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Microbial population dynamics and the role of sulfur reducing bacteria genes in stabilizing Pb, Zn and Cd in the terrestrial subsurface

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Abstract

Milling and mining metal ores are major sources of heavy metal contamination. The Spring River and its tributaries in southeast Kansas are contaminated with Pb, Zn, and Cd as a result of 120 years of mining activities. Trace metal transformations and cycling in mine waste materials greatly influence their mobility and toxicity and affect plant productivity and human health. It has been hypothesized that under reduced conditions in sulfate-rich environments, these metals can be transformed into their sulfide forms, thus limiting mobility and toxicity. We studied biogeochemical transformations of Pb, Zn and Cd in flooded subsurface mine waste materials, natural or treated with organic carbon (OC) and/or sulfur (S), by combining advanced microbiological and X-ray spectroscopic techniques to determine the effects of treatments on the microbial community structure and identify the dominant functional genes involved in the biogeochemical transformations, especially metal sulfide formation over time. Samples collected from medium-, and long-term submerged columns were used for microarray analysis via functional gene array (GeoChip 4.2). The total number of detected gene abundance decreased under long-term submergence, but major functional genes abundance was enhanced with OC plus S treatment. The microbial community exhibited a substantial change in structure in response to OC and S addition. Sulfur-reducing bacteria genes dsrA/B were identified as key players in metal sulfide formation via dissimilatory sulfate reduction. Uniqueness of this study is that microbial analyses presented here in details are in agreements with molecular-scale synchrotron-based X-ray data supporting that OC-plus-S treatment would be a promising strategy for reducing metal toxicity in mine waste materials.

Keywords

Mine waste, lead, Zinc, Cadmium, Microbial role, Sulfur-Reducing Bacteria

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1 Introduction

Generation of large amounts of mine waste containing several heavy metals is the main environmental concern associated with milling and mining activities (Baker et al., 2003, Bhattacharya et al., 2008). Heavy metals are dispersed via different pathways such as wind, surface water runoff, and metal-laden sediments are transported to neighboring water bodies (Almendras et al., 2009 and Johnson et al., 2005). The Tri-State mining district in parts of southeast Kansas, southwest Missouri, and northeast Oklahoma was one of the largest Pb and Zn ore-mining districts in the world for 120 years (until 1970). The movement of soluble metals and metal-laden sediments from the landscape into surface waters via surface runoff are the primary ecological concerns for both aquatic and terrestrial organisms (Pierzynski et al., 2006). The US Environmental Protection Agency (US EPA) has suggested wetland construction as a remediation strategy for soils highly contaminated by abandoned mine waste materials with the hypothesis that these metals could be transformed into their sulfide forms under reduced conditions in sulfate-rich environments, thus limiting their mobility and toxicity.

Several challenges are associated with this strategy. Mine waste material with low dissolved OC content could have significant effects on redox processes (Hayes et al., 2006, Stein et al., 2007 and Zhang et al., 2005) because OC is the main driver of biogeochemical cycling of major and trace elements (Borch et al., 2009 and Evans et al., 2006). Limited S in mine waste could limit sulfide formation and promote carbonate precipitation, depending on pH and carbonate concentration (Toevs et al., 2006). Therefore, the addition of OC and S could facilitate these metals to be transformed back into their sulfide forms under reduced conditions, thereby limiting their mobility and toxicity. A generalized sulfate reduction reaction using organic matter (OM) as an electron donor is:

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$SO_4^{2-} + 2CH_2O + 2H^+ \rightarrow H_2S + 2H_2O + 2CO_2$ (pH<7.0): Stein (10)

At high metal concentrations, metals tend to precipitate as metal sulfides around circumneutral pH because the rate of H_2S formation increases at a pH of 7.0 to a maximum of 8.0 (Burton et al., 2008 and Chen et al., 1997):

$H_2S + M^{2+} \rightarrow MS + 2H^+$

The above-mentioned reaction is the result of dissimilatory sulfate metabolism that has been tested and successfully removed contaminants via biostimulation. Of all the metal sulfide minerals, iron sulfide mineralization is most often attributed to microbial activity (McLean et al., 2007), especially to the activity of dissimilatory sulfur-reducing bacteria (SRB). Environmentally important activities displayed by SRBs are the result of metabolic production of high levels of sulfides that are reactive and participate in subsequent mineral formation (Bazylinski et al., 2003 and Lovely et al., 1995).

Using a culture-dependent technique would not be feasible to study the complex microbial community because 99% of microorganisms have not been cultured (Whiteman et al., 2004), therefore, culture-independent techniques such as functional gene arrays (FGA) are required (He et al., 2007 and Van Nostrand et al., 2011). GeoChip 4.2 is a functional gene array that contains 83,992 oligonucleotides (50-mer) probes targeting 152, 414 genes in 410 gene categories from more than 5200 microbial strains including bacteria, archaea, fungi, and viruses. These genes are involved in the biogeochemical processes and functional activities of microbial communities important to human health, ecosystem management, agriculture, energy, global climate change, and environmental cleanup and restoration, including N, C, S and P cycling; metal reduction and resistance; and organic contaminant degradation (Tu et al., 2014). This technique enables detection, characterization, and quantification of microorganisms in mine

96 waste and links microbial diversity to ecosystem processes and functions (He et al., 2007 and
97 Loick et al., 2014). The approach has been used successfully to track the dynamics of metal-
98 reducing bacteria and associated communities for an *in situ* bioremediation study (Lu et al.,
99 2012, Wu et al., 2001, Van Nostrand et al., 2009 and Zhou et al., 2008).

100 Phospholipid fatty acid analysis (PLFA) is another rapid, inexpensive, and an efficient
101 way to determine the structure, and the effect of treatments on microbial community (Frostegård
102 et al., 2011). Certain PLFAs markers can serve as unique signatures for a particular group.
103 However, such biomarkers cannot detect individual microbial species due to overlapping PLFA
104 patterns; nevertheless, whole PLFA pattern is used to elucidate the shift in community
105 composition, and their relation to specific metabolic and environmental conditions (Olsson et al.,
106 1999).

107 Few studies have combined microbial analysis with solution chemistry and microscopic
108 and X-ray spectroscopic techniques to develop a complete molecular-scale understanding of
109 complex biogeochemical processes affecting soil and water (Brantley et al., 2007 and Brown et
110 al., 1999). This study attempted to explore the interplay between geochemical and biological
111 processes in the transformation of Pb, Zn and Cd in natural subsurface environments
112 biostimulated by the addition of OC and S. Stimulating the systems with OC and S would favor
113 SRB growth and activities. We expect that OC-plus-S treatment would result in a higher
114 abundance of SRB genes compared with natural, OC alone, or S alone treatments. Study
115 objectives were to: a) characterize the microbial community playing a role in the biogeochemical
116 transformation of Pb, Zn and Cd under reduced conditions; b) measure the change in microbial
117 community structure with OC and/or S treatment over medium- and long-term incubation; and c)

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identify the most dominant genes and associated mechanisms involved in effective immobilization of Pb, Zn and Cd.

2 Materials and Methods

2.1 Sample collection and characterization

Contaminated mine waste materials were collected from a secured repository area in Baxter Springs, KS, a part of the Tri-State mining district that has a 120-year history of Pb- and Zn-ore mining related activities. The material was sieved to 2-mm size, and 0.5-g sample was digested in triplicate following the aqua-regia reflux tube soil-digestion method to determine the concentrations of selected elements (Zarcinas et al., 1996). Total N, and C content was measured using LECO TruSpec CN Carbon/Nitrogen combustion analyzer (LECO Corporation, St. Joseph, MI). The pH of a water extract (water: mine waste ratio of 2:1) was determined using Orion Ag/AgCl pH electrode. Particle-size distribution was determined using a modification of the pipet method of Kilmer et al. (Kilmer et al., 1949), and method 3A1 from the Soil Survey Laboratory Method Manual (1996).

2.2 Treatment application and experimental setup

For S-treatment application, sodium sulfate (Na_2SO_4) solution was added to the mine waste material to provide S at a ratio of 1:2 mM of sum of metals present in material: mM of S. The metal concentrations used for the summation were Pb, Zn, Cd, Fe, and Mn. The treated materials were equilibrated for 10 days at room temperature on a reciprocating shaker (6010, Eberbach Corporation, Ann Arbor, MI) at 192 reciprocates/min for 3 days, and at 92 reciprocates/min for the remaining 7 days. After equilibration, S-treated mine waste was leached with deionized (DI) water to reduce salinity until a target electrical conductivity of <2 mS cm^{-1} was achieved, then the material was air-dried. Both S-treated and untreated mine waste materials were inoculated

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141 with 0.5 g $100g^{-1}$ of soil slurry (Ivan, Kennebec, and Kahola silt loams) collected from the North
142 Agronomy Farm at Kansas State University, Manhattan, KS. The serial dilution of soil slurry
143 was cultured on a Petri dish using Postgate's medium and incubated overnight at 34 ºC in an
144 anaerobic jar (AG0025A used with oxygen absorber; OXAN0025A, Fisher Scientific,
145 Pittsburgh, PA). The black patches observed on the plate indirectly confirmed the presence of
146 SRB in the soil slurry. The method used for SRB culturing was adapted from Luptakova et al.
147 (Luptakova et al., 2005). The mine waste materials (non-treated or treated with S) were well
148 mixed with soil slurry and used to pack Plexiglas columns (20 cm length, 3.2 cm ID with 3
149 windows milled at 2.8 cm, 9.84 cm, and 16.94 cm) to achieve a bulk density of about 1.7 g cm^{-3}.
150 The packed columns were saturated slowly with DI water using a Mariotte's bottle that delivered
151 a constant flow rate before the eluent solution was supplied. The eluent consisted of a base of
152 simulated groundwater (1 mM NaCl, 1mM $MgCl_2$, 1 mM KCl, 1 mM $CaCl_2$ adjusted to pH 7.2)
153 with or without 10.7 mM Na-lactate (32 mM OC). This eluent provided four treatments for the
154 columns designated as C0S0, C1S0, C0S1, and C1S1, where C0 and C1 designated simulated
155 groundwater without OC and with OC, respectively; S0 designated simulated groundwater
156 applied to columns without added S; and S1 designated simulated groundwater applied to
157 columns with added S. Each treatment combination had two replicates due to limited space
158 available in the glovebox. The eluent solution was supplied using a syringe pump (KD Scientific
159 Inc., Holliston, MA) at the rate of 13 mm day^{-1} to simulate a slow groundwater discharge rate
160 (Wan et al., 2005). Three series of column experiments, short (32-day), medium (119-day), and
161 long-term (252-day), were conducted at room temperature ~25 °C at different times due to the
162 lack of space in the anaerobic chamber to conduct them all simultaneously. All three series of
163 experiments were conducted based on a completely randomized design with a two-way factorial

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experiment (factor 1: OC with two levels, 0 and 10.7 mM L^{-1}; factor 2: S with two levels; 0 and 252.7 mg kg^{-1}). Effluent samples were collected weekly for medium-term and biweekly for long-term submergence, and analyzed for pH, redox potential, total dissolved elements measurements for Pb, Cd, Zn, Fe, S, Mn, K, Ca, Mg, Na, anions including sulfate, nitrate, nitrite, chloride, phosphate, and dissolved organic carbon (DOC) measurements. At the end of each column experiment, samples (about 20 g) were collected from three windows located on the columns and frozen at -80 °C for DNA extraction, and x-ray absorption spectroscopy (XAS). More details on solution chemistry data collection, and approaches used in synchrotron-based X-ray analysis, and their outcomes can be found in Karna et al. (2016).

2.3 Phospholipid fatty acid (PLFA) analysis

The PLFA analysis was performed as an initial measurement to determine the microbial community changes with OC and S treatment prior to microarray analysis was performed. For this, PLFA extraction was done on the original mine waste materials, and submerged C0S0, and C1S1 treatments from medium term study only based on single phase extraction of lipids, which was then methylated to give fatty acids methyl esters (FAME) and analyzed by gas chromatograph. The PLFA extraction was performed by following the method of Bligh and Dyer (1959) as modified by White and Ringlberg (1998). The resulting FAMES were analyzed using a Thermo Scientific Trace GC-ISQ mass spectrometer (Thermo Scientific, Germany) with helium as a carrier gas. Analysis was conducted in the electron impact (70 eV) mode. Peaks were identified based on retention times of commercially available bacterial acid methyl esters (BAME; Matreya 1114) standard mix. The methyl ester peaks that were not present in the BAME mix were tentatively assigned through mass spectral interpretation by comparison with spectra from a library (Wiley 138K mass spectral database). Sample peaks were quantified based

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187 on comparison of the abundance with an internal standard - nonadecanoic acid methyl ester
188 (19:0). The abundance was expressed as nmoles/gram. Fatty acids (FA) are designated a:b,
189 where 'a' represents total number of carbons and b the number of double bonds. An 'ω' indicates
190 the position of a double bond from the aliphatic end of the FA. The prefixes 'a' and 'i' refer to
191 anteiso and iso branching, while the suffixes 'c' and 't' refers to cis and trans 33 isomers
192 (conformations). Presence of methyl groups are indicated by aMe, where 'a' indicates the
193 position of the methyl group. Fatty acids were grouped based on criteria by McKinley et al.
194 (2005) whereby Gram positive bacteria were branched monounsaturated cyclopropane (i-15:0, a-
195 15:0, i-16:0, i-17:0 and a-17:0). Gram negative biomarkers are cyclopropane PLFAs (2-OH 12:0,
196 3-OH 12:0, 2-OH 14:0, 3-OH 14:0, 2-OH 16:0, C16_1_9_cis, C16_0_2-OH, 16:1ω7c, 18:1ω7c,
197 cy17:0, cy19:0), while actinomycetes is 10Me18:0. Fungi biomarkers are polyunsaturated
198 PLFAs (18:1ω5c, 18:2ω9, 12C, 18:2ω6,9,12), while arbuscular mycorrhizae fungi (AMF) is
199 16:1ω5c, and *Desulfoviobrio* biomarker is i_17_1 (McKinley et al., 2005).

200 2.4 GeoChip analysis

201 Microarray analysis was performed on all the treatment combinations; C0S0, C0S1, C1S0, C1S1
202 from medium term study, whereas only C0S0 and C1S1 treatment combinations were used from
203 long-term study. The samples selection for long-term study was done based on the geochemical
204 and spectroscopic results obtained from medium-term study. Due to lack of space issue in the
205 glovebox chamber, we used two replicates for each treatment combination. Rather than running
206 microbial analysis for those two replicates, we did generate an additional replicate by mixing
207 equal portions materials of 1 and 2 column replicates and ran that as an independent
208 confirmation sample/third replicate.

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2.5 DNA extraction, labeling, hybridization, scanning, and data processing

About 5 g of soil was used for genomic DNA extraction using the PowerMax soil DNA isolation kit (Mo Bio, Carlsbad, CA). Raw DNA extracts were purified using Wizard Plus *SV* Minipreps purification system (Promega Biosciences, San Luis Obispo, CA). Purified DNA was quantified using the Quant-iT PicoGreen dsDNA assay kit (Promega Biosciences, San Luis Obispo, CA). DNA was labeled then hybridized at 42 °C on GeoChip 4.2 as described in Lu et al. (2012). The hybridized arrays were scanned with a NimbleGen MS 200 Microarray Scanner, and scanned images were extracted and quantified using Nimble Scan software (Roche NimbleGen, Madison, WI), followed by data preprocessing (Lu et al., 2012). Positive and negative controls, including (i) 8 degenerate probes targeting 16S rRNA sequences for positive controls, (ii) 563 strain-specific probes targeting 7 hyper-thermophile genomes for negative controls, and (iii) a common oligonucleotide reference standard for data normalization and comparison was included for grid alignment and data normalization and comparison (Liang et al., 2011). Statistical analyses were performed using SAS for Windows version 9.2 (SAS Institute Inc., 2009). The data were analyzed using PROC ANOVA. Tukey's Honestly Significant Difference (HSD) test was used for means separation ($\alpha = 0.05$). Dissimilarity test was also conducted by using the software available at Institute of Environmental genomics (IEG) website, OU, OK (Table S3). All hybridization data are available at http://www.agronomy.k-state.edu/research/soil-and-environment/soil-environment-chem/Research%20Data.html.

3 Results

3.1 General characterization of mine waste materials

The mine waste material consisted of 85% sand (2000 to 50 µm), 11.3% silt (50 to 2 µm), and 3.4% clay (<2 µm). Total N and C were 0.03 g kg^{-1} and 1.56 g kg^{-1}, respectively. The pH of the

232 water extract (DI water: geomaterial mass ratio, 2:1) was 7.2, and the electrical conductivity was
233 2.31 mS cm^{-1}. Selected total elemental concentrations of Pb, Zn, and Cd in the material were
234 5048, 23,468, and 67 mg kg^{-1}, respectively (Table S2). The standard reference material 2711a
235 (National Institute of Standards and Technology, Gaithersburg, MD) was digested along with the
236 geomaterial to ensure a recovery percentage of each element that ranged from 79 to 109%.

237 **3.2.** Preliminary microbial community characterization

238 The PLFA analysis results on starting original mine tailings, inoculum, non-amended control,
239 and amended soils submerged for 119-day indicated the presence of biomarkers for various
240 microbial groups (Gram-, Gram+, AMF, fungi, and Actinomycetes). Total PLFA in starting mine
241 waste materials was 2.42 nmole/g, whereas it was 6.18 nmole/g in the submerged sediment that
242 was used as inoculum (Table S1). Once the materials were inoculated and submerged, no
243 significant increase in summed abundance of PLFA biomarkers was observed in non-amended
244 control (C0S0), whereas it was significantly increased in the samples treated with both OC plus S
245 (C1S1). Specifically, Gram-, and Gram+ biomarkers abundance was significantly increased in
246 amended soil, with respect to starting mine waste materials, whereas there was no noticeable
247 difference in non-amended soil. Fungi biomarker abundance was decreased with varied amount
248 in both untreated and treated soils under submergence. AMF and Actinomycetes PLFA
249 biomarkers were also decreased, however remains same in both non-amended and amended
250 samples. More interestingly, total PLFA for *Desulfovibrio* biomarkers was significantly
251 increased in OC plus S treated soil only (Table S1).

252 3.3. X-ray absorption spectroscopy

253 Multiple synchrotron-based techniques have been used to enhance quantitative mineral species
254 identification (Heald et al., 2007 and Manceau et al., 2002). Micro-, and bulk-XAS as well as μ-

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255 XRD techniques were used to identify the minerals in the original mine waste materials in this
256 study. The results in agreement between μ-XRD and bulk XAFS techniques indicated presence
257 of carbonates, sulfates, silicates, and oxides minerals, which are supported by other studies
258 conducted on smelter-impacted soils (Manceau et al., 2000a, Nachtegaal et al., 2005 and
259 Scheinost et al., 2002). Bulk-XAFS speciation conducted for Pb, Zn and Cd in starting mine
260 waste materials that was used in this study indicated none sulfide minerals, whereas it was
261 dominant with silicates, carbonates, sulfates, phosphates, nitrates and hydroxides minerals (Fig.
262 1a, 1b and 1c). Speciation changed after the mine waste material was treated with OC and/or S,
263 and submerged for different time period. Bulk XAS data indicated about 62% galena (PbS), 31%
264 sphalerite (ZnS) and 39% Cd-sulfide formation in C1S1 compared to none in C0S0 (Fig. 1a, 1b
265 and 1c), respectively under long-term incubation. Instead, more carbonates were formed in non-
266 amended (C0S0) flooded materials (Karna et al., 2016). Functional gene diversity
267 Functional gene richness, indicated by the total number of genes detected, was significantly
268 increased in C1S1 compared to C0S0 under medium-term submergence (Fig. 2). In contrast,
269 under long-term submergence, the total number of detected genes significantly decreased in both
270 C0S0, and C1S1 treatment (Fig. 2).

271 ### 3.4. Relationships among microbial communities

272 Detrended correspondence analysis (DCA) was used to examine the overall functional structure
273 changes in microbial communities with the OC-plus-S treatment under medium- and long-term
274 submergence. In the DCA ordination plot, similar samples cluster closely (Ramette et al., 2007).
275 The overall DCA ordination plot obtained from all detected genes resulted in clear clustering of
276 samples from medium- and long-term submergence (Fig. 3).

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When samples from medium- and long-term submergence were plotted individually, separate clusters for each treatment were formed (Fig. S1), indicating an overall effect of OC and/or S treatments and time on the community structure in relation to geochemistry dynamics and enhanced reduction (Fig. 3). DCA analysis with metal resistance genes showed a separate cluster for C1S1 but there was some overlap among the rest of the treatments under medium-term submergence (Fig. S2), however clearer clusters were formed for both C0S0 and C1S1 under long-term submergence. Interestingly, the DCA ordination plot of C-cycling genes indicated clear cluster for C1S0 when only OC was added (Fig. S3). Similarly, the DCA plots of S-cycling category, and S-genes such as dsrA and dsrB segregated much clearly for C0S1 and C1S1 when S was added, whereas no overlapping was observed with rest of the other treatments (Fig. S4, S5, S6). Under longer submergence, both treatments, C0S0 and C1S1 samples made separate clusters under each category. Overall, DCA results for metal resistance and S-cycling genes showed clear clusters for the treatments submerged for both medium and long term, but the DCA ordination plot for C-cycling genes showed slight overlapping. The DCA of individual S-cycling genes: dsrA, dsrB (Fig. S5, S6) revealed clearer clusters with dsrB compared with dsrA genes.

3.5. Total abundance of functional gene categories

The shifts that were observed in the DCA ordination plots were likely the result of changes in total abundance of functional genes. Results from individual gene categories revealed that S- and C-cycling functional gene abundance was enhanced by 35% and 27%respectively, in C1S1 compared with C0S0 over time (Fig. 4a). On the other hand, metal resistance and organic

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299 remediation functional genes decreased by 26% (Fig. 4a) and 21% (Fig. 4b), respectively, in
300 C1S1 compared with C0S0.

301 Thus, significant enrichment of S- ($\rho = 0.01$) and C-cycling genes ($\rho = 0.01$) and a large
302 decrease in metal resistance ($\rho = 0.001$) and organic remediation genes by 50 to 60% ($\rho = 0.001$)
303 within both treated and untreated samples over time could have resulted in community structure
304 changes. Functional genes involved in S- and C-cycling were significantly enhanced in C1S1
305 despite the fact that the total number of detected genes decreased under long-term submergence,
306 indicating direct involvement of S- and C-cycling genes in biogeochemical transformation
307 processes.

308 ### 3.6 Changes in S-, C-cycling, and metal resistance genes

309 To better understand the differences observed in the categories above, changes in individual
310 genes were examined. Sulfate-reducing bacteria mediate the direct and indirect reduction of
311 heavy metals and metalloids (Chen et al., 1997 and White et al., 2000), and have been considered
312 key players in anaerobic bioremediation for contaminated soils, waters, and subsurface (Janssen
313 et al., 2004 and Kirk et al., 2002). In SRB, the dsr gene encodes the dissimilatory sulfite
314 reductase enzyme with subunits, and A/B is a key enzyme in reducing sulfite to sulfide and is
315 required by all sulfate reducers (Klein et al., 2004). Thus, dsr genes provide insight into SRB
316 activities and their functional role in sulfate reduction. Under S-cycling, dsrA, dsrB, and csyJ
317 were more abundant by 31% ($\rho = 0.01$), 35% ($\rho = 0.01$), and 40% ($\rho = 0.002$), respectively, in
318 C1S1 compared with C0S0 under long-term submergence (Fig. 5a), indicating their major role in
319 dissimilatory sulfate reduction. Similarly, among C-cycling functional genes, phenol oxidase and
320 endochitinase were the most dominant genes and were 35% ($\rho = 0.002$) and 30% ($\rho = 0.017$)
321 more abundant, respectively, in C1S1 than in C0S0 (Fig. 5b). Metal resistance genes for Cd, Zn,

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and Pb were examined, and cadA (Cd resistance gene), czcA (Cd, Zn, and Co resistance gene), and pbrA (Pb resistant gene) decreased by 29% (ρ=<0.001), 24% (ρ =0.002), and 15% (ρ = 0.002), respectively, in C1S1 compared with C0S0 over time (Fig. 5c).

Canonical correspondence analysis (CCA) was performed to examine the relationship between microbial community structure and geochemistry (Fig. 6) to correlate environmental variables with the functional community structure and determine the most significant variable causing the change in community structure. Environmental variables such as dissolved organic carbon (DOC), SO_4^{2-}, total S, and NO_3^- were used to perform CCA.

In CCA, environmental variables are represented as arrows starting at the origin and pointing outward. Our CCA results show that DOC and S are closer, with a small angle indicating these variables have a stronger correlation and have similar influence on microbial communities. Dissolved organic carbon and NO_3^- had longer arrows with larger angles, indicating these variables have a stronger influence on the microbial community but in a different manner. The SO_4^{2-} and total S vectors are in opposite directions, indicating that these factors are negatively correlated. This could be explained as the total difference between total sulfur and sulfate is sulfide indicating that under high S concentrations, sulfide formation has been favored.

4.0. Discussions

4.1. Preliminary microbial community characterization

The contaminants effects on *in situ* microbiota are generally continuous, and may trigger the loss or emergence of a particular genera or species of microorganism (Smith et al., 1986). The higher abundance of gram+, and fungi biomarkers were present in the starting materials as these communities are more successful in resource limited situations like mine impacted soils with

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very less nutrients. On addition of inoculum followed by OC and S treatment, changes in PLFA composition and biomass was detected compared to non-amended soil in medium-term submergence. This suggests that the OC and S additions in this study favored microbial growth pattern and composition resulting in change of microbial community structure. Specifically, branched monounsaturated cyclopropane PLFAs, characteristic of Gram+ bacteria, and cyclopropane PLFAs, characteristic of Gram- bacteria abundance, and branched fatty acid, i17:1, characteristics of *Desulfovibrio* were increased on OC and S amendment indicating that these PLFA biomarkers could be the main contributors in microbial community structure change in amended soil. The increased abundance of those microbial communities could be due to added OC and S, their prior presence, and their capability to survive in adverse situation, and difference in substrate utilization (Bossio et al., 1998 and Ibekwe et al., 1998). Another reason could be due to increased metal resistance genes. Several gram+, and gram- soil bacteria isolated from a Pb-contaminated sites have exhibited resistance to a range of metal ions such as Pb, Zn, Cu, Cd, Co, and Hg (Trajanovska et al., 1997). The significant increase in *Desulfovibrio* biomarker could be result of dissimilatory sulfate reduction happening in the system due to OC and S addition.

4.2. Relationships among microbial communities

Detrended correspondence analysis conducted based on time effect indicated that there was not very clear clustering of microbial community based on OC and/or S treatments under medium-term submergence. Relatively more overlapping among the samples from C1S0, C0S1, and C0S0 systems were observed compared to C1S1 (Fig. S2), and that suggests some closer associated microorganisms from these treatments. The closely associated microbes could be due to common, and flexible substrate utilization preference. Comparatively, lesser or no overlap was observed among the samples from C1S1 and C0S0 under longer submergence. The segregation

among the clusters from these two treatments under different category increased with time depending on their involvement in microbial community structure changes. This supports the fact that time was another dominant factor in determining the microbial community structure. The positive effect of OC, S, and N via increase in corresponding functional genes abundance and the impact on change in microbial community structure has been observed by several studies (Fuhrman et al., 2009, Kleikemper et al., 2002 and Tokunaga et al., 2003). Overall, DCA results indicated that the decreases in metal resistance and organic remediation functional genes and enrichment in S- and C-cycling functional genes were mainly involved in the observed community shift.

4.3. Functional gene diversity

The significant increase in microbial community abundance in C1S1 followed by a significant decline may indicate rapid oxidation of added OC coupled with a reduction in available terminal electron acceptors (TEAs) and a subsequent decline as suitable TEAs were exhausted. This result could be explained by the trend that was observed with DOC concentration in the current study. Initial concentration of DOC in the eluent was 32 mM but was reduced to 30 mM in effluent at 7-day submergence and further decreased to <detection limit (DL) under long-term submergence in OC-added treatments. On the other hand, non-OC-treated columns showed <3 mM DOC, with no significant change during long-term submergence (Table 1). A similar result was reported by Brodie et al. (2006), in which initial enrichment in total functional genes was observed with OC addition and subsequently declined, but no such enhancement in functional gene richness was observed without OC addition. Therefore, we speculate that this results could be owing to decreased availability of OC (<3 mM) (Table 1).

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390 Table 1. Chemical data for the effluent samples collected after medium- (119-day) and long-
391 (252-day) term submergence. The soil samples collected at these time points were used for
392 microarray analysis.

393 Previous studies revealed that the addition of OC stimulated biomass and microbial
394 activity in these typically nutrient-poor environments and had a significant effect on microbial
395 biomass, microbial community structure, and functional genes (Holmes et al., 2002, Martin et al.,
396 2002 and Yergeau et al., 2007). Sufficient labile OC must be available for sulfate reduction and
397 is a key rate-limiting factor in metal sulfide formation (Ku et al., 2008 and Morse et al., 1999).
398 This process can be accelerated by the action of indigenous microorganisms fueled through the
399 addition of exogenous carbon (Khan et al., 2010). The change in microbial community structure
400 was observed because of direct and indirect involvement of certain functional genes that was also
401 reported in the study, bioremediation of U using the microarray conducted by Van Nostrand et
402 al. (2011). Several other studies conducted using other techniques, such as phospholipid fatty
403 acid analysis (PLFA) and polymerase chain reactions-denaturing gradient gel electrophoresis
404 (PCR-DGGE), reported changes in microbial community structure with the addition of OC as a
405 substrate (Calbrix et al., 2007, Griffiths et al., 1998 and Eiler et al., 2003).

406 As previously mentioned, some of these genes represent background populations,
407 whereas others may be directly involved in bio-reduction (Van Nostrand et al., 2011). For
408 example, if organic remediation genes are considered to represent background functional genes,
409 their significant decrease (Fig. 4b) is probably owing to an increase in genes directly involved in
410 bio-reduction (i.e., dsrA/B) rather than a true reduction in organic remediation genes, because
411 they are likely not involved in bio-reduction; the similar result was also reported by Van
412 Nostrand et al. (2011).

4.4. Total abundance of functional gene categories

The abundance of stress-related functional genes and metal resistance genes decreased during long-term submergence, with both C1S1 and C0S0 indicating that in addition to OC and S, submergence time played a role in decreasing toxicity in these systems. Heavy metals are predicted to represent a major stress on the microbial community, and adaptation to metal stress may be of particular importance in shaping microbial community structure (Hemme et al., 2010). Several studies have indicated the impact of heavy metals on microbial activities and their community structure (Khan et al., 2010 and Hemme et al., 2010). A study conducted on the effects of Pb and Cd on soil microbial activities and their community structure via denaturing gradient gel electrophoresis (DGGE) indicated that Pb and Cd together decreased the number of bacteria when no nutrients were supplied and revealed a significant impact on community structure dynamics, particularly at high Pb and Cd concentrations (Khan et al., 2010). Increased activity of S-cycling functional genes could be owing to readily available sulfate as TEA under more reduced conditions, thereby favoring dissimilatory sulfate reduction as reported before by Brodie et al. (2006) and Muyzer et al. (2008). In the current study, the relationship observed between enhanced dissimilatory sulfate reduction and increased S-cycling functional genes can be further supported by the decreased sulfate-S concentration in effluent samples (Table 1) and the increased metal sulfide formation (Fig. 1a, 1b and 1c). Direct involvement of C-cycling and S-cycling genes in dissimilatory S reduction via rapid consumption of OC followed by sulfate reduction were also reported before by Huerta-Diaz et al. (1998). The changes in microbial communities' result in changes in functional gene abundances.

Metal precipitation is one of the most significant processes involved in the long-term retention of metals in artificial and natural wetlands. Such processes may be accompanied by other indirect

436 reductive metal precipitation (such as redox transformation), including dissimilatory sulfate
437 reduction and the subsequent precipitation of metal sulfides (Huerta-Diaz et al., 1998). As
438 reported in Karna et al. (2016), our results also suggest that appropriate microbial communities
439 were stimulated by OC- and/or S- treatments and resulted in rapid immobilization of Pb, Zn, and
440 Cd in both C1S0 and C1S1 under medium- and long-term submergence. The reduction of metals
441 concentration in solution were most likely due to biogeochemical transformations of Pb, Zn and
442 Cd under reduced conditions. This was supported by bulk XAS, indicating increasing galena
443 (PbS), sphalerite (ZnS), and cadmium sulfide formations in C1S1over time (Karna et al., 2016).
444 Similar amount of galena formation was also observed in C1S0. Limited S concentration and
445 enhanced pH in C1S0 treatment, however, could lead metal carbonates to be more stable in long
446 run, which are not as stable as sulfide minerals, and controlling metal solubility. Therefore,
447 treatment with both OC and S will be more promising as metal sulfides are more resistant to
448 oxidation, and less sulfide formation is needed to maintain permissibly low metal concentrations
449 in water for a longer period of time.
450 A handful of studies have examined non-redox-sensitive element removal via constructed
451 wetland treatment systems (Almendras et al., 2009 and White et al., 2000). Earlier studies by
452 Almendras et al. (6) tested Pb, Cu, and Zn stability via sulfide formations and showed that
453 biostimulation plays a vital role in stabilizing Pb, Zn and Cd in the subsurface environment. The
454 results from our study also suggest that wetland construction can be a better alternative for
455 stabilizing non-redox-sensitive elements such as Pb, Zn and Cd in mine waste materials or
456 similar geomaterial. Uniqueness of this study is that microbial analyses presented here in details
457 are in agreements with molecular-scale synchrotron-based X-ray data (Karna et al., 2016).
458 Combining advanced microbiological techniques with synchrotron based speciation enhances

459 our understanding of the biogeochemical processes involved in Pb and Zn removal via
460 dissimilatory sulfate reductions under reduced conditions. The results obtained from the current
461 study indicate that OC and S addition stimulated microbial growth and activities, causing
462 changes in the functional microbial community structure via enhancement or reduction of
463 functional genes in saturated mine waste materials enriched with Pb and Zn. The decrease in
464 metal resistance genes indicated reduced toxicity over time. Correspondingly, enrichment in S-
465 and C-cycling genes in OC- and/or -S-treated samples corroborated that these members made
466 significant contributions to the metal stability in the highly contaminated mine waste in a
467 subsurface environment. Sulfur-reducing bacteria gene dsrA/B appeared to be a key player in
468 forming metal sulfides and was significantly enhanced in C1S1 during long-term submergence.
469 On the other hand, no significant difference was detected in functional gene richness in any
470 C0S0 treatment category over time. The information obtained from this study help us conclude
471 that biostimulation would be beneficial for inducing metal sulfide formations in mine waste
472 materials and that SRBs can be used as key players in *in situ* bioremediation of Pb and Zn in
473 subsurface treatment wetlands.

474 **Supplementary Materials**

475 There are six supplementary figures and three supplementary **tables** provided in this document.

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Author's contributions

R.R.K. conceived, designed, and performed the experiments with the support of G.M.H., and wrote the paper with input from G.M.H. T.Y. assisted in collecting the microarray data at the institute of Environmental Genomics. J.D.V., C.W.R., and J.Z. assisted in data analysis and data interpretation. Y.M.A. assisted in statistical analysis.

Conflicts of Interest

The authors declare no conflicts of interest.

References

Almendras M., Wiertz, J., & Chamy R. (2009). Heavy metals immobilization in contaminated smelter soils using microbial sulfate reduction. Advanced Materials Research, **71**, 577-580.

Bazylinski, D. A., & Frankel, R. B. (2003). Biologically controlled mineralization in prokaryotes. Reviews in Mineralogy and Geochemistry, **54**, 217-247.

Bhattacharya, J., Ji, S. W., Lee, H. S., Cheong, Y. W., Yim, G. J., & Min, J. S. (2008). Treatment of acidic coal mine drainage: Design and operational challenges of successive alkalinity producing systems. Mine Water and the Environment, **27**, 12-19.

Bligh, E. G., & Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. Canadian Journal of Biochemistry and Physiology, **37**, 911-917.

Borch, T., Kretzschmar, R., Kappler, A., Cappellen, P. V., Ginder-Vogel, M., & Voegelin, A. (2009). Biogeochemical redox processes and their impact on contaminant dynamics. Environmental Science and Technology, **44**, 15-23.

Bossio, D. A., & Scow, K. M. (1998). Impacts of carbon and flooding on soil microbial communities: phospholipid fatty acid profiles and substrate utilization patterns. Microbial Ecology, **35**, 265-278.

Brantley, S. L., Goldhaber, M. B., & Ragnarsdottir, K. V. (2007). Crossing disciplines and scales to understand the critical zone. Elements, **3**, 307-314.

Brodie, E. L., Desantis, T. Z., Joyner, D. C., Baek, S. M., Larsen, J. T., & Andersen, G. L. (2006). Application of a high-density oligonucleotide microarray approach to study bacterial population dynamics during uranium reduction and reoxidation. Applied Environmental and Microbiology, **72**, 6288-6298.

 doi:10.20944/preprints201808.0082.v1

514 Brown, G. E., Foster, A. L., & Ostergen, J. D. (1999). Mineral surfaces and bioavailability of
515 heavy metals: A molecular perspective. Current Issue, **96**, 3388-3395.
516 Burton, E. D., Bush, R. T., Sullivan, L. A., & Mitchell, D. R. (2008). Schwertmannite
517 transformation to goethite via the Fe (II) pathway: Reaction rates and implications for iron–
518 sulfide formation. Geochimica et Cosmochimica Acta, **72**, 4551-4564.
519 Calbrix, R., Barray, S., Chabrerie, O., Fourrie, L., & Laval, K. (2007). Impact of organic
520 amendments on the dynamics of soil microbial biomass and bacterial communities in cultivated
521 land. Appllied Soil Ecology, **35**, 511-522.
522 Chen, X., Wright, J. V, Conca, J. L., & Peurrung, L. M. (1997). Effects of pH on heavy metal
523 sorption on mineral apatite. Environmental Science and Technology, **31**, 624-631.
524 Eiler, A., Langenheder, S., Bertilsson, S., & Tranvik, L. J. (2003). Heterotrophic bacterial growth
525 efficiency and community structure at different natural organic carbon concentrations. Applied
526 and Environmental Microbiology, **69**, 3701-3709.
527 Evans, M., Warburton, J., & Yang, J. (2006). Eroding blanket peat catchments: Global and local
528 implications of upland organic sediment budgets. Geomorphology, **79**, 45-57.
529 Frostegård, Å., Tunlid, A., & Bååth, E. (2011). Use and misuse of PLFA measurements in
530 soils. Soil Biology and Biochemistry, **43**, 1621-1625.
531 Fuhrman, J. A. (2009). Microbial community structure and its functional implications. Nature,
532 **459**, 193-199.
533 Griffiths B, Ritz K, Ebblewhite N, & Dobson G. (1998). Soil microbial community structure:
534 Effects of substrate loading rates. Soil Biology and Biochemistry, **31**, 145-153.
535 Hayes, J. M., & Waldbauer, J. R. (2006). The carbon cycle and associated redox processes
536 through time. Philosophical Transactions of the Royal Society B, **361**, 931-950.

He, Z., Gentry, T. J., Schadt, C. W., Wu, L., Liebich, J., & Chong, S. C. (2007). GeoChip: A comprehensive microarray for investigating biogeochemical, ecological and environmental processes. International Society of Microbial Ecology Journal, **1**, 67-77.

Heald, S. M., J. O. Cross, D. L. Brewe, & R. A. Gordon. (2007). Nuclear instruments and methods in physics research section a: accelerators, spectrometers, detectors and associated equipment. Proceedings of the National Conference of Synchrotron Radiation Research, **582**, 215-217.

Hemme, C. L., Deng, Y., Gentry, T. J., Fields, M. W., Wu, L., & Barua, S. (2010). Metagenomic insights into evolution of a heavy metal-contaminated groundwater microbial community. International Society of Microbial Ecology Journal, **4**, 660-672.

Holmes, D. E., Finneran, K. T., O'Neil, R. A., & Lovley, D. R. (2002). Enrichment of members of the family *Geobacteraceae* associated with stimulation of dissimilatory metal reduction in uranium-contaminated aquifer sediments. Applied and Environmental Microbiology, **68**, 2300-2306.

Huerta-Diaz, M. A., Tessier, A., & Carignan, R. (1998). Geochemistry of trace metals associated with reduced sulfur in freshwater sediments. Applied Geochemistry, **13**, 213-233.

Ibekwe, A. M., & Kennedy, A. C. (1998). Phospholipid fatty acid profiles and carbon utilization patterns for analysis of microbial community structure under field conditions. FEMS Microbiol Ecology, **26**, 151-163.

Janssen, G. M., & Temminghoff, E. J. (2004). In situ metal precipitation in a zinc- contaminated, aerobic sandy aquifer by means of biological sulfate. Environmental Science and Technology, pp. 4002-4011.

559 Johnson, D. B., & Hallberg, K. B. (2005). Acid mine drainage remediation options: A review.
560 Science of the Total Environment, **338**, 3-14.
561 Karna, R. R., Hettiarachchi, G. M., Newville, M., Sun, C. J. & Ma, Q. (2016). Synchrotron-
562 Based X-Ray Spectroscopy Studies for Redox-Based Remediation of Lead, Zinc, and Cadmium
563 in Mine Waste Materials. Journal of Environmental Quality, **45**, 1883-1893.
564 Khan, S., Hesham, A. E., Qiao, M., Rehman, S., & He, J. (2010). Effects of Cd and Pb on soil
565 microbial community structure and activities. Environmental Science and Pollution Research,
566 **17**, 288-296.
567 Kilmer, V. J., & Alexander, L. T. (1949). Methods of making mechanical analyses of soils. Soil
568 Science Society of America Journal, **68**, 15-24.
569 Kirk, M. F., Holm, T. R., Park, J., Jin, Q. S., Sanford, R. A., Fouke, B. W., & Bethke, C. M.
570 (2002). Bacterial sulfate reduction limits natural arsenic contamination in groundwater. Geology.
571 **32**, 953–956.
572 Kleikemper, J., Schroth, M. H., Sigler, W. V., Schmucki, M., Bernasconi, S. M., & Zeyer, J.
573 (2002). Activity and diversity of sulfate-reducing bacteria in a petroleum hydrocarbon-
574 contaminated aquifer. Applied and Environmental Microbiology, **68**, 1516-1523.
575 Klein, M., Friedrich, M., Roger, A. J, Hugenholtz, P., Fishbain, S., Abicht, H., Blackall, L. L.,
576 Stahl, D. A., & Wagner, M. (2004). Multiple lateral transfers of dissimilatory sulfite reductase
577 genes between major lineages of sulfate-reducing prokaryotes. Journal of Bacteriology, **183**,
578 6028-6035.
579 Ku, T. C. W., Kay, J., Browne, E., Martini, A. M., Peters, S. C., & Chen, M. D. (2008).
580 Pyritization of iron in tropical coastal sediments: Implications for the development of iron,

581 sulfur, and carbon diagenetic properties, Saint Lucia, Lesser Antilles. Mar. Geology, **249**, 184-
582 205.
583 Liang, Y. T., Van Nostrand, J. D., Deng, Y., He, Z. H., Wu, L. Y., Zhang, X., Li, G. H., & Zhou,
584 J. Z. (2011). Functional gene diversity of soil microbial communities from five oil-contaminated
585 fields in China. International Society of Microbial Ecology Journal, **5**, 403-413.
586 Loick, N., & Weisener, C. (2014). Novel molecular tools to assess microbial activity in
587 contaminated environments. Geomicrobiology and Biogeochemistry, pp. 17-35.
588 Lovley, D. R. (1995). Bioremediation of organic and metal contaminants with dissimilatory
589 metal reduction. Journal of Industrial Microbiology and Biotechnology, **14**, 85-93.
590 Lu, Z., Deng, Y., Van Nostrand, J. D., He, Z., Voordeckers, J., & Zhou, A. (2012). Microbial
591 gene functions enriched in the deep water horizon deep-sea oil plume. International Society of
592 Microbial Ecology Journal, **6**, 451-460.
593 Luptakova, A., & Kusnierova, M. (2005). Bioremediation of acid mine drainage contaminated by
594 SRB. Hydrometallurgy, **77**, 97-102.
595 Manceau, A., Marcus, M. A., & Tamura, N. (2002). Quantitative speciation of heavy metals in
596 soils and sediments by synchrotron x-ray techniques. In: P.A. Fenter et al., editors, Applications
597 of synchrotron radiation in low-temperature geochemistry and environmental sciences. Reviews
598 in Mineralogy and Geochemistry, **49**, 341-428.
599 Manceau, A. B., Lanson, B., Schlegel, M. L., Harge, J. C., Musso, M., Eybert-Berard, L.,
600 Hazemann, J. L., Chateigner, D., & Lamble, G. M. (2000a). Quantitative Zn speciation in
601 smelter-contaminated soils by EXAFS spectroscopy. American Journal of Science, **300**, 289-
602 343.

603 Martin, T., Oswald, O., & Graham, I. A. (2002). Arabidopsis seedling growth, storage lipid
604 mobilization, and photosynthetic gene expression are regulated by carbon: Nitrogen availability.
605 Plant Physiology, **128**, 472-481.
606 McKinley, V. L., Peacock, A. D., & White, D. C. (2005). Microbial community PLFA and PHB
607 responses to ecosystem restoration in tallgrass prairie soils. Soil Biology and Biochemistry, **37**,
608 1946-195.
609 McLean, L., Pray, T., Onstott, T., Brodie, E., Hazen, T., & Southam, G. (2007). Mineralogical,
610 chemical and biological characterization of an anaerobic biofilm collected from a borehole in a
611 deep gold mine in South Africa. Geomicrobiology Journal, **24**, 491-504.
612 Morse, J., & Luther, I. G. (1999). Chemical influences on trace metal-sulfide interactions in
613 anoxic sediments. Geochimica et Cosmochimica Acta, **63**, 3373-3378.
614 Muyzer, G., & Stams, A. J. (2008). The ecology and biotechnology of sulfate-reducing bacteria.
615 Natural Reviews Microbiology, **6**, 441-454.
616 Nachtegaal, M., Marcus, M. A., Sonke, J. E., Vangronsveld, J., & Van Der Lelie, D. (2005).
617 Effects of *in situ* remediation on the speciation and bioavailability of zinc in a smelter-
618 contaminated soil. Geochimica et Cosmochimica Acta, **69**, 4649-4664.
619 Olsson, P. A. (1999). Signature fatty acids provide tools for determination of the distribution and
620 interactions of mycorrhizal fungi in soils. FEMS Microbiol Ecology, **29**, 303-310.
621 Pierzynski, G. M., & Vaillant, G. C. (2006). Remediation to reduce ecological risk from trace
622 element contamination: A decision case study. Journal of Natural Resources and Life Sciences
623 Education, **35**, 85-94.
624 Ramette, A. (2007). Multivariate analyses in microbial ecology. FEMS Microbiol Ecology, **62**,
625 142-160.

626 Scheinost, A. C., Kretzschmar, R., Pfister, S., & Roberts, D. R. (2002). Combining selective
627 sequential extractions, x-ray absorption spectroscopy, and principal component analysis for
628 quantitative zinc speciation in soil. Environmental Science and Technology, **36**, 5021-5028.
629 Smith, G. A., Nickels, J. S., Kerger, B. D., Davis, J. D., & Collins, S. P. (1986). Quantitative
630 characterization of microbial biomass and community structure in subsurface material: a
631 prokaryotic consortium responsive to organic contamination. Canadian Journal of Microbiology,
632 **32**, 104-111.
633 Soil Survey Laboratory Staff, 1996. Soil survey laboratory manual. Soil Survey Investigation
634 Report no. 42. USDA-NRCS. U.S. Gov. Printing Office, Washington, D.C.
635 Stein, O. R., Borden-Stewart, D. J., Hook, P. B., & Jones, W. L. (2007). Seasonal influence on
636 sulfate reduction and zinc sequestration in subsurface treatment wetlands. Water Research, **41**,
637 3440-3448.
638 Toevs, G. R., Morra, M. J., Polizzotto, M. L., Strawn, D. G., Bostick, B. C., & Fendorf, S.
639 (2006). Metal (loid) diagenesis in mine-impacted sediments of Lake Coeur d'Alene, Idaho.
640 Environmental Science and Technology, **40**, 2537-2543.
641 Tokunaga, T. K., Wan, J., Firestone, M. K., Hazen, T. C., Olson, K. R., & Herman, D. J. (2003).
642 In situ reduction of chromium (VI) in heavily contaminated soils through organic carbon
643 amendment. Journal of Environmental Quality, **32**, 1641-1649.
644 Trajanovska, M. L., & Britz, M. B. (1997). Detection of heavy metals ion resistance in Gram-
645 positive and Gram-negative bacteria isolated from lead contaminated sites. Biodegradation, **8**,
646 113-124.

647 Tu, Q., Yu, H., He, Z., Deng, Y., Wu, L., & Nostrand, J. D. (2014). GeoChip 4: A functional
648 gene-array-based high-throughput environmental technology for microbial community analysis.
649 Molecular Ecology Resources, **14**, 914-928.
650 Van Nostrand, J. D., Wu, L., Wu, W. M., Huang, Z., Gentry, T. J., & Deng, Y. (2011). Dynamics
651 of microbial community composition and function during in situ bioremediation of a uranium-
652 contaminated aquifer. Applied and Environmental Microbiology, **77**, 3860-3869.
653 Van Nostrand, J. D., Wu, W., Wu, L., Deng, Y., Carley, J., & Carroll, S. (2009). GeoChip-based
654 analysis of functional microbial communities during the reoxidation of a bioreduced uranium-
655 contaminated aquifer. Environmental Microbiology, **11**, 2611-2626.
656 Wan, J., Tokunaga, T. K., Brodie, E., Wang, Z., Zheng, Z., & Herman, D. (2005). Reoxidation of
657 bioreduced uranium under reducing conditions. Environmental Science and Technology, **39**,
658 6162-6169.
659 White, C., & Gadd, G. (2000). Copper accumulation by sulfate-reducing bacterial biofilms.
660 FEMS Microbiology Letters, **183**, 313-318.
661 White, D. C., & Ringlberg, D. B. (1998). Signature lipid biomarker analysis. pp. 255-272, IN: R.
662 S. Bulage et al. (Eds.), Techniques in microbial ecology, Oxford University Press, New York.
663 Whiteman, M., Armstrong, J. S., Chu, S. H., Jia-Ling, S., Wong, B., & Cheung, N. S. (2004).
664 The novel neuromodulator hydrogen sulfide: An endogenous peroxynitrite 'scavenger'? Journal
665 of Neurochemistry, **90**, 765-768.
666 Wu. L. (2001). Development and evaluation of functional gene arrays for detection of selected
667 genes in the environment. Applied and Environmental Microbiology, **67**, 5780-5790.

668 Yergeau, E., Kang, S., He, Z., Zhou, J., & Kowalchuk, G.A. (2007). Functional microarray
669 analysis of nitrogen and carbon cycling genes across an Antarctic latitudinal transect.
670 International Society of Microbial Ecology Journal, **1**, 163-179.
671 Zarcinas, B. A., McLaughlin, M. J., & Smart, M. K. (1996). The effect of acid digestion
672 technique on the performance of nebulization systems used in inductively coupled plasma
673 spectrometry. Communications in Soil Science and Plant Analysis, **27**, 1331-1354.
674 Zhang, S., Xue, X., Liu, R., & Jin, Z. (2005). Current situation and prospect of the
675 comprehensive utilization of mining tailings. Journal of Mining and Metallurgy, **3**, 44-48.
676 Zhou, J., Kang, S., Schadt, C., & Garten, Jr. C. (2008). Spatial scaling of functional Gene
677 diversity across various microbial taxa. Proceedings of the National Academy of Sciences of the
678 United States of America, 105, 7768-7773.

679 **List of Tables**

680 Table 1. Chemical data for the effluent samples collected after medium- (119-day) and long-
681 (252-day) term submergence. The soil samples collected at these time points were used for
682 microarray analysis.
683

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684

	(µg/L)				(mg/L)		
Sample	**Zn**	**Cd**	**Pb**	**pH**	**DOC**	**Sulfate-S**	**Nitrate-N**
C0S0 119-day	723±40.9	432±10.9	<DL	7.57±0.02	5±0.03	474±10.25	2.0±0.1
C0S0 252-day	517±30.9	28±0.9	<DL	8.41±0.03	62±2.6	571±5.64	2.0±0.2
C0S1 119-day	30±1.7	2±0.01	<DL	8.00±0.02	4±0.1	468±6.78	1.8±0.5
C0S1 252-day	<DL	1±0.006	36±1.6	6.39±0.005	65±0.8	¶	2.2±0.02
C1S0 119-day	<DL	1±0.001	<DL	8.18±0.012	5±0.02	503±7.34	1.9±0.01
C1S0 252-day	<DL	<DL	<DL	7.58±0.015	<DL	474±3.95	2.0±0.05
C1S1 119-day	<DL	1±0.004	<DL	7.40±0.01	4±0.1	437±10.02	1.8±0.04
C1S1 252-day	<DL	<DL	<DL	7.02±0.01	<DL	288±8.64	1.9±0.14

*DL corresponds to detection limit. Detection limit of 0.6 for Cd, and 0.7 μgL^{-1} for Pb was determined.

¶ indicates data not collected

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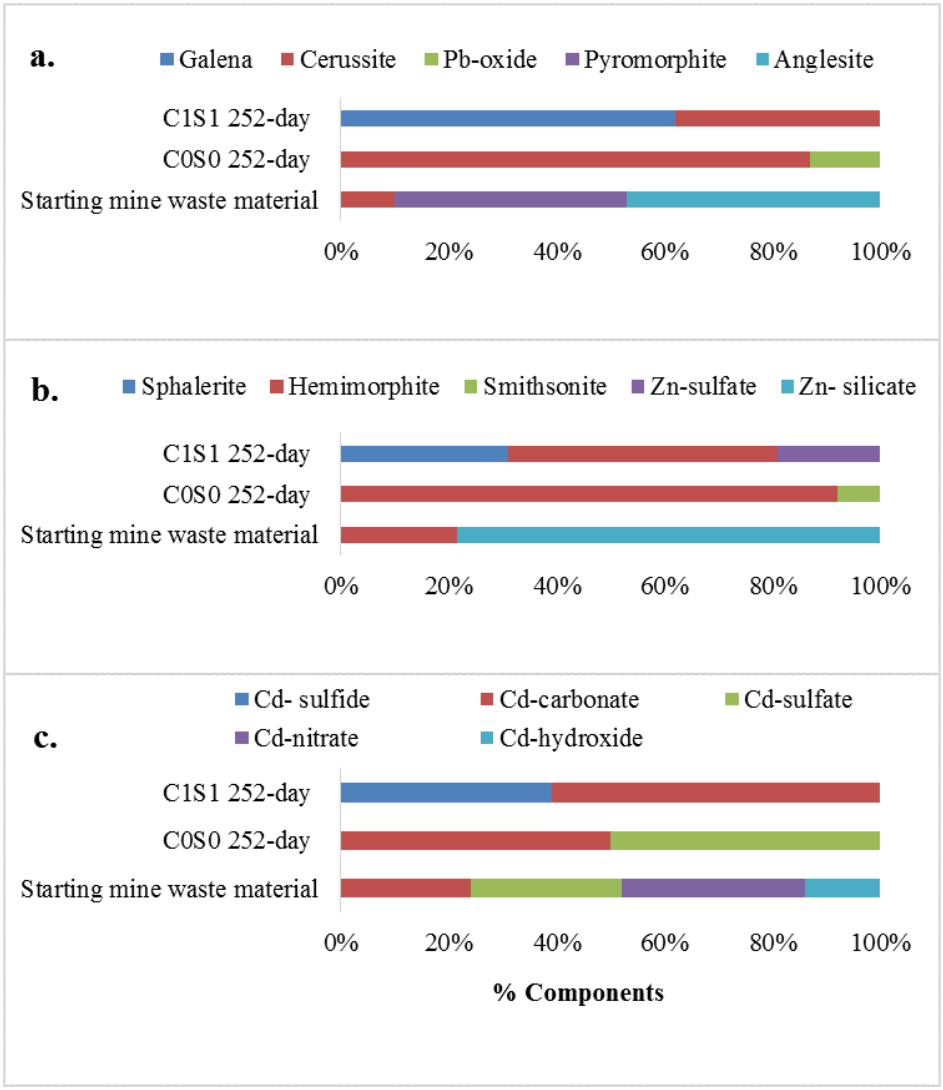
686 **List of Figures:**



687

688 **Figure 1:** X-ray absorption fine structure spectroscopy results showing % components for a)
689 Lead, b) zinc and c) cadmium in starting mine waste material, control (C0S0) and OC-plus-S-
690 treated sample (C1S1) under long-term (252-day) submergence. The phase identified as less than
691 10% may not be significant due to error associated with smaller estimations.

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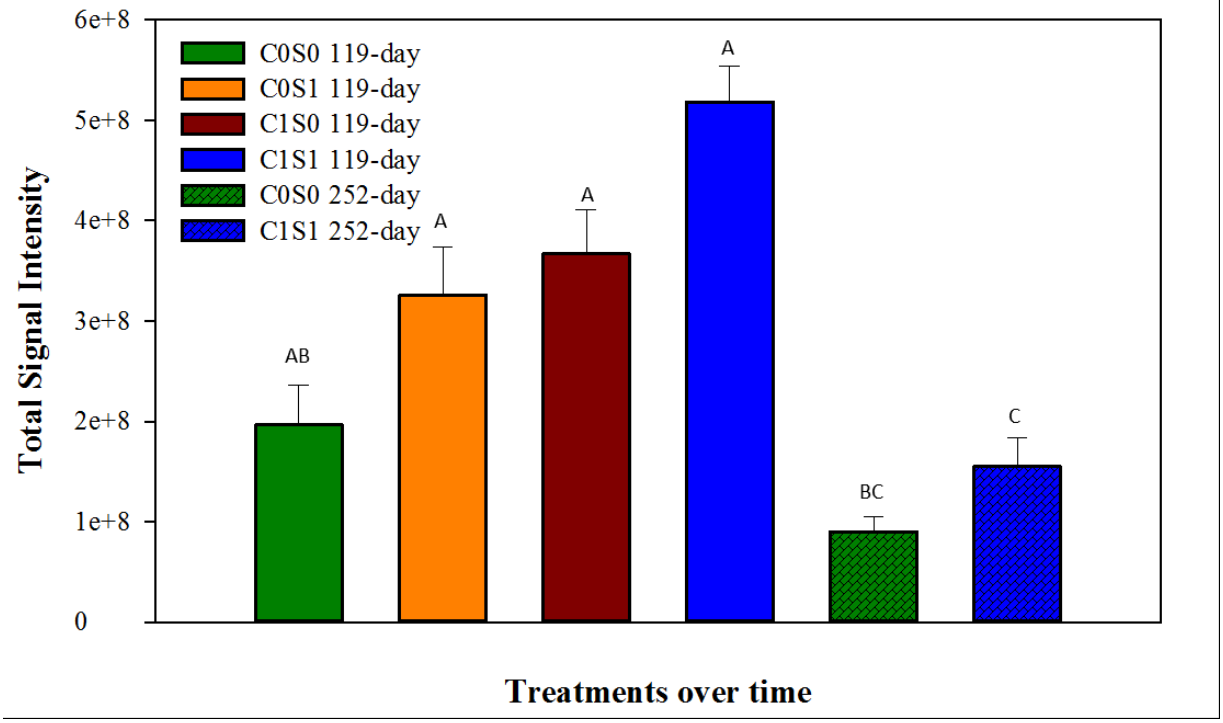

692

693 **Figure 2:** Functional Gene richness under medium- (119-day), and long-term (252-day)
694 submergence. All the treatments; C0S0, C0S1, C1S0, and C1S1 (solid filled bars) from medium-
695 term submergence, and only C0S0, and C1S1 (pattern filled bars) from long-term submergence
696 are plotted. Vertical bars represent the mean of three replicates; 2 replicates from individual
697 column, and 1 replicate from the mixture of two columns. Bars with the same letters are not
698 significantly different. Different letters within a category indicate significance difference
699 (α=0.05).

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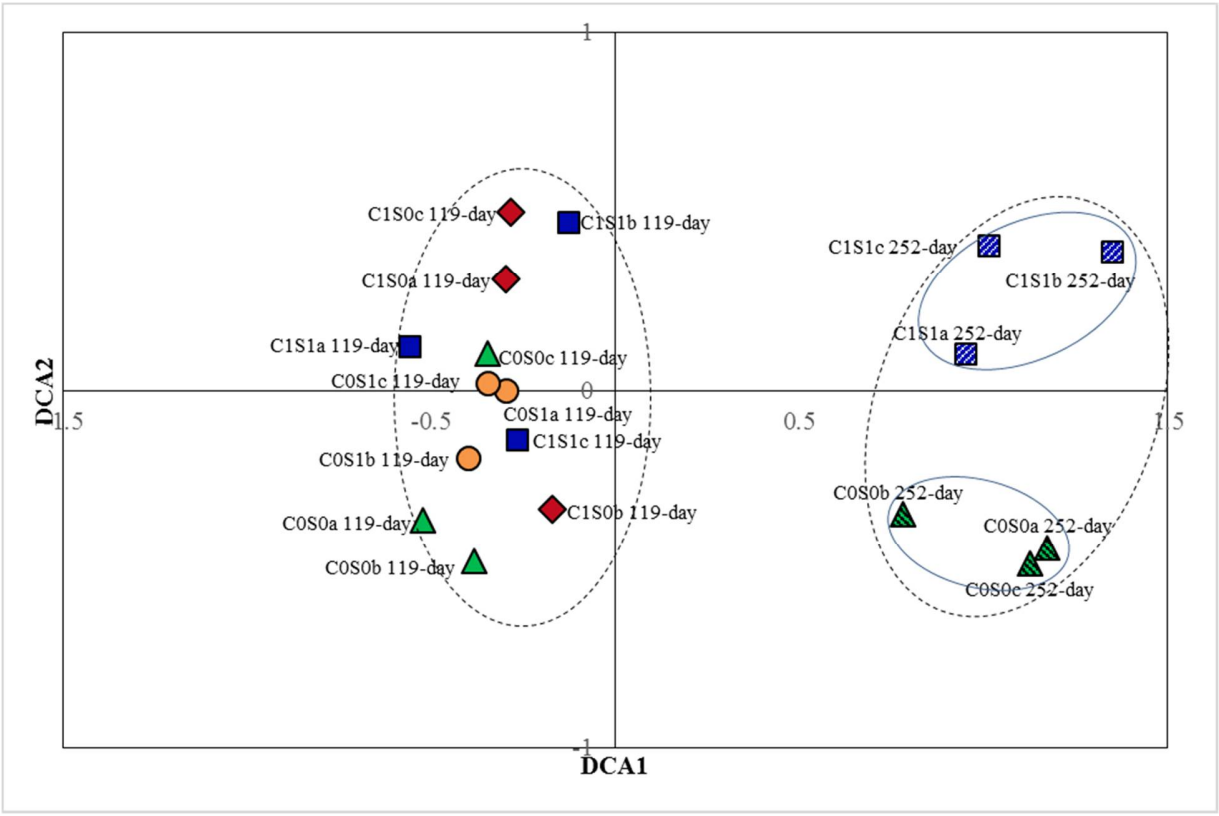

Figure 3: Detrended correspondence analysis (DCA) for the total number of detected genes under medium- (119-day) and long-term (252-day) submergence, indicating community structure changes. All the treatments; C0S0, C0S1, C1S0, and C1S1 (solid filled markers) from medium-term submergence, and only C0S0, and C1S1 (pattern filled markers) from long-term submergence are plotted.

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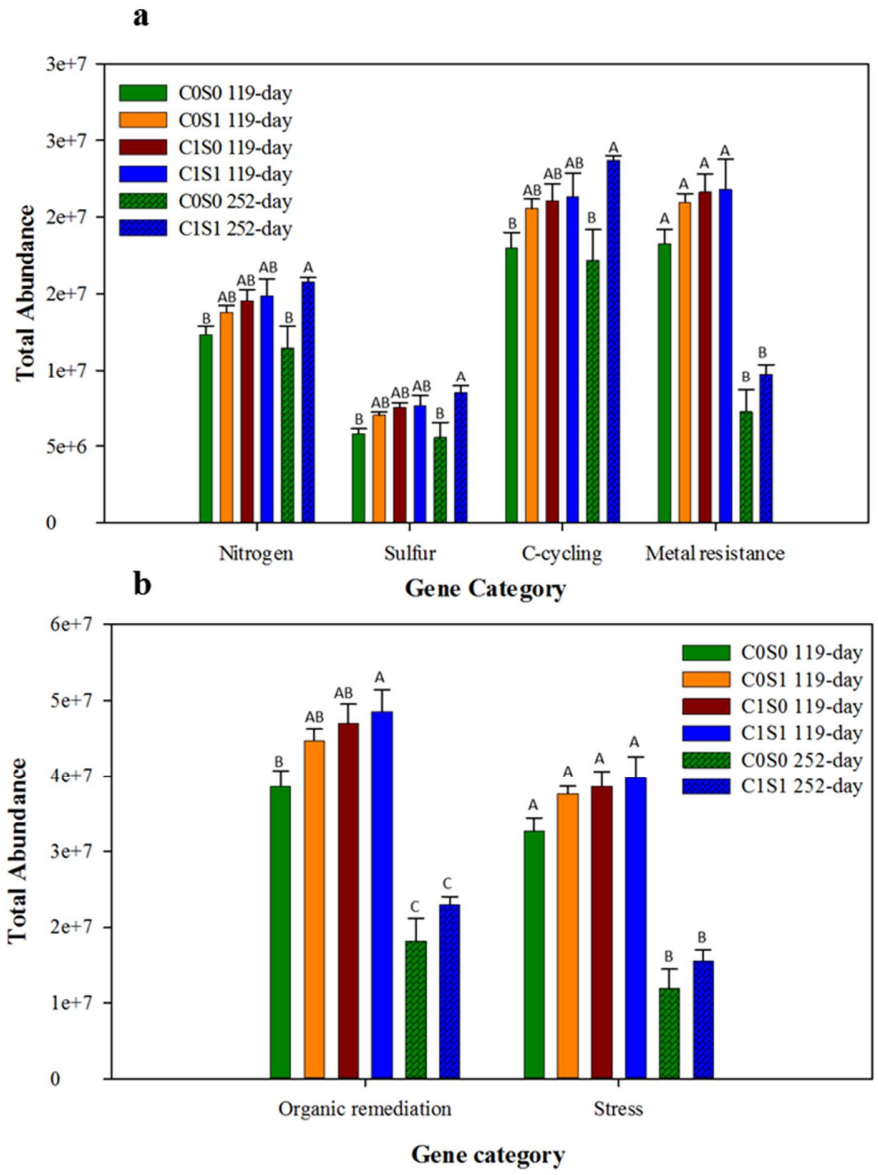

706

707 **Figure 4:** Total abundance of function genes in selected categories for the samples submerged
708 for both medium- (119-day) and long-term (252-day) submergence. All the treatments; C0S0,
709 C0S1, C1S0, and C1S1 (solid filled bars) from medium-term submergence, and only C0S0, and
710 C1S1 (pattern filled bars) from long-term submergence are plotted. Vertical bars represent the
711 mean of three replicates; 2 replicates from individual column, and 1 replicate from the mixture of
712 two columns. Bars with the same letter are not significantly different. Different letters within a
713 category indicate significance difference (α=0.05).

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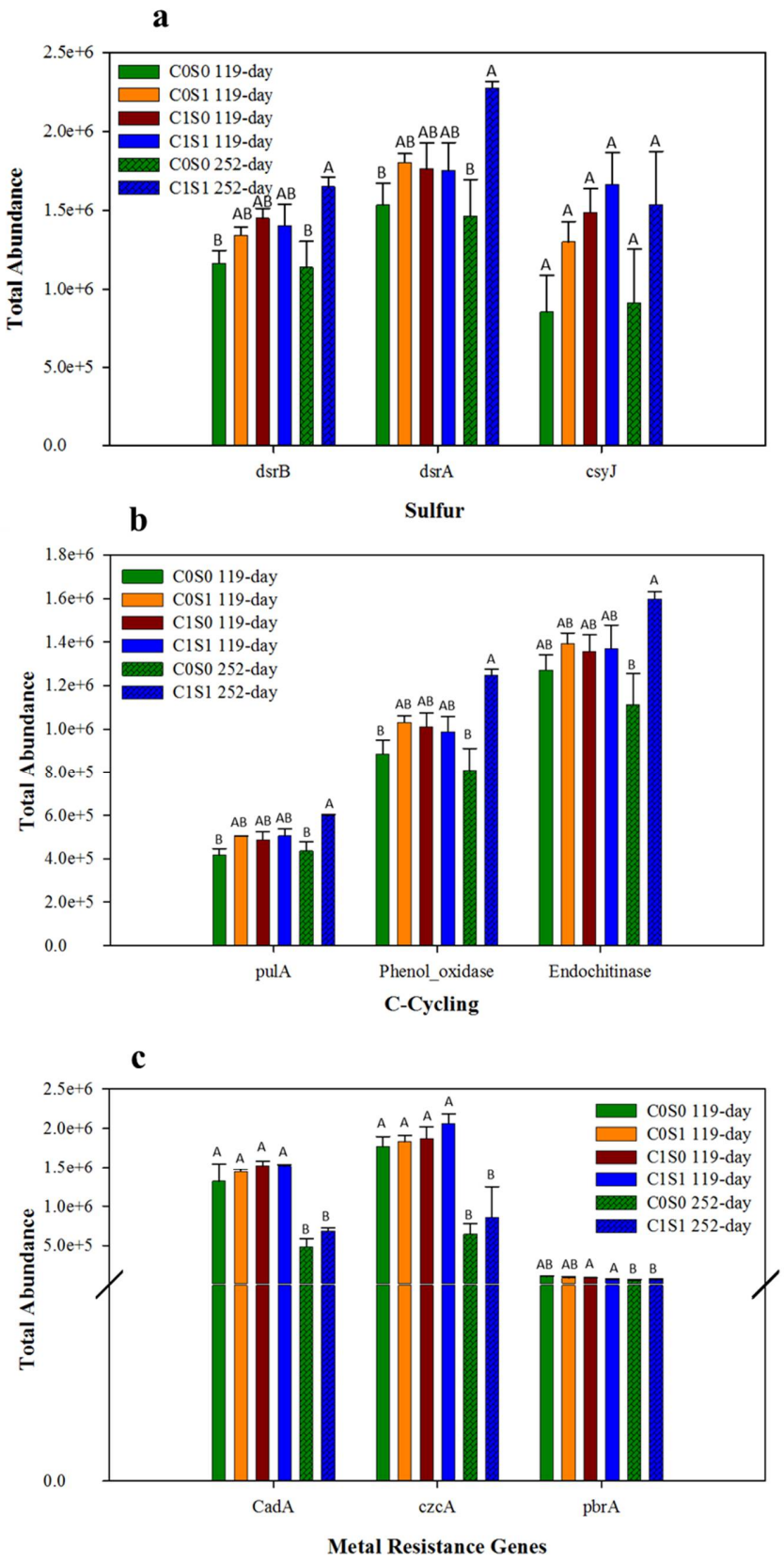

714

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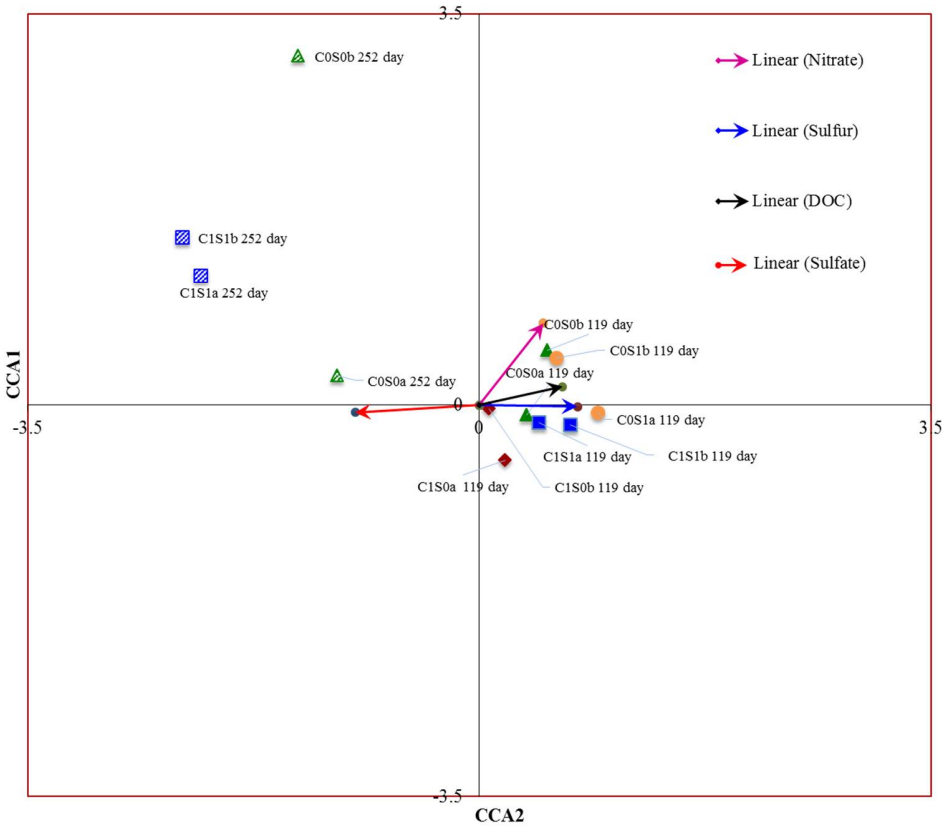
715 **Figure 5:** Total abundance of a) dsrA/dsrB, and csyJ in the sulfur category, b) pulA,
716 Phenol_oxidase, Endochitinase under the C-cycling category, and c) Cd resistance gene (CadA),
717 Zn resistance gene (czcA), and Pb resistance gene (pbrA). Vertical bars represent the mean of
718 three replicates. All the treatments; C0S0, C0S1, C1S0, and C1S1 (solid filled bars) from
719 medium-term submergence, and only C0S0, and C1S1 (pattern filled bars) from long-term
720 submergence are plotted. Vertical bars represent the mean of three replicates; 2 replicates from
721 individual column, and 1 replicate from the mixture of two columns. Bars with the same letter
722 are not significantly different. Different letters within a category indicate significance difference
723 (α=0.05).



724

725 **Figure 6:** Canonical correspondence analysis (CCA) indicating the relationship between
726 microbial communities with environmental factors.