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# Microbial communities in riparian soils of a settling pond for mine drainage treatment

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ABSTRACT

Mine drainage leads to serious contamination of soil. To assess the effects of mine drainage on microbial communities in riparian soils, we used an Illumina MiSeq platform to explore the soil microbial composition and diversity along a settling pond used for mine drainage treatment. Non-metric multi-dimensional scaling analysis showed that the microbial communities differed significantly among the four sampling zones (influent, upstream, downstream and effluent), but not seasonally. Constrained analysis of principal coordinates indicated heavy metals (zinc, lead and copper), total sulphur, pH and available potassium significantly influenced the microbial community compositions. Heavy metals were the key determinants separating the influent zone from the other three zones. Lower diversity indices were observed in the influent zone. However, more potential indicator species, related to sulphur and organic matter metabolism were found there, such as the sulphur-oxidizing genera *Acidiferrobacter*, *Thermithiobacillus*, *Limnobacter*, *Thioprofundum* and *Thiovirga*, and the sulphur-reducing genera *Desulfotomaculum* and *Desulfobulbus*; the organic matter degrading genera, *Porphyrobacter* and *Paucimonas*, were also identified. The results indicated that more microorganisms related to sulphur- and carbon-cycles may exist in soils heavily contaminated by mine drainage.



## 1. Introduction

Heavy metal pollution caused by the minerals industry is a serious environmental problem worldwide. Mining of metal ores and coal creates by-products and mine drainage often pollutes nearby aquatic ecosystems and the groundwater (Bernhardt et al., 2012; Bier et al., 2015). Underground and opencast mine products and wastes (including tailings) are potential sources of acidic or alkaline and metal-rich effluents, known as 'acid mine/rock drainage' (AMD/ARD) or 'alkaline mine drainage' (AlkMD). To reduce the environmental damage, active and passive treatment processes of mine drainage, done before it enters the stream, are often applied.

Active treatment of mine drainage generally consists of continuous adding of alkaline chemicals to raise the water pH, neutralizing the acidity and precipitating any metals, while passive treatment utilizes natural and constructed wetland ecosystems (Johnson and Hallberg, 2005; Skousen et al., 1998). The active treatment process is the most common and traditional method used to mitigate acidic effluents. However, with the development of technology, low costs and maintenance requirements, most bioremediation options for mine drainage comprise passive systems (Coulton et al., 2003; Johnson and Hallberg, 2005). In China, many mine areas and smelting industries have adopted passive processes to treat mine drainage using the complex terrain and form natural settling ponds or wetlands.

Microbial diversity, abundance and composition in soils, sediments and waters are strongly influenced by mine drainage (Bier et al., 2015; Kuang et al., 2013; Sun et al., 2015). In areas where there is erosion caused by mine drainage, microbial communities tolerant to acid or alkaline, high sulphate and high metal conditions are often found, such as the sulphate-reducing Desulfobacteraceae and acid-tolerant Euryarchaeota, Gammaproteobacteria and Nitrospira (Bier et al., 2015; Kuang et al., 2013).

Some studies have considered how the passive treatment of mine drainage affects microbial communities in waters and

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0043-1354/

sediments (DeNicola and Stapleton, 2014; Santelli et al., 2010; Weber et al., 2008). However, little is known about how the passive treatment of mine drainage affects the microbial community in soils surrounding settling ponds; these ponds capture precipitated metal oxyhydroxides after lime treatment and hold mine effluents during treatment until they reach the appropriate standards for discharge (Skousen et al., 1998). Soils surrounding settling ponds are in the transition zone between terrestrial and aquatic ecosystems and perform vital ecological functions, including water storage and nutrient cycling (Naiman et al., 2010).

Based upon the function and structure of settling ponds, it could be expected that there would be a gradient of heavy metal concentrations, from the influent to the effluent across the pond. Such gradients might be a selective factor for the microbial communities in the surrounding soils. Numerous studies have indicated changes in microbial diversity, abundance and composition along environmental gradients, such as pH gradients, created by acid or alkaline mine drainage and heavy metal polluted soils (Azarbad et al., 2015; Bier et al., 2015; Feris et al., 2003).

Microbial indicators have been identified to help predict the responses of microbial communities to these environmental conditions (Feris et al., 2003; Fierer et al., 2007; Sims et al., 2013). Indicators related to pH gradients include *Ferrovum* spp., *Leptospirillum* groups and *Acidithiobacillus ferrooxidans* (Kuang et al., 2013); those related to trace heavy metals include *Hydrogenophaga* and *Rhodobacter* (Bier et al., 2015). These indicators have been found in water environments contaminated with mine drainage (Bier et al., 2015; Kuang et al., 2013). The microbial community could further be used as an indicator for evaluating the cumulative effects of different mine drainage contaminants in riparian soils.

This study aimed to reveal the microbial community dynamics in mine drainage-contaminated riparian soils. We speculated that soil exposure to slightly AlkMD would drive important shifts in the microbial assemblages. At the study site, weak AlkMD pollution had resulted from mineral processing during Pb and Zn mining, by the spreading of limestone to precipitate metals and increase alkalinity. The microbial communities in the soils of four zones between the influent and effluent of a settling pond were analysed (by sequencing microbial 16S rRNA gene amplicons with an Illumina MiSeq).

The main objectives of the present study were to investigate: (1) how weak AlkMD alters microbial community composition (α-diversity); (2) which environmental factors influence the community composition; and (3) which microbial taxa can be used as indictors for evaluating the cumulative effects of pollution by weak AlkMD in riparian soils of settling ponds. The community variations we observed might have been driven by niche-based processes in the riparian soils that were contaminated by mine drainage.

## 2. Materials and methods

### 2.1. Sampling sites and strategy

The sampling sites were located in a metal-polluted valley in the southeast of Mianxian County (12 km from Hanzhong City), Shaanxi Province, China. The riparian soil samples were collected adjacent to a mine drainage settling pond of a Pb–Zn smelter that had been running for almost 10 years. The riparian soil was polluted by mine drainage flooding on and off with the production of wastewater. The texture of the soil was clay (clay, 50–60%; silt, 30–40%; sand, 10–20%).

Thirty-two samples were collected from the influent (Zone I), upstream (Zone II), downstream (Zone III) and effluent (Zone IV) areas, along the settling pond over three seasons in 2013 (spring: April, 12 samples; summer: August, 10 samples; and winter: December, 10 samples) (Fig. 1). The location of 32 sampling sites was recorded using a global positioning system; the distances between sampling sites ranged from approximately 10 to 50 m. Soil samples (0–15 cm) were collected in sterile plastic bags using a shovel after removing the topsoil and immediately kept on ice for transport to the laboratory within 3 h of collection. Once in the laboratory, the soil samples to be used for microbial analysis were immediately stored at −80 °C. The soil samples for physical and chemical characterization were air-dried for 2 weeks before used.

### 2.2. Soil physical and chemical characterization

Soil pH (soil/water = 1:2.5, w/v); total nitrogen (N), phosphorus (P) potassium (K) and sulphur (S); and available P and K were measured using routine methods (Rayment and Higginson, 1992). Soil organic matter content was analysed according to the method of Walkley and Black (1934). Total metal concentrations of zinc (Zn), copper (Cu), lead (Pb), cadmium (Cd), calcium (Ca) and iron (Fe) were measured with atomic absorption spectroscopy (Welz and Sperling, 2008), after sieving the soil samples through a 0.25 mm nylon mesh sieve.

### 2.3. DNA extraction, PCR and 16 rRNA gene sequencing

DNA was extracted from 0.5 g of soil using the Fast DNA SPIN Kit for Soil (MP Biomedicals, Santa Ana, CA, USA), according to the manufacturer's instructions. The universal primer set 515F/926R (Yu et al., 2015) was used to amplify the V4–V5 region of the 16S rRNA genes, with a 12 bp bar code on the reverse primer. Each 25 μL PCR mixture contained 0.625 units of ExTaq polymerase (Takara, Shanghai, China), 1×PCR buffer, 800 μM of each dNTP and 300 nM of each primer. Cycling conditions involved an initial 3 min denaturing step, followed by 30 cycles of 30 s at 94 °C, 30 s at 50 °C and 30 s at 72 °C, and a final extension phase of 5 min at 72 °C.

PCR amplification was conducted in triplicate and the products were pooled for each subsample. A composite DNA sample for sequencing was created by combining equimolar ratios of the amplification products from the individual subsamples, as described previously (Fierer et al., 2008). The composite DNA was gel-purified and sequenced using an Illumina MiSeq platform (Caporaso et al., 2012).

### 2.4. Bioinformatic analysis of 16S rRNA gene sequences

Raw Illumina fastq files were de-multiplexed, quality filtered, and analysed using QIIME v1.7.0 (Caporaso et al., 2010). Reads containing more than three consecutive bases, receiving a quality score <20, or containing >6 nt ambiguous nucleotides ("N") in the homopolymeric regions were discarded. Chimeric sequences were identified and removed using USEARCH (Edgar et al., 2011). Operational taxonomic units (OTU) were assigned using UCLUST, with a threshold level of 97% similarity (Edgar, 2010).

Phylogenetic classification of the reads was determined based on the Ribosomal Database Project (RDP), using a threshold of 80% (Wang et al., 2007). Microbial phylotypes with corresponding read counts were imported into METAGENassist (Arndt et al., 2012; www.metagenassist.ca) to map the microbial metabolic features. The alpha microbial biodiversity of each sample was estimated using the abundance-based diversity indices of Shannon, Simpson, Chao1 and Pielou (Pagaling et al., 2014).

### 2.5. Statistical analyses

All statistical analyses were implemented using various

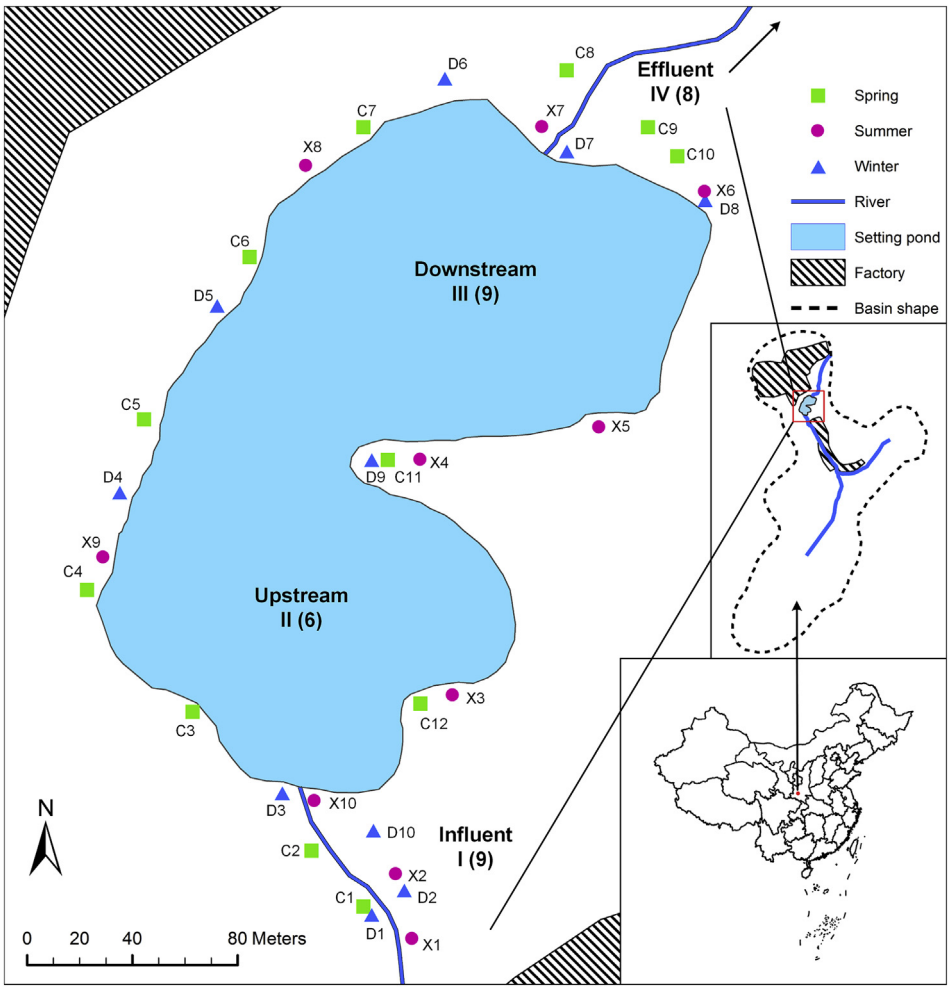


**Fig. 1.** The locations of riparian soil sample sites between the mine drainage influent and effluent of a settling pond. Four sampling zones were influent (I), upstream (II), downstream (III) and effluent (IV).

packages in R (version 3.1.3) (R Development Core Team, 2014). Normality of the distributions of the residual of models was checked with the Shapiro-Wilk test. Depending on the distribution of the estimated parameters, either ANOVA or the Kruskal-Wallis rank sum test was used to check for significant differences between the variances of parameters. Comparisons between each sample pair were conducted using either Student's *t*-tests or Wilcoxon Rank Sum tests. Correlations between the diversity indices and measured soil parameters, or between different measured soil parameters, were calculated based on Pearson's product moment correlation coefficients. Significant differences in the microbial community compositions among samples were tested by Permutational multivariate ANOVA (PERMANOVA) using the adonis() function of the vegan package in R (version 2.2.1) (Oksanen et al., 2013).

Nonmetric multidimensional scaling (NMDS) was conducted to visualize differences in the OTU-based community composition using the metaMDS() function of the vegan package in R. Hierarchical cluster analyses were performed, based on Bray-Curtis distances, using Ward's method (Wishart, 1969), with the agnes() function of the cluster package (version 1.14.3) in R (Maechle, 2012). Constrained analysis of principal coordinates (CAP) analysis, based on the capscale() function of the vegan package in R, was used to illustrate the correlations between the microbial community and environmental factors. Distance matrices of community data were based on Bray-Curtis and weighted UniFrac distances. Indicator species analysis was conducted using the multipatt() function of the indicspecies package (version 1.7.4) in R (De Caceres and Legendre, 2009).

## 3. Results

### 3.1. Site characteristics and environmental conditions

The riparian soil samples were slightly alkaline, with the average pH ranging from 7.38 to 7.77 in the four zones (Table 1; further details are given in Supplementary Material (SM) Tables S1 and S2). The soils from zones II, III and IV presented similar physicochemical properties, although there were minor differences among them (Table 1). In contrast, significantly lower pH values, total K and available K contents, and higher contents of metals (Cu, Zn, Pb, Cd and Ca), total N, total S, total P and available P were observed in the soils of zone I, compared with those in the other three zones ($P < 0.05$; Table 1). The only significant seasonal differences detected in the soil parameters were for available P and Zn contents (SM Table S2).

### 3.2. Sequencing and taxa identification

The sequencing run of 16S rRNA amplicons yielded 1,226,061

**Table 1**
Physicochemical properties of the riparian soils sampled from the influent (Zone I), upstream (Zone II), downstream (Zone III) and effluent (Zone IV) zones of a settling pond (mean ± standard variation).

| | Zone | | | |
|---|---|---|---|---|
| | I (n = 9) | II (n = 6) | III (n = 9) | IV (n = 8) |
| pH | 7.38 ± 0.18$^{A}$ | 7.77 ± 0.11$^{B}$ | 7.71 ± 0.31$^{B}$ | 7.62 ± 0.27$^{AB}$ |
| TN (%) | 0.11 ± 0.03$^{A}$ | 0.09 ± 0.03$^{A}$ | 0.05 ± 0.01$^{BC}$ | 0.09 ± 0.04$^{AC}$ |
| TP (%) | 0.08 ± 0.02$^{A}$ | 0.06 ± 0.02$^{AC}$ | 0.04 ± 0.01$^{B}$ | 0.05 ± 0.01$^{BC}$ |
| TK (%) | 0.70 ± 0.34$^{A}$ | 1.87 ± 0.15$^{B}$ | 1.80 ± 0.13$^{B}$ | 1.78 ± 0.19$^{B}$ |
| SOM (%) | 1.67 ± 0.70$^{A}$ | 1.63 ± 0.92$^{AB}$ | 0.81 ± 0.34$^{B}$ | 1.40 ± 0.89$^{AB}$ |
| AP (%) | 28.41 ± 14.89$^{A}$ | 11.27 ± 3.92$^{B}$ | 10.40 ± 6.50$^{B}$ | 10.30 ± 8.80$^{B}$ |
| AK (%) | 78.93 ± 28.65$^{A}$ | 156.14 ± 23.83$^{B}$ | 160.07 ± 25.43$^{B}$ | 232.66 ± 30.81$^{C}$ |
| TS (%) | 0.39 ± 0.28$^{A}$ | 0.02 ± 0.03$^{B}$ | 0.02 ± 0.03$^{B}$ | 0.04 ± 0.07$^{B}$ |
| Cu (mg kg$^{-1}$) | 1107.7 ± 610.9$^{A}$ | 74.5 ± 41.3$^{B}$ | 34.3 ± 12.6$^{C}$ | 85.6 ± 60.1$^{B}$ |
| Zn (mg kg$^{-1}$) | 13,790.6 ± 3055.4$^{A}$ | 3198.3 ± 3218.3$^{B}$ | 1399.6 ± 1798.7$^{B}$ | 5875.5 ± 8132.8$^{B}$ |
| Pb (mg kg$^{-1}$) | 12,651.2 ± 8996.1$^{A}$ | 479.6 ± 370.9$^{B}$ | 107.0 ± 158.2$^{C}$ | 548.6 ± 681.6$^{B}$ |
| Cd (mg kg$^{-1}$) | 544.1 ± 256.2$^{A}$ | 137.5 ± 124.8$^{B}$ | 44.3 ± 60.0$^{B}$ | 279.5 ± 415.6$^{B}$ |
| Ca (mg kg$^{-1}$) | 156,202.8 ± 29,299.7$^{A}$ | 75,913.2 ± 16,724.7$^{B}$ | 67,654.9 ± 11,813.0$^{B}$ | 78,950.1 ± 25,979.3$^{B}$ |
| Fe (mg kg$^{-1}$) | 27,683.1 ± 8806.9$^{A}$ | 30,545.2 ± 2829.4$^{A}$ | 29,803.7 ± 1960.8$^{A}$ | 37,625.5 ± 6663.4$^{B}$ |

TN: total nitrogen; TP: total phosphorus; TK: total potassium; SOM: soil organic matter; AP: available phosphorus; AK: available potassium; and TS: total sulphur.
$^{ABCD}$Data for each physicochemical parameter that do not share a letter are significantly different ($P < 0.05$).

raw reads, with 934,813 quality reads (after filtering), from the 32 samples. The number of sequences per sample ranged from 18,647 to 44,534, with an average of 29,211 ± 6045 reads. A total of 39,324 different OTUs were clustered from the reads and were classified into 683 genera, 272 families, 148 orders, 90 classes and 37 phyla (Table 2). Of these OTUs, 227 (0.58%) were assigned to Archaea. The coverage ranged between 85.86% and 88.13%, demonstrating that most of the bacterial taxa in the soil samples were detected (Table 2).

Across all the samples, Proteobacteria, Chloroflexi, Bacteroidetes and Acidobacteria represented the most dominant phyla, accounting for 41.0%, 11.9%, 10.1% and 8.7% of all OTUs, respectively. Some less abundant phyla were also detected in most of the samples, including Firmicutes (4.0%), Actinobacteria (2.6%) and Verrucomicrobia (1.8%). The most abundant classes were Betaproteobacteria (15.1%), Gammaproteobacteria (14.8%) and Anaerolineae (11.1%). At the family level, the most abundant phylotypes were Anaerolineaceae (11.1%), followed by Enterobacteriaceae (8.7%) and Chitinophagaceae (3.9%).

### 3.3. Microbial communities in the riparian soils along the settling pond

Mean relative abundances of the dominant lineages in four zones (Fig. 2) indicate that the dominant species in the influent zone were different from those in the other zones (also SM

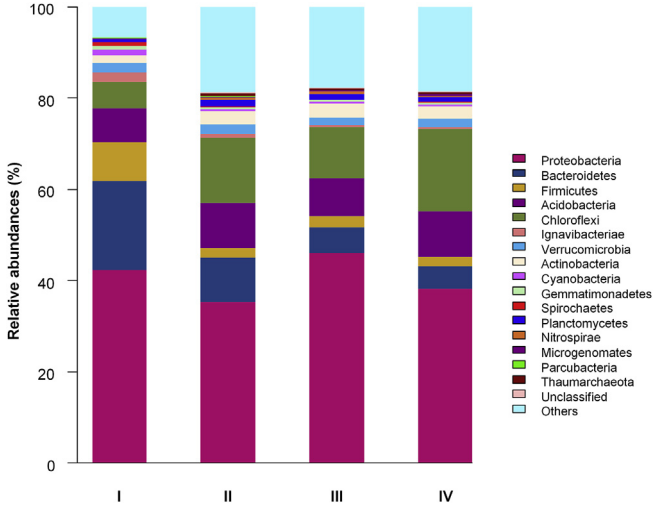


**Fig. 2.** Mean relative abundances (%) of dominant lineages (phylum level) in the four zones of a settling pond (I, influent; II, upstream; III, downstream; and IV, effluent).

**Table 2**
Summary of the pyrosequencing data and alpha diversity indices by sampling zone.

| | Zone | | | |
|---|---|---|---|---|
| | I (n = 9) | II (n = 6) | III (n = 9) | IV (n = 8) |
| Total number of raw reads | 364,230 | 213,657 | 343,365 | 304,809 |
| Mean number of Raw reads (per sample) | 40,470 ± 5870 | 35,609 ± 6143 | 38,151 ± 7625 | 38,101 ± 8766 |
| Total number of high-quality reads | 264,015 | 165,004 | 265,469 | 240,325 |
| Mean number of high-quality reads (per sample) | 29,335 ± 4745 | 27,500 ± 5476 | 29,496 ± 6182 | 30,040 ± 7236 |
| Observed $OTU_{97}$ | 20,510 | 19,032 | 23,159 | 21,398 |
| Simpson diversity | 0.965 ± 0.051 | 0.990 ± 0.006 | 0.928 ± 0.129 | 0.976 ± 0.022 |
| Shannon diversity | 5.998 ± 0.670 | 6.902 ± 0.362 | 6.138 ± 1.134 | 6.432 ± 0.408 |
| Pielou diversity | 0.704 ± 0.066 | 0.796 ± 0.039 | 0.713 ± 0.1541 | 0.748 ± 0.040 |
| Chao1 diversity | 11,615.47 ± 1985.52 | 13,301.14 ± 1566.79 | 12,455.07 ± 2908.44 | 12,852.61 ± 1434.61 |
| Coverage (%) | 88.13 ± 2.21 | 85.86 ± 1.39 | 87.56 ± 2.53 | 87.56 ± 2.27 |
| Number of genera | 602 | 482 | 530 | 506 |
| Number of families | 249 | 231 | 241 | 232 |
| Number of orders | 139 | 127 | 133 | 132 |
| Number of classes | 83 | 84 | 86 | 86 |
| Number of phyla | 37 | 35 | 35 | 35 |

Table S3). The NMDS analyses show that the samples did not cluster according to sampling period, but according to sampling zone (Fig. 3); the samples from the influent zone were clearly separated from the samples from the other zones.

Using the Bray-Curtis distances, which do not incorporate phylogenetic relatedness in the community differences, all the sampling sites in the four zones were separated into two groups by the hierarchical cluster analysis (Ward's method): group A and group B (Fig. 4). Group A included the sites in the influent zone of the settling pond, which were characterized by higher concentrations of Cu, Zn, Pb, Cd, Ca, total N, total S, total P and available P (Wilcoxon Rank Sum tests, $P < 0.05$; SM Table S2). Group B consisted of the sites in the upstream, downstream and effluent zones. The microbial community composition of the two groups differed significantly ($F = 7.41$, $P < 0.001$; SM Table S4, Figs. S1 and S2).

### 3.4. Relationships between the environmental variables and microbial community

Correlations between the α-diversity indices (Shannon, Chao1, Simpson and Pielou) and environmental variables were determined for the samples from each zone and for the three seasons (SM Table S5). In general, the correlations between the diversity indices and environmental variables within each season were not significant, except for the correlations between pH and Chao1 in spring and summer, pH and Shannon in summer and available K and Simpson in winter (in spring, Chao1: $P = 0.046$, $r = 0.58$; in summer, Chao1: $P = 0.018$, $r = 0.72$; Shannon: $P = 0.049$, $r = 0.63$, in winter, Simpson: $P = 0.019$, $r = -0.72$). In the influent zone, Chao1 and Simpson were significantly correlated with soil organic matter and Pb, respectively (Chao1: $P = 0.023$, $r = -0.73$; Simpson: $P = 0.011$, $r = -0.79$). There were no consistently significant correlations between any diversity index and environmental variable among all four zones. However, the correlations between Shannon diversity and available K in the upstream and effluent zones were both significant (upstream zone: $P = 0.014$, $r = -0.90$; effluent zone: $P = 0.023$, $r = -0.77$).

No significant differences in microbial diversity were detected among the three seasons (Kruskal-Wallis; $P > 0.05$; SM Table S6). However, the α-diversity (Shannon and Pielou indices) was significantly different (Kruskal-Wallis; $P = 0.047$ and $P = 0.040$, respectively) among the four sampling zones, especially between the influent zone (lower diversity) and the upstream zone (Wilcoxon; $P = 0.005$ and $P = 0.003$, respectively). Also, lower Shannon and Pielou diversities were calculated for group A (Kruskal-Wallis; $P = 0.022$ and $P = 0.015$, respectively).

In the CAP analysis, the constrained axes explained 36.4% of the total variance. The two first constrained axes explained 51.5% and 12.7%, respectively. The first axis, CAP1, characterized by changes in Cu, Zn, Pb, total S, available K and pH, separated the influent zone from the other three zones (heavily and moderately polluted sites, according to the standard of industrial soils, GB15618-2008). Of all the environmental factors considered (SM Table S1), the Cu, Zn, Pb, total S and available K contents, and the soil pH, significantly ($P < 0.05$) shaped the microbial community structures (Fig. 5).

### 3.5. Possible metabolic features in the microbial community of the two post hoc groups

Microbial community composition in the two *post hoc* groups (group A and group B) was significantly different at the phylum level (SM Fig. S1). In group A, there were higher relative abundances of Betaproteobacteria, Bacteroidetes, Firmicutes and Ignavibacteriae ($P < 0.05$) and lower relative abundances of Chloroflexi, Deltaproteobacteria, Actinobacteria and Planctomycetes ($P < 0.01$). There also appeared to be slightly higher relative abundances of Alphaproteobacteria and Verrucomicrobia and slightly lower relative abundances of Gammaproteobacteria and Acidobacteria in group A, but the differences were not significant ($P > 0.05$).

At the family level (SM Fig. S2a), the dominant taxa (>5%) were Chitinophagaceae (8.3%), Burkholderiales_incertae_sedis (6.6%) and Anaerolineaceae (5.7%) in group A, while Anaerolineaceae (13.2%) and Enterobacteriaceae (11.5%) were dominant in group B. At the genus level, the most abundant genera within the different zones were determined (SM Fig. S2b); *Aquabacterium*, *Ohtaekwangia*, *Geothrix*, *Ferruginibacter*, *Thiobacillus* and *Ignavibacterium* (>2%) were the most abundant genera in the influent zone, while *Escherichia*, *Gp6* and *Thiobacillus* (>2%) were the most abundant genera in the other three zones. While *Escherichia* were dominant in group B (11.3%), they only constituted 1.6% of the community in group A. Similarly, *Gp6* accounted for 1.3% and 3.7% of the community in group A and group B, respectively. Furthermore, slight differences were observed in the relative abundance of *Thiobacillus* (2.3% and 2.0%) and *Subdivision3_genera_incretae_sedis* (1.3% and 1.5%), in group A and group B, respectively.

To provide a functional understanding of the microbial active-microbiome in the two *post hoc* groups, the metabolic functions of the identified genera were elucidated from the METAGENassist database (Arndt et al., 2012). The data suggest that most metabolic

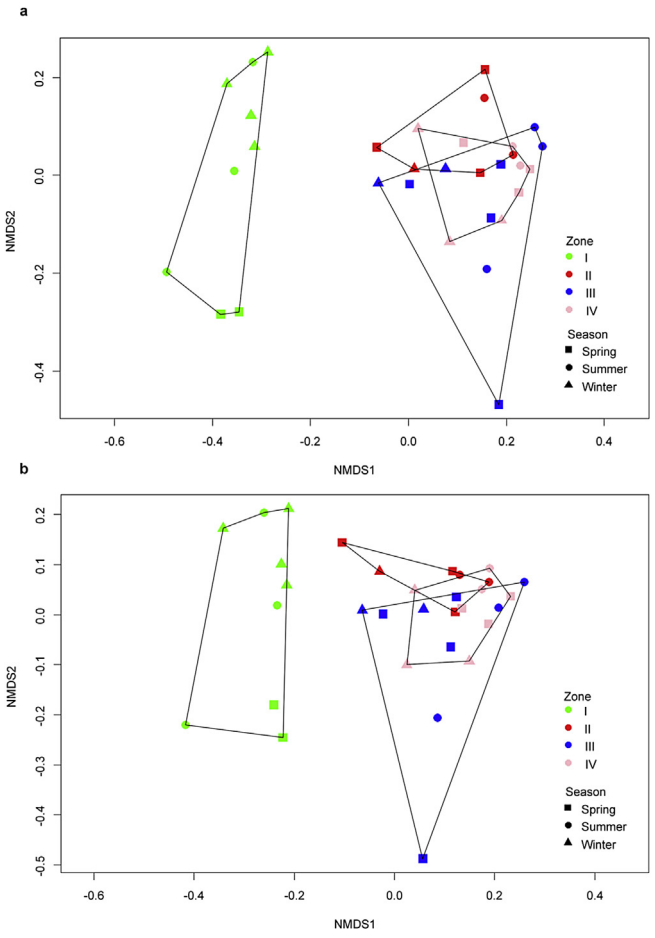


**Fig. 3.** NMDS ordination plots derived from the (a) Bray-Curtis distance matrix and (b) Unifrac distance matrix. The distance matrices were based on the relative abundances of the microbial OTUs. The configuration stresses were (a) 0.115 and (b) 0.104. Samples are clustered together by sampling zone (I, influent; II, upstream; III, downstream; and IV, effluent), but not by sampling period (spring, square; summer, circle; winter, triangle).

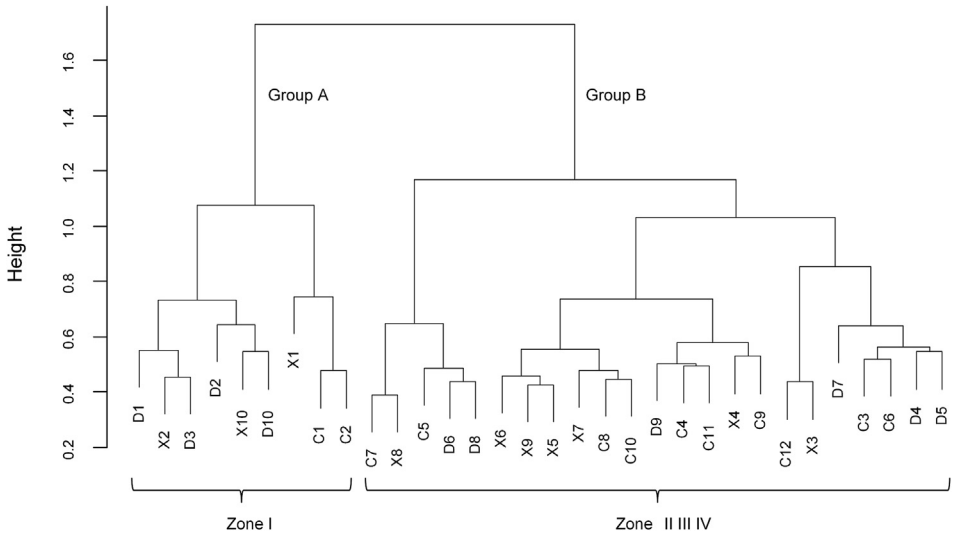


**Fig. 4.** *Post hoc* grouping of sample sites, based on hierarchical cluster analysis of Bray-Curtis distance, using Ward's method. Agglomerative coefficient = 0.71, average silhouette width = 0.20.

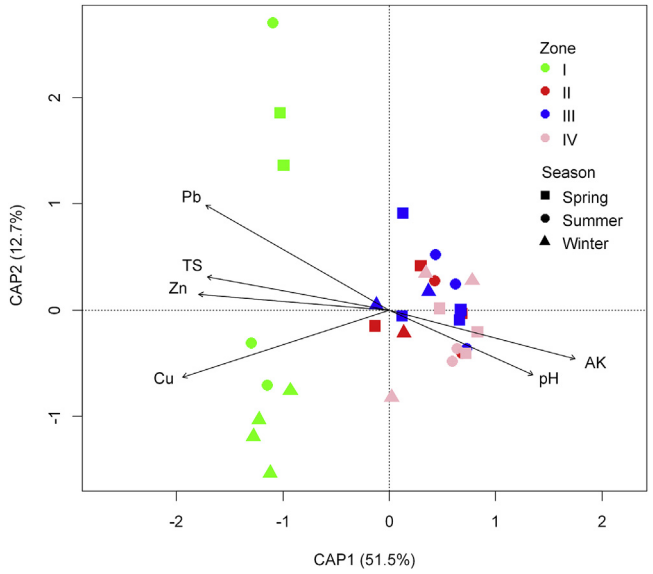


**Fig. 5.** Constrained analysis of principal coordinates (CAP) based on the relative abundances of microbial operational taxonomic units and measured environmental soil parameters that had significant correlations with the microbial community structure in four zones of a setting pond. Green, red, blue and pink colours represent four zones; square, circle and triangle shapes represent three sampling periods (I, influent zone; II, upstream zone; III, downstream zone; IV, effluent zone; TS: total sulphur; and AK, available potassium). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

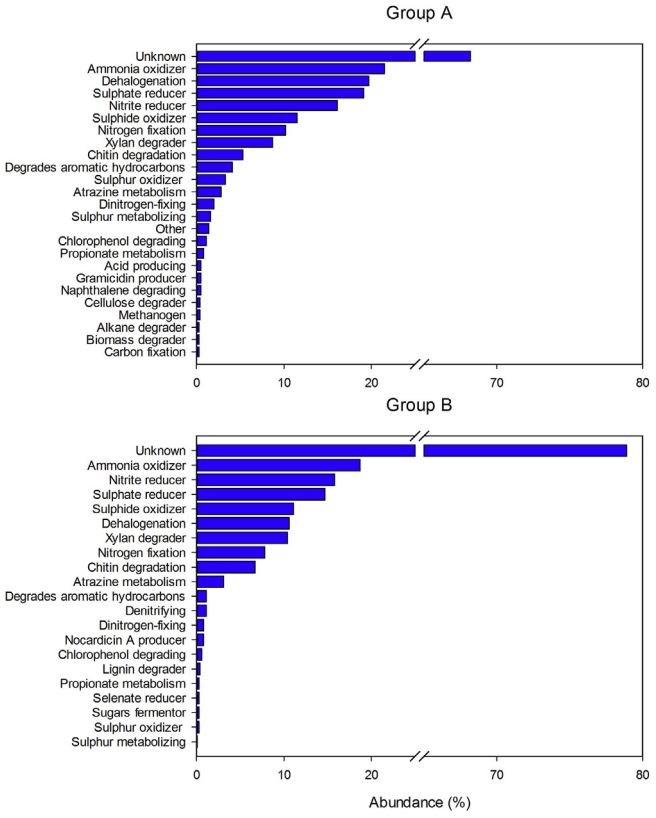


**Fig. 6.** Comparison of the metabolic groups in the bacterial communities among the soil samples in the influent (group A) and other zones (group B) of a setting pond, according to the METAGENassist analysis.

activities were higher in group A than in group B (Fig. 6). The influent zone contained some ammonia oxidizers (21.5%), nitrogen-fixing microbes (10.2%), sulphate reducers (19.1%), sulphide oxidizers (11.5%), sulphur metabolizers (1.6%) and sulphur oxidizers (3.3%). It is interesting that carbon-fixing microbes (0.3%) were only found in group A, and denitrifying (1.1%) and sugar fermenters (0.3%) were only found in group B.

Acid producers (0.5%), alkane degraders (0.3%), biomass degraders (0.3%), cellulose degraders (0.4%), methanogens (0.4%) and naphthalene degraders (0.5%) were only found in the influent zone. In addition, chlorophenol degraders (1.1%), aromatic hydrocarbon degraders (4.1%), dehalogenation microbes (19.7%) and propionate metabolisers (0.8%) were higher in the influent zone than in the other zones. Atrazine metabolisers, chitin degraders and xylan degraders were found in both groups.

### 3.6. Indictor taxa in microbial community of two post hoc groups

Comparing the heavily polluted (influent zone; group A) and

moderately polluted (other zones; group B) soils, we found 3119 OTU-based taxa that were strongly associated with only one of the groups (Table 3 and SM Table S7). Most OTU indicators (n = 2311) were closely associated with the group in the heavily polluted zone (group A) and 808 indicators were associated with the group in the moderately polluted zone (group B). Of the classified OTUs, 37 classes (out of 90), 64 orders (out of 148), 101 families (out of 273), and 164 genera (out of 687) were potential indicator taxa for the two *post hoc* groups. However, only some indicators were found for summer and winter (SM Table S8).

## 4. Discussion

Settling ponds are traditional structures used to remove suspended matter from wastewater across several industries, including mining. However, the efficiency of contaminant removal varies, according to particle size and chemical characteristics (Simanjuntak et al., 2009). In the present study, we observed significant differences in soil physicochemical properties, especially the concentrations of heavy metals, along the gradient, between the influent and other zones (Table 1 and SM Table S1). This demonstrated that the settling pond was an effective system that reduced heavy metal contamination in the riparian soil affected by mine drainage water. The slightly alkaline condition and high heavy metal concentrations in the riparian soils along the settling pond (Table 1 and SM Table S1) were similar to the results of Central Appalachian streams that responded to a gradient of AlkMD (Bier et al., 2015).

It could be hypothesized that the change in heavy metal concentrations and other soil characteristics might affect the microbial flora in the soils, due to natural selection; in turn, the microbial community could play a role in the transformation and removal of heavy metals from the mine drainage (Sheoran and Sheoran, 2006), such as Acidiferrobacter metabolizing Fe and S and producing sulphuric acid (Hallberg et al., 2011). There have been a few studies of microbial communities in riparian soils along settling ponds used for passive treatment of mine drainage, but they have not considered how the microbial community could respond to the mine drainage treatment relative to normal ecosystems. Therefore, in the present study, we gave consideration to the interactions among microbial communities and the effects of wastewater treatment procedure in the riparian soil surrounding the settling pond.

### 4.1. Variations in the microbial communities and their diversity

The current results provide evidence that the microbial communities varied in the soils surrounding the settling pond, and these variations occurred in relation to the heavy metal contents and pH values of the soils; thus, these changes likely reflect the effects of the wastewater treatment procedure on the soil microorganisms. The effects were clear both from the diversity indices and the community compositions. The microbial community composition of the riparian soils was significantly different between the influent zone and the other three zones, following the direction of flow of the slightly alkaline mine drainage (Figs. 1 and

**Table 3**
Taxa identified at the order or family levels as potential indicators through indicator taxa analysis of the two *post hoc* groups.

| Site classification | IndVal | P | Taxon (phylum, class, order) |
|---|---|---|---|
| **Identified to order** | | | |
| Group A | 0.95 | 0.0001 | Firmicutes; Erysipelotrichia; Erysipelotrichales |
| | 0.94 | 0.0001 | Tenericutes; Mollicutes; Acholeplasmatales |
| | 0.93 | 0.0001 | Proteobacteria; Gammaproteobacteria; Chromatiales |
| | 0.88 | 0.0001 | Elusimicrobia; Elusimicrobia; Elusimicrobiales |
| | 0.88 | 0.0018 | Acidobacteria; Acidobacteria_Gp1; Candidatus Koribacter |
| | 0.86 | 0.0002 | Proteobacteria; Gammaproteobacteria; Acidithiobacillales |
| | 0.72 | 0.0007 | Deinococcus-Thermus; Deinococci; Thermales |
| Group B | 0.91 | 0.0001 | Armatimonadetes; Armatimonadetes_Gp2; Armatimonadetes_Gp2 |
| | 0.87 | 0.0001 | Armatimonadetes; Armatimonadetes_Gp5; Armatimonadetes_Gp5 |
| | 0.77 | 0.0147 | Chloroflexi; Ktedonobacteria; Ktedonobacterales |
| | 0.77 | 0.0037 | Acidobacteria; Acidobacteria_Gp23; Gp23 |
| | 0.72 | 0.0021 | Acidobacteria; Acidobacteria_Gp1; Gp1 |
| | 0.70 | 0.0071 | Elusimicrobia; Endomicrobia; Candidatus Endomicrobium |
| **Identified to family** | | | |
| Group A | 0.99 | 0.0001 | Proteobacteria; Gammaproteobacteria; Chromatiales; Ectothiorhodospiraceae |
| | 0.98 | 0.0011 | Firmicutes; Bacilli; Bacillales; Paenibacillaceae |
| | 0.95 | 0.0001 | Firmicutes; Erysipelotrichia; Erysipelotrichales; Erysipelotrichaceae |
| | 0.95 | 0.0064 | Firmicutes; Bacilli; Bacillales; Alicyclobacillaceae |
| | 0.94 | 0.0002 | Tenericutes; Mollicutes; Acholeplasmatales; Acholeplasmataceae |
| | 0.93 | 0.0001 | Firmicutes; Clostridia; Clostridiales; Clostridiales Family XIII. Incertae Sedis |
| | 0.88 | 0.0001 | Proteobacteria; Alphaproteobacteria; Rhizobiales; Beijerinckiaceae |
| | 0.88 | 0.0001 | Elusimicrobia; Elusimicrobia; Elusimicrobiales; Elusimicrobiaceae |
| | 0.87 | 0.0018 | Acidobacteria; Acidobacteria_Gp1; Candidatus Koribacter; Candidatus Koribacter |
| | 0.87 | 0.0003 | Actinobacteria; Actinobacteria; Actinomycetales; Propionibacteriaceae |
| | 0.86 | 0.0002 | Proteobacteria; Gammaproteobacteria; Acidithiobacillales; Thermithiobacillaceae |
| | 0.85 | 0.0027 | Bacteroidetes; Cytophagia; Cytophagales; Flammeovirgaceae |
| | 0.84 | 0.0022 | Firmicutes; Clostridia; Clostridiales; Clostridiales Family XI. Incertae Sedis |
| | 0.80 | 0.0007 | Actinobacteria; Actinobacteria; Actinomycetales; Kineosporiaceae |
| | 0.77 | 0.0016 | Proteobacteria; Gammaproteobacteria; Alteromonadales; Alteromonadaceae |
| | 0.74 | 0.0004 | Firmicutes; Negativicutes; Selenomonadales; Acidaminococcaceae |
| | 0.72 | 0.0008 | Deinococcus-Thermus; Deinococci; Thermales; Thermaceae |
| Group B | 0.91 | 0.0001 | Armatimonadetes; Armatimonadetes_Gp2; Armatimonadetes_Gp2; Armatimonadetes_Gp2 |
| | 0.87 | 0.0001 | Armatimonadetes; Armatimonadetes_Gp5; Gp5; Gp5 |
| | 0.77 | 0.0047 | Acidobacteria; Acidobacteria_Gp23; Gp23; Gp23 |
| | 0.72 | 0.0029 | Acidobacteria; Acidobacteria_Gp1; Gp1; Gp1 |
| | 0.70 | 0.0070 | Elusimicrobia; Endomicrobia; Candidatus Endomicrobium; Candidatus Endomicrobium |

The data show the top indicators, with ≥70% perfect indication, based on their relative abundances and frequencies.

2) and related to the contamination levels (Fig. 5). Similar changes were found in other contaminated systems, including sediment and mine drainage environments (Bier et al., 2015; Feris et al., 2003; Kang et al., 2013; Tan et al., 2009).

Previously, contamination by heavy metals, hydrocarbons and polychlorinated biphenyls has been shown to decrease the bacterial diversity and shape community composition (Bier et al., 2015; Hu et al., 2007; Quero et al., 2015; Saul et al., 2005; Singh et al., 2014). Additionally, environmental factors, including pH, organic matter content, salinity and nutrient contents, were also shown to determine diversity and the community composition of soil microorganisms (Fierer and Jackson, 2006; Zhang et al., 2012). Similar to these previous studies, pH was a major driver of the microbial communities in the soils surrounding the settling pond (Fig. 5). However, this is surprising because the pH range was very small and around pH 7; thus, it was not very selective. It is possible that the variable pH was not independent but linked to another variable such as metals concentrations. The total S also emerged as one of the most significant factors separating the influent zone from the other three zones. Similar studies have proved that the S-based metabolic processes are important in metal-rich extreme environments such as mine tailing (Chen et al., 2013) and AMD (Hua et al., 2015).

Our result, that available K was one of the major determinants of the microbial community in the riparian soil along the settling pond, contrasted with some previous results, which only found available K to be a minor factor driving the microbial community, in agricultural soils (Li et al., 2011; Zhang et al., 2012). The strong correlations between the available K and the microbial communities might be related to the low available K content observed in Zone I (influent or group A) and the high available K in the other zones (group B) (Table 1).

The compositional shifts found were mainly in terms of the relative abundance, rather than turnover, of the microbial taxa. In the weak AlkMD-affected sites from the three different seasons, the community changes in the two *post hoc* groups (Fig. 4) were also strongly associated with the metal pollution levels; this is similar to previous reports that high concentrations of heavy metals negatively affect microbial diversity (Bier et al., 2015; Chodak et al., 2013; Hu et al., 2007). However, studies have suggested that microbial diversity might be high in metal contaminated environments, especially after long-term contamination (e.g., >10 years) (Bouskill et al., 2010; Feris et al., 2003; Sorci et al., 1999). These conflicting results may be related to the metal levels and the soil characteristics in the different studies, and the history of contamination.

In our study, we did not find significant relationships among the soil microbial communities or α-diversity and the three seasons. In contrast, most studies show seasonal fluctuations in the microbial communities of streams or sediments contaminated by mine drainage (Bouskill et al., 2010; Feris et al., 2004; Tan et al., 2009). Seasonal changes generally affect the soil microbial communities through temperature and precipitation (which affects soil moisture); however, the soil moisture surrounding the settling pond was always stable, while the average temperature varying between 0 °C and 27 °C had little effect on the soil microbial communities (data not shown). Therefore, it is reasonable that season did not markedly influence the soil microbial communities surrounding the settling pond.

### 4.2. Dominant microbial species and possible metabolic features of the microbial community in the two post hoc groups

Analysing the possible metabolic functions of the microbial communities in this study offered some explanation as to why the physicochemical characteristics of the soils affected the microbial flora. The relative abundances of the dominant phyla, Bacteroidetes and Firmicutes (Fig. 2), and class, Betaproteobacteria (SM Fig. S1), were lower in group B (zones II, III and IV), while the class Gammaproteobacteria and phylum Chloroflexi were higher in group B, compared with group A (zone I; influent zone). The changes in Betaproteobacteria and Gammaproteobacteria relative abundances in our study were different from results previously obtained, which showed decreases in the proportion of Betaproteobacteria across contamination gradients in soil and mine drainage environments (Bier et al., 2015; Sandaa et al., 1999), and increases in the abundance of Gammaproteobacteria in heavy metal contaminated sediments (Bouskill et al., 2010; Feris et al., 2003, 2009). These differences indicate that the microbial community in the contaminated environments might be affected by both heavy metals and other factors such as pH values and nutrients (Fig. 5).

Bacteroidetes are Gram-negative bacteria, with aerobic or anaerobic traits; they are composed of a wide range of physiologically diverse species (Bauer et al., 2006; Dhal et al., 2011; Fernández-Gómez et al., 2013). Firmicutes are also frequently reported in extreme environments, such as hypersaline sediments and heavy metal polluted soils (Emmerich et al., 2012; Sánchez-Andrea et al., 2011; Sitte et al., 2010). Therefore, the dominance of Proteobacteria, Bacteroidetes and Firmicutes in soils surrounding the settling pond could be related to their ability to adapt to the metal-rich environment.

Similar metabolic functions were found in the soils of group A and group B (Fig. 6), mainly the degraders, nitrogen metabolizers, and those bacteria involved in the sulphur cycle. However, there were higher relative abundances of ammonia oxidizers, dehalogenation bacteria, nitrite reducers, nitrogen-fixing bacteria and sulphate reducers, among others, in group A than group B. There was also a lower proportion of bacteria with unknown function (68%) in group A than in group B. These results might be related to the response of the microflora to environmental toxicity (Wakelin et al., 2014). That is, the heavily contaminated sites needed the microbiome to accumulate carbon, owing to the high metabolic rates (Chodak et al., 2013; Nakatsu et al., 2005). The heavily polluted environment could severely inhibit the activity of the microbiome during denitrification and sugar metabolism, as shown in previous studies (Bier et al., 2015; Giller et al., 1998).

There were also higher abundances of bacteria with sulphur-related functions in the influent zone than in group B (especially sulphur metabolizing and sulphur oxidizing bacteria). These metabolic functions could be used to predict that the influent zone contained a higher sulphur content, arising from the smelter (Allison and Martiny, 2008). In fact, higher total S was measured in the influent zone than in the group B (Table 1 and SM Tables S1 and S2). It is clear that the microbiome that was exposed to the contaminated soil (resulting from mine drainage) possessed special functions and had adapted to the habitat (Kuang et al., 2013).

### 4.3. Indicator taxa in the microbial communities of the two post hoc groups

Taxa in the microbial communities were identified as potential indicators that respond to the existing environmental variations. Due to the significant differences in environmental parameters between the two *post hoc* groups, we could select microbial taxa that were different between the groups and may help predict soil contamination by mine drainage. Bacteria related to sulphur oxidation and reduction are promising indicators, including *Acidiferrobacter*, *Thermithiobacillus*, *Limnobacter*, *Thioprofundum* and *Thiovirga* (sulphur-oxidizing bacteria), and *Desulfotomaculum* and *Desulfobulbus* (sulphur-reducing bacteria); these were

characteristic of the soils in group A. Only *Desulfopila* (in relation to sulphur reduction) was specific to the soils in group B.

Genera related to degrading organic compounds may also prove to be good indicators; those found in group A soils including *Limnobacter*, *Desulfitobacterium*, *Porphyrobacter* and *Paucimonas*. *Zoogloea*, *Sphaerotilus*, *Belnapia* and *Meiothermus*, which are highly resistant to extreme environments, were also observed in the group A soils. Interestingly, *Denitratisoma*, which belongs to the family Rhodocyclaceae could degrade aromatic compounds by denitrification (Hesselsoe et al., 2009). Overall, the potential indicators could have responded to the existing environment. The detection and quantification of the indicator bacteria in this study could be used to assess the impact of mine drainage on the surrounding soil environment.

## 5. Conclusions

Our Miseq sequencing survey, based on the 16S rRNA gene, showed significant differences in the microbial communities present in the riparian soils, along the flow direction of the mine drainage. Potential indicator species related to sulphur and carbon metabolism were identified in the heavily contaminated influent zone. There were trends in the relative abundances of Betaproteobacteria (which increased with increasing contamination) and Gammaproteobacteria (which decreased). The observed variations in the microbial community were likely driven by niche-based processes in the riparian soils contaminated by mine drainage. In other words, mine drainage had a profound influence on the riparian ecological environment. The potential bioremediation functions of the sulphur- and carbon-cycle related bacteria found in the settling pond riparian soils should be further investigated. This could include employing metatranscriptomic or metagenomic approaches.

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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.watres.2016.03.061.

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