestion under batch conditions to determine *aqua regia* soluble fraction. Each digested fraction collected was stabilized in 0.5% HCl-1% HNO_3 and analyzed using inductively coupled plasma mass spectrometry (Agilent 7500ce ICP-MS).

3. Results and discussion

3.1. Phase association and geochemistry

Total elemental concentrations (Table 1) compare well with previously reported literature from the CDAR delta and St. Joe River (STJOE) delta as the control site (Harrington et al., 1998b). Decreased Pb and Zn concentrations were observed with increased distance from the CDAR channel. Additionally higher concentrations of all metals were observed in the CDAR samples as compared to STJOE control site ($Pb_{CDAR}/Pb_{STJOE} = 94$, $Mn_{CDAR}/Mn_{STJOE} = 60$, $Zn_{CDAR}/Zn_{STJOE} = 22$, $Fe_{CDAR}/Fe_{STJOE} = 8$). pH and dissolved O_2 varied very little between the four sites.

Figs. 1 and 2 show the phase association of sub-samples from CDAR and STJOE sites. Concentrations of Zn and Pb decrease with distance from the center channel of CDAR as is shown in Fig. 2. Fig. 1-A and B show redox active elements (Fe, Mn) with similar profiles between contaminated and uncontaminated sites, however the concentrations between contaminated and control sites differ substantially (Fig. 2A, B). Approximately 60% of the totals of both Fe and Mn were primarily associated with oxyhydroxide phases (Fig. 2A, B). The greater prevalence of sulfidic/organic phases in CDAR compared to STJOE appears to reflect the primary mineralogy and deposition of sulfidic host material from the mining district upstream. Greater than 50% of the total Zn was associated with the sulfidic/organic fraction (Fig. 2-C) while greater than 70% of Pb was associated with the exchangeable/carbonate phase (Fig. 2-D). The exchangeable/carbonate phase is thought to be the most bioavailable fraction since heavy metals in this fraction are easily released with changes in pH (Traina and Laperche, 1999).

In studies of surface sediments from Coeur d'Alene, Horowitz el al. (Horowitz et al., 1992) reported that 95% of Pb and 80% of Zn were associated with the reducible oxide/oxyhydroxide phases, while Harrington et al. (Harrington et al., 1998b) reported Pb and Zn to be primarily associated with the sulfidic phase. Our study shows Pb to be primarily associated with the more bioavailable exchangeable/carbonate fraction. Observed differences in phase partitioning in these three studies may be due to the method of extraction (batch vs. continuous) to identify phase association, extractant scheme (BCR, Tessier), sample handling and treatment, or seasonal/depositional effects.

Table 2 provides CSE data for all core samples. Comparison of CSE to batch *aqua regia* digests show CSE underestimated Fe by about 10% and Pb by approximately 30% while overestimating Zn by 10%. Some variability exists between *aqua regia* and CSE data, and has been noted by others using the revised BCR extraction technique (Mossop and Davidson, 2003). Additionally, an important factor that may have contributed to differences in phase partitioning for this study when compared previous studies was that the samples were collected in July soon after a sizeable seasonal flooding event. Monthly discharge rates were approximately 3.5 times the previous three year's mean monthly discharge rate for June and greater than 4.5 times the previous three year's mean monthly discharge rate for July (http://waterdata.usgs.gov/id/nwis/) which presumably transported, mixed, and deposited new sediments from stream beds, floodplains, and surrounding soils onto these river deltas. In the lateral lakes surrounding the CDAR, Bostick et al. (2001) found that seasonal changes affected the partitioning of Zn with sulfidic and carbonate phases predominating in flooded areas, while (hydr)oxides were found in oxic, drier soils and sediments. The metal (hydr)oxide species reported by Bostick were transformed to carbonate and sulfidic species during submersion

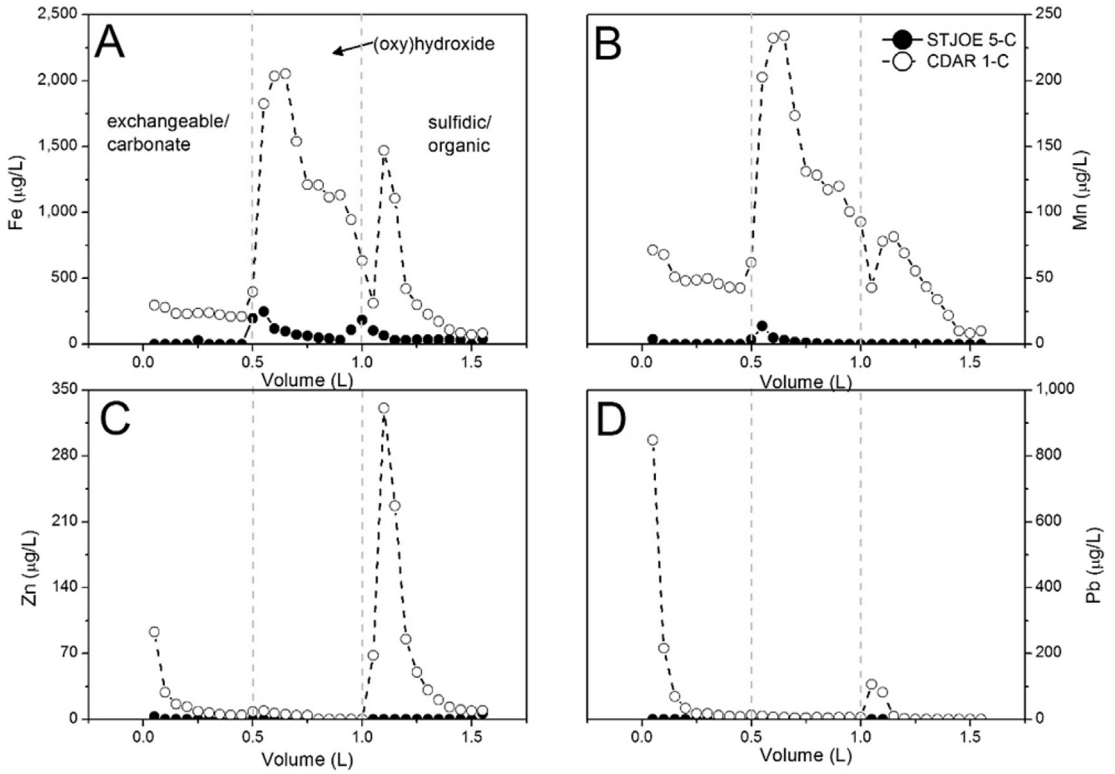

Fig. 1. Extractogram of (A) Fe, (B) Mn, (C) Zn, and (D) Pb phases from CDAR 1-C (○) and STJOE 5-C (●). Extractogram shows heavy metal phases as defined by chemical extraction.

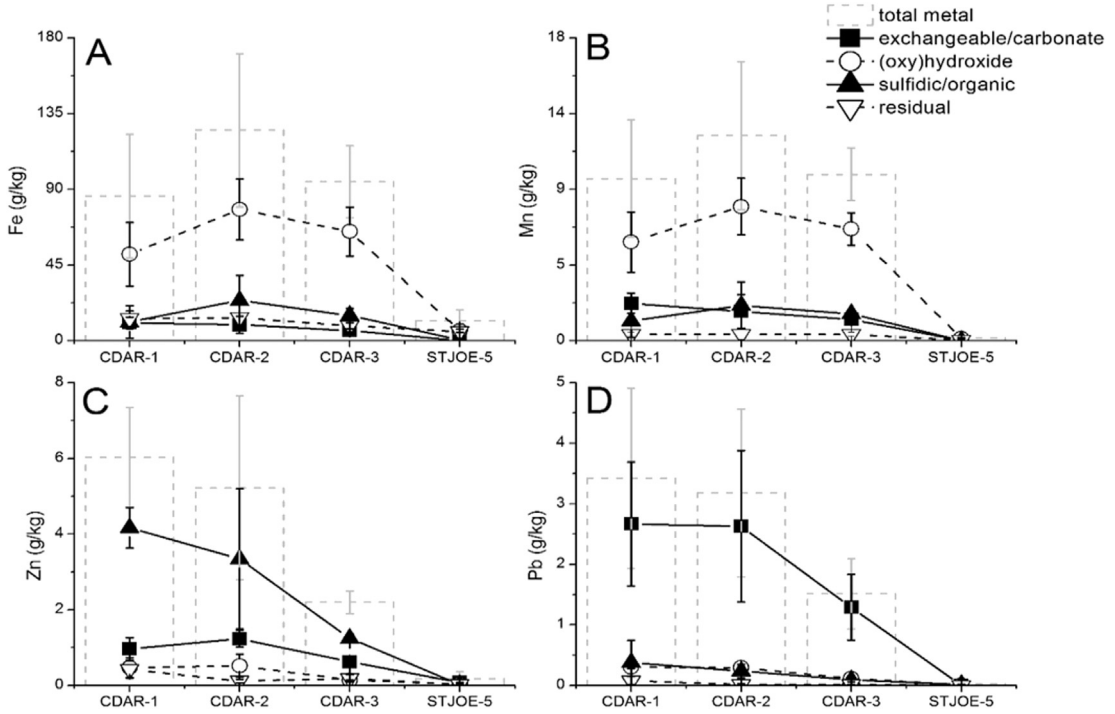

Fig. 2. Total metal (bar) and phase association (lines) for Fe, Mn, Zn, and Pb as defined from chemical extraction for metal contaminated CDAR and uncontaminated STJOE sites.

due to reducing conditions, however a small portion remained as metal oxyhydroxides (Bostick et al., 2001). In LCDA, La Force et al. (La Force et al., 1999) found that Zn and Pb increased in the operational carbonate and exchangeable phases during oxygenation. As our sampling occurred after a significant high flow event, transport and oxygenation from the riparian zone, riverbed, and lateral lakes most likely deposited additional sediments onto the CDAR delta and may explain the predominance of Pb exchangeable/carbonate.

3.2. Microbial communities and heavy metal phases

Out of 63 demarcated phyla and 445 families on the Phylochip, 40 phyla and 246 unique families were detected in core samples. Unclassified members of orders of *Bacteroidetes KSA1* and *marine group A mgA-2* were present as single detections in every sample and 17 families were only detected on one of two duplicate Phylochips; 12 in CDAR and 5 in STJOE. Phyla of these single detections were represented as follows, *Actinobacteria* (6), *Firmicutes* (3), *Proteobacteria* (3), *Acidobacteria* (2), and single detections of *Aquaficae*, *Cyanobateria*, and candidate phyla *LD1PA* group. Due to single detections these taxa were removed from further statistical analyses.

To visually examine the partitioning of remaining taxa between the uncontaminated and contaminated sites, a measure of environment affinity (E_a) was constructed to quantitatively compare taxa across the two environments. Environment affinity is defined as:

Using this measure, positive scores denote taxa affinity to the STJOE site and negative scores show affinity to CDAR. The affinity score cannot be used to compare between different taxonomies (e.g. phylum vs. family). An increase of one E_a unit indicates a doubling of diversity of the taxa of interest with respect to its environment. An equal distribution of taxa diversity between environments gives an E_a of zero.

Fig. 3 shows the E_a which indicates phyla signatures changed from the uncontaminated STJOE to the heavily contaminated CDAR site. Candidate phyla *NC10*, *OP8* and *LD1PA* were only present in metal contaminated cores and the diversity of *Natronoanaerobium* appear to double in CDAR cores compared to the control site. This increase in diversity suggests an increased fitness of these phyla in the metal contaminated sediments of the CDAR site. In contrast, diversity of phyla *Aquificae*, *Coprothermobacteria*, and *Synergistes* appear at least two times greater in the uncontaminated control site.

3.2.1. Metal/microbial interactions

GGE biplots were used to give graphical representations of the correlations between the geochemical variables and the microbial communities. Figs. 4 and 5 show GGE biplots of phyla to the dominant phases for Zn and Pb, respectively. For comparison, environmental variables were included in Figs. 4 and 5 as filled diamonds while phyla are represented with filled squares. Abbreviations used in Figs. 4 and 5 are as follows: Acidobacteria (ACID), Actinobacteria (ACTI), AD3 (AD3), Bacteriodetes (BACT), BRC1 (BRC1), Caldithrix (CALD), Chlorobi (CHLB), Chloroflexi (CHLO), Coprothermobacteria (CPTB), Cyanobacteria (CYAN), Deinococcus/Thermus (DETH), Firmicutes (FIRM), Gemmatimonadetes (GEMA), LD1PA group (LD1P), Lentisphaerae (LENT), marine group A (MGA), Natronoanaerobium (NATA), NC10 (NC10), Nitrospira (NITR), OD1 (OD1), OP10 (OP10), OP3 (OP3), OP8 (OP8), OP9/JS1 (OPJS), Planctomycetes (PLAN), Proteobacteria (PROT), SPAM (SPAM), Spirochaetes (SPIR), Synergistes (SYN), termite group 1 (TG1), Thermodesulfobacteria (THDB), TM7 (TM7), Unclassified (UN), Verrucomicrobia (VERR), WS3 (WS3), WS5 (WS5), Fe exchangeable/carbonate (FeEx), Mn exchangeable/carbonate (MnEx), Pb exchangeable/carbonate (PbEx), Zn exchangeable/carbonate (ZnEx), Fe oxyhydroxides (FeOx), Mn oxyhydroxides (MnOx), Pb oxyhydroxides (PbOx), Zn oxyhydroxides (ZnOx), Fe sulfidic/organic (FeS), Mn sulfidic/organic (MnS), Pb sulfidic/organic (PbS), Zn sulfidic/organic (ZnS), Fe residual (FeR), Mn residual (MnR), Pb residual (PbR), and Zn residual (ZnR). The two principle axes in Figs. 4 and 5 capture 89.7% of the variability in GGE plot, indicating that the biplot adequately describes the linear relationship between the geochemical variables and the communities. Microbial

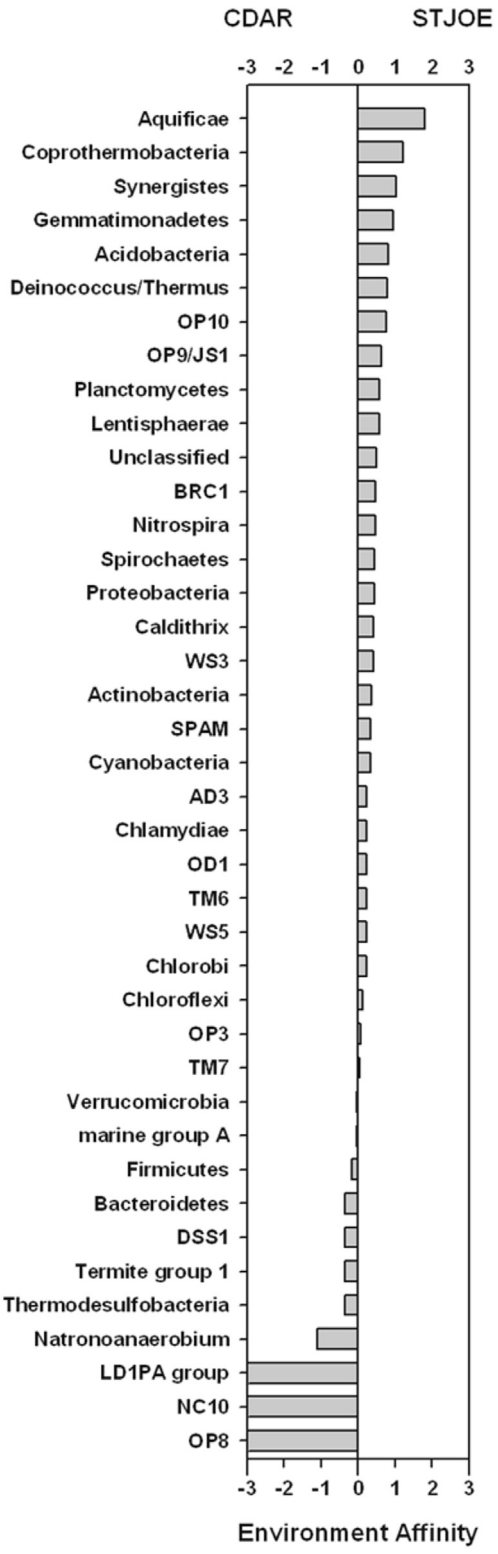

Fig. 3. Environment affinity $\log_2$([# taxa detected/g wet sediment] $_{STJOE}$/[# taxa detected/g wet sediment] $_{CDAR}$) comparison of phyla between CDAR and STJOE sites.

$$E_a = log_2 \frac{(\#\ of\ taxa/gram\ wet\ sediment)_{STJOE}}{(\#\ of\ taxa/gram\ wet\ sediment)_{CDAR}} \quad (1)$$

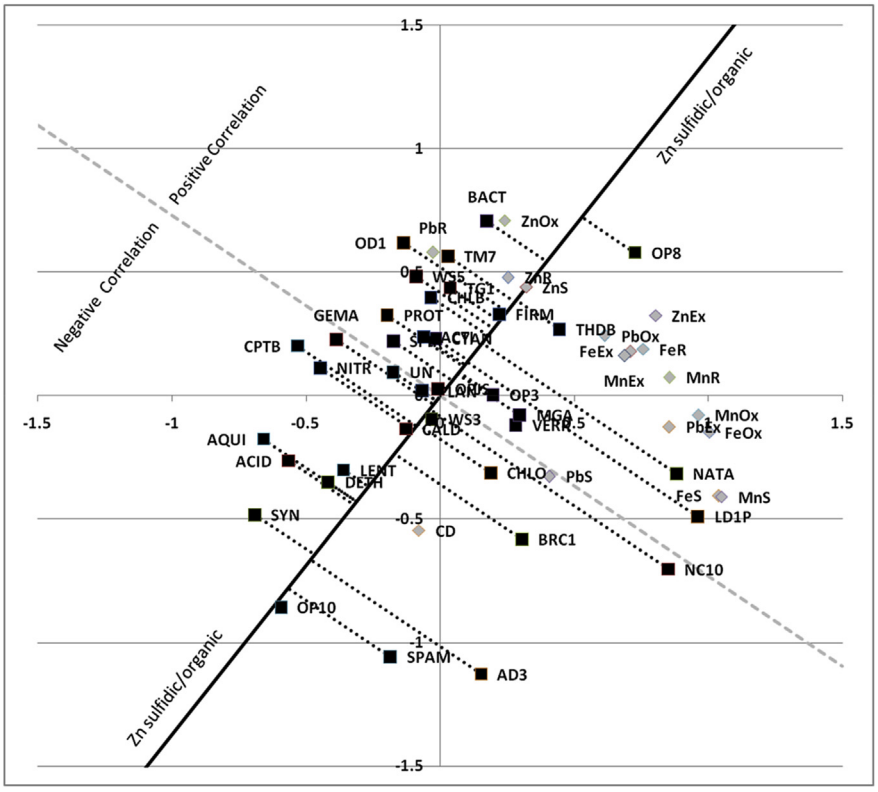

Fig. 4. Mean subtracted and standard deviation normalized "mean performance vs. stability" GGE biplot of Zn sulfidic/organic phase. X and Y axes account for 89.7% of the variance. Markers for phyla (boxes) and environmental variables (diamonds).

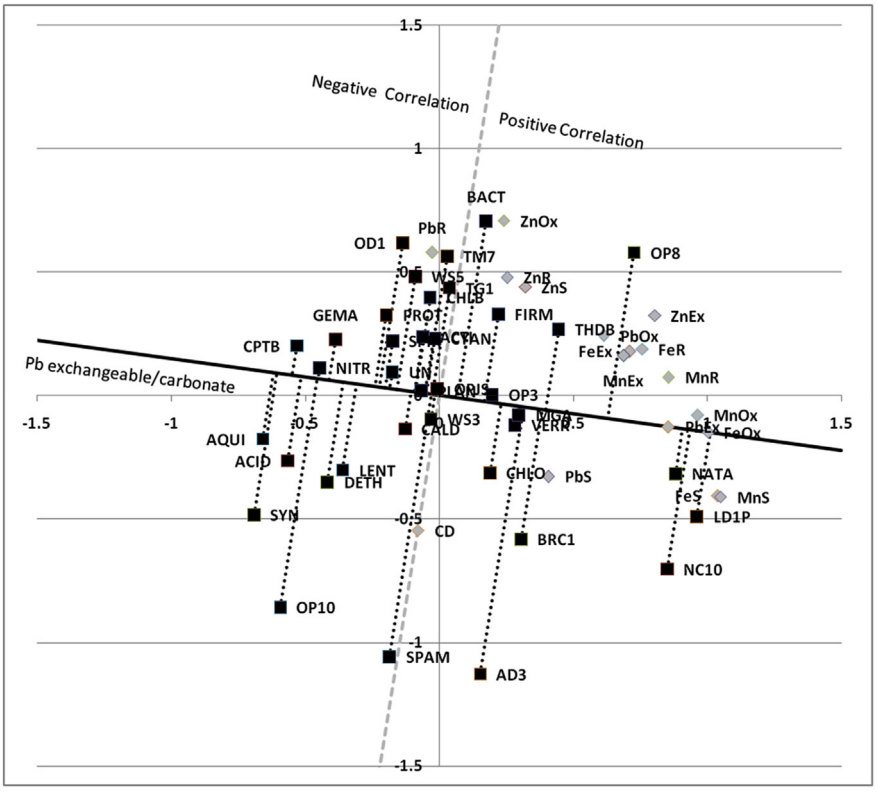

Fig. 5. Mean subtracted and standard deviation normalized "mean performance vs. stability" GGE biplot of Pb exchangeable/carbonate phase. X and Y axes account for 89.7% of the variance. Markers for phyla (boxes) and environmental variables (diamonds).

communities which lie outward along a geochemical variable axis (bold line) have more correlation (either positive or negative) with that geochemical variable. For example, Fig. 4 shows that the diversity of candidate phyla *OP8* (r = 0.64) is most positively correlated with geochemical variable Zn in the sulfidic/organic fraction, while diversity of *OP10* (r = −0.48) is most negatively correlated with Zn.

Similarly in Fig. 5, *Aquificae* (r = −0.52) and *Synergistes* (r = −0.49) are most negatively correlated with Pb in the exchangeable/carbonate fraction, while candidate phylum *LD1PA* (r = 0.49) is most positively correlated. Dominant phases from Fe and Mn (e.g., oxyhydroxides) lie approximately on the same ray from the origin as Pb exchangeable/carbonate and show very similar results and interpretation and were not included as additional figures. As these three geochemical variables lie closely along the first principle axis (x-axis), these variables account for a large part of the variability in the correlation dataset.

Linear models were used to better understand the correlations between the environmental predictors and the microbial responses. Supplemental Table 1 presents the best linear regression models predicting phylum diversity (least number of terms (up to two) with a p-value <0.05). However, some variables were capable of giving nearly identical p-values if substituted in the model, and are included. Most often these variables were highly correlated with one another and/or were predicted to behave similarly geochemically (e.g. Fe-exchangeable/CO_3 and Mn-exchangeable/CO_3). As suggested by the GGE plots (since these phyla were not at the extreme ends of any of the geochemical variable axes in Figs. 4 and 5), phyla *Acidobacteria*, *Calithrix*, *Coprothermobacteria*, *Cyanobacteria*, *Firmicutes*, *Gemmatimonadetes*, *Lentisphaerae*, *Spirochaetes*, *Thermodesulfobacteria*, *Verrucomicrobia*, unclassified phyla, and candidate phyla from *AD3*, *BRC1*, *DSS1*, *marine group A*, *OP3*, *OP9/JS1*, *and WS3* did not have a significant linear relationship with any singleton or pair of environmental variables.

Fig. 6 presents the number of phylum represented by a given model (Fig. 6-A) and the number of OTUs represented by a given model (Fig. 6-B). For example, the Pb-exchangeable/carbonate (negative coefficient) and Pb-oxyhydroxide (positive coefficient) linear model were a significant predictor for 2273 OTUs (Fig. 6-B) from nine phyla (Fig. 6-A), representing greater than 50% of total OTUs and approximately 20% of phyla, respectively. As can be seen from Fig. 6, some models were more prominent than others in predicting diversity of multiple phyla. Eighteen phyla (Fig. 6-A) or approximately one third (1406 OTUs) of the OTU diversity (Fig. 6-B) could not be described by two or less component models. Some phyla could be nearly equally predicted by more than one type of linear model and were included in Fig. 6. To illustrate, candidate phylum *NC10* could be predicted by three different models, 1) Fe-oxyhydroxide and Zn-sulfidic organic, 2) Fe-sulfidic/organic and Pb-sulfidic/organic, and 3) Mn-oxyhydroxide and Zn-sulfidic organic. The regression model with predictors Mn and Fe-sulfidic/organic and the model with Zn and Pb-residual each described approximately 6% of the OTUs measured in this study while the Pb-exchangeable/carbonate and Pb-oxyhydroxide model accounts for over 50% (Fig. 6-B).

The operationally defined Mn and Fe sulfidic/organic phases also appear to be important in multiple linear models. In the linear models that contain Mn and Fe sulfidic/organic, the coefficients from Mn sulfidic/organic phases were always negative while the coefficients for the Fe sulfidic/organic phases were always positive. This may indicate that increases in sulfidic/organic phases of Mn decrease diversity while increases in Fe sulfidic/organic increase diversity. Thus the ratio of Fe sulfidic/organic to Mn sulfidic/organic may be an important predictor of diversity for some taxa. Mn sulfides are not likely to form in this system due to competition from carbonate ions (Krauskopf, 1967), thus Mn is most likely present as the organically associated form. These sulfidic/organic phases were present almost solely in CDAR samples as compared with STJOE samples.

The residual fraction of Pb (positive coefficient) and Zn (negative coefficient) also appear to be an important model for multiple phyla. Though the biological availability of the residual fraction is not well defined in terms of chemistry, it may suggest that the ratio of Pb residual to Zn residual is important in increasing diversity or that Zn is more bioavailable to exert selective pressure. Another explanation may be that geochemistry which favors Pb residual

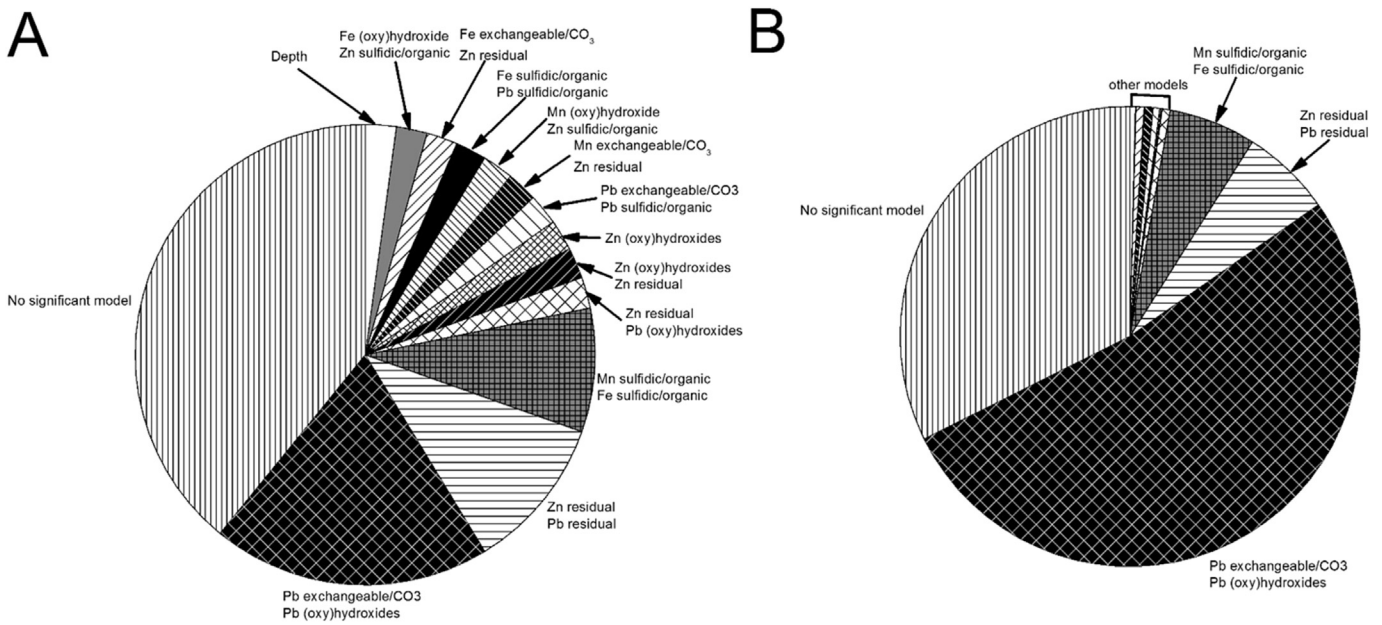

Fig. 6. A) Fraction of phylum represented by a given model B) fraction of OTUs represented by a given model. These pie charts contain some duplication as some geochemical models were equally ranked based on *p*-value (see Supplemental Table 1).

over Zn residual heavy metal phases favors higher diversity.

Pb exchangeable/carbonate and Pb oxyhydroxide appeared in multiple models with Pb exchangeable/carbonate phase coefficients consistently negative when present in the same linear model with Pb oxyhydroxide phases. This may indicate that the partitioning of Pb into more bioavailable phases was responsible for a decrease in diversity (as # OTU detected), while greater Pb partitioning into the oxyhydroxide phases allowed diversity to increase. This highlights the importance of Pb partitioning in predicting and understanding diversity in the LCDA system and suggests that increased Pb bioavailability (as Pb in exchangeable/carbonate phases) increases selective pressure and reduces microbial diversity (see Supplemental Table 1).

Because iron reducing bacteria (IRB) can release heavy metals through reductive dissolution and SRB can precipitate heavy metals as insoluble metal sulfides, IRB (Cummings et al., 1999, 2000, 2003) and SRB (Ramamoorthy et al., 2009; Sass et al., 2009) have been the focus of multiple studies in LCDA. The interplay between IRB and SRB appear to influence the fate and transport of heavy metals in LCDA (Sengör et al., 2007; Spycher et al., 2008). The presumed SRB lineages detected in this study correlate with Pb and Zn in the residual fraction, including members of orders *Desulfobacterales* ($R^2 = 0.755$; $p = 0.007$), *Desulfuromonadales* ($R^2 = 0.488$; $p = 0.094$), *Desulfotomaculum* ($R^2 = 0.599$; $p = 0.039$), and *Syntrophobacterales* ($R^2 = 0.604$; $p = 0.038$). Orders from most *Desulfovibrionales* ($R^2 = 0.517$; $p = 0.078$) and *Bdellovibrionales* ($R^2 = 0.551$; $p = 0.06$) correlate with Pb in the exchangeable/carbonate and oxyhydroxide phases. Some exceptions are families from unclassified members of the order *Myxococcales* (Zn sulfidic/organic; $R^2 = 0.526$; $p = 0.017$) and families *Desulfuromonaceae* (Mn oxyhydroxide, Fe sulfidic/organic; $R^2 = 0.622$; $p = 0.032$), and *Desulfomicrobiaceae* (Pb sulfidic/organic; $R^2 = 0.454$; $p = 0.032$).

The environment affinity presented in Fig. 3 shows *Desulfuromonaceae* was detected in greater diversity in STJOE samples. With the exception of the model for *Desulfohalobiaceae*, coefficients for Pb exchangeable/carbonate are consistently negative while oxyhydroxides coefficients are consistently positive, suggesting more labile Pb phases decrease diversity. Models for SRB lineages are negative for Zn residual and positive for Pb residual phases. Families from IRB lineages *Shewanellaceae*, *Geobacteraceae*, and *Rhodocyclaceae* show correlation to Pb in the exchangeable/carbonate and oxyhydroxide phases with decreases in diversity predicted by increases in phase partitioning to the more bioavailable Pb exchangeable/carbonate.

4. Conclusions

Metal contamination from decades of upstream mining has impacted LCDA in both sediment and aqueous chemistries. This study reflects the complexity of natural systems and interactions between microbes and their environment. Total metal concentrations measured in this study compare well with previous studies (Harrington et al., 1998b; Horowitz et al., 1992), although phase association differs, specifically in this study with respect to Pb. These differences may be due to techniques employed for phase characterization, or influenced by environmental factors, such as fresh deposition and/or oxygenation resulting high river flow conditions (see Section 3.1).

Microbial signatures appear to change with sediment metal content and associated metal phase. Candidate phyla *NC10*, *OP8* and *LD1PA* were only detected in metal contaminated cores and diversity among *Natronoanaerobium* was double that found in the uncontaminated control site. In contrast, diversity within the phyla *Aquificae*, *Coprothermobacteria*, and *Synergistes* was at least twice as high in the uncontaminated control site. Partitioning of Pb into more bioavailable phases correlated with decreases in diversity (as # OTU detected), while greater partitioning of Pb into oxyhydroxide phases correlated with increased diversity for multiple lineages for approximately 50% of OTUs detected in this study. Dynamic changes in Pb speciation, such as seasonal changes during changes in flow conditions, could impact microbial diversity. This emphasizes the importance of Pb speciation in influencing microbial diversity in the LCDA system and advances the knowledge of the LCDA system.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.apgeochem.2015.12.013.

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