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# The dynamic development of bacterial community following long-term weathering of bauxite residue

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**ABSTRACT:** Bauxite residue disposal areas (BRDAs) are typically bare of vegetation with high salinity and alkalinity. However, spontaneous encroachment of vegetation indicated that natural weathering processes had transformed the residue tailings into a soil-like material. Here we investigate the development of bacterial communities and their geochemical drivers in bauxite residue, using Illumina high-throughput sequencing technology. Bauxite residue from weathered sites had lower pH and exchangeable sodium percentages (ESP), but greater organic carbon and nitrogen concentrations. Following natural processes, the diversity of the bacterial community significantly increased and hierarchical clustering separated un-weathered from older weathered sites. Taxonomic analysis revealed that long-term weathering processes encouraged populations of *Proteobacteria*, *Chloroflexi*, *Acidobacteria* and *Planctomycetes*, whilst reducing populations of *Firmicutes* and *Actinobacteria*. LefSe analysis revealed that the biomarker changed considerably in older weathered residues compared with that of un-weathered residue. Amongst all the residue properties, variation in total organic carbon (TOC), total nitrogen (TN), available phosphorus (AP) and pH, showed significant effects on the diversity and structures of the bacterial community. The results have demonstrated that natural weathering processes stimulate the dynamic development of bacterial communities in bauxite residue, and may provide a hypothetical basis for the application of microbes at disposal areas to improve soil conditions.

## HIGHLIGHT:

- ▶ Development of microbial communities in bauxite residue disposal areas following long-term natural processes was investigated.
- ▶ Natural processes promoted the diversity of bacterial communities in bauxite residue.
- ▶ Bacterial community structure significantly differed in weathered disposal areas
- ▶ pH, TOC, TN and AP may be the most important factors influencing bacterial community structure in bauxite residue.

**KEY WORD:** Bauxite residue; Weathering; Bacterial diversity; Bacterial community; Illumina high-throughput sequencing

## 1 Introduction

Bauxite residue, an industrial waste produced during the production of alumina, represents a large and increasing global problem (Gomes et al., 2016). Currently, the worldwide inventory reached an estimated 4.2 billion tons, increasing by approximately 200 million tons per annum (Xue et al., 2019). Bauxite residue is rarely recycled and is frequently stored in bauxite residue disposal areas (BRDAs) (Power et al., 2011). Therefore, the growing mass of stored bauxite residue highlights the need for strategies to manage this growing problem, and in situ revegetation may be a promising way towards this (Xue et al., 2016).

Bauxite residue often presents high alkalinity and salinity, as well as a lack of nutrients, which largely hinder plant growth (Jones and Haynes, 2011). Currently, various amendments are applied prior to vegetation establishment to render the residue more hospitable to plants (Courtney and Harrington, 2012; Courtney and Kirwan, 2012; Fuller et al., 1982; Wong, 1993). However, no study has documented long-term successful revegetation on BRDAs.

Successful revegetation must involve the development of microbially-driven nutrient cycling for the long-term provision of plant nutrients (Grandlic et al., 2008; Rashid et al., 2016). Different microbes perform diverse functions during soil nutrient cycling such as, decomposition of organic matter and leaf litter (Schweinsberg-Mickan and Muller, 2009), mobilization and translocation of inorganic mineral nutrients (Nassal et al., 2018) and fixation of atmospheric carbon and nitrogen (Lynn et al., 2017). In addition, many plant species benefit from symbiotic associations with soil microorganisms such as mycorrhizal fungi and rhizobia (Kuiper et al., 2004). Studies on mine tailings have emphasized a strong association between the establishment of a stable plant community and the abundance and composition of soil microbiota (Chen et al., 2008; Dhawi et al., 2016; Mendez and Maier, 2008). For instance, arbuscular mycorrhizal fungi (AMF) commonly assist in the recovery of soil organic carbon and nitrogen in coal mine districts. Plant growth-promoting bacteria (PGPB) are also successful in promoting the establishment of vegetation on metallic mine tailings.

For rehabilitation on bauxite residues, microbial communities also appear to be essential. For example, the addition of organic amendments increased soluble organic C, microbial biomass C, basal respiration and promoted enzyme activities (Jones et al., 2010; Jones et al., 2011). The diversity of bacterial and fungal communities developed rapidly and was similar to that in a coastal sand analog (Banning et al., 2011). In addition, long-term restoration also created diverse soil-like microbial communities as environmental conditions became less extreme indicating the potential for feedback to exist between microbial communities and their environment which may be exploited in the development of microbially-driven remediation strategies (Schmalenberger et al., 2013). However, we know of no published studies investigating the successional development of microbial community structure and function in bauxite residue following weathering over time. Recently, spontaneous vegetation growth on abandoned BRDAs suggested that natural weathering processes could improve bauxite residue and support vegetation establishment. Although natural weathering processes improved the properties of bauxite residue and promoted plant establishment (Kong et al., 2017b; Zhu et al., 2016), microbial diversity and functional community structure remained elusive. In this study we use high-throughput sequencing and real-time quantitative PCR technology in an attempt to identify the microbial community structure and functional genes involved in C and N cycling within bauxite residue at their different stages of weathering.

We hypothesize that natural weathering processes have changed the diversity and structural composition of the microbial communities and in doing so have improved the properties of bauxite residue over time in order to support plant establishment. Thus, the objectives of this investigation were to, (a) investigate the effect of weathering processes on bacterial community diversity, 2) identify the changes in bacterial community structure at the weathered disposal area and, 3) reveal potential influencing factors which affect bacterial community structure in bauxite residue.

## 2 Materials and methods

### 2.1 Site description and sampling

The selected bauxite residue disposal area (BRDA) is located in Central China (35°24'N, 113° 25'E) and was in operation for 20 years from 1993 to 2013. The climate is temperate continental monsoon, with an average temperature of 12.8-14.8 °C and average precipitation of 874 mm. Residue samples were collected during October, 2018. Based on the time of deposition and vegetation cover, four sampling sites were selected; unweathered site (UW), young weathered site (YW), old weathered site (OW) and old weathered site with grass (OWV). These locations were used to evaluate the influence of natural regeneration on the bacterial community (Table 1). The OWV sites were mainly dominated by *Cynodon dactylon*. For each site, residue samples were collected to a depth of 0–20 cm in triplicate using an auger with a 10 cm diameter (Fig. 1).

All samples were brought back to the laboratory and divided into two parts. One part was dried at room temperature and then sieved (<2 mm) for physicochemical analyses. The second part was prepared under –80 °C in the laboratory for microbial analyses.

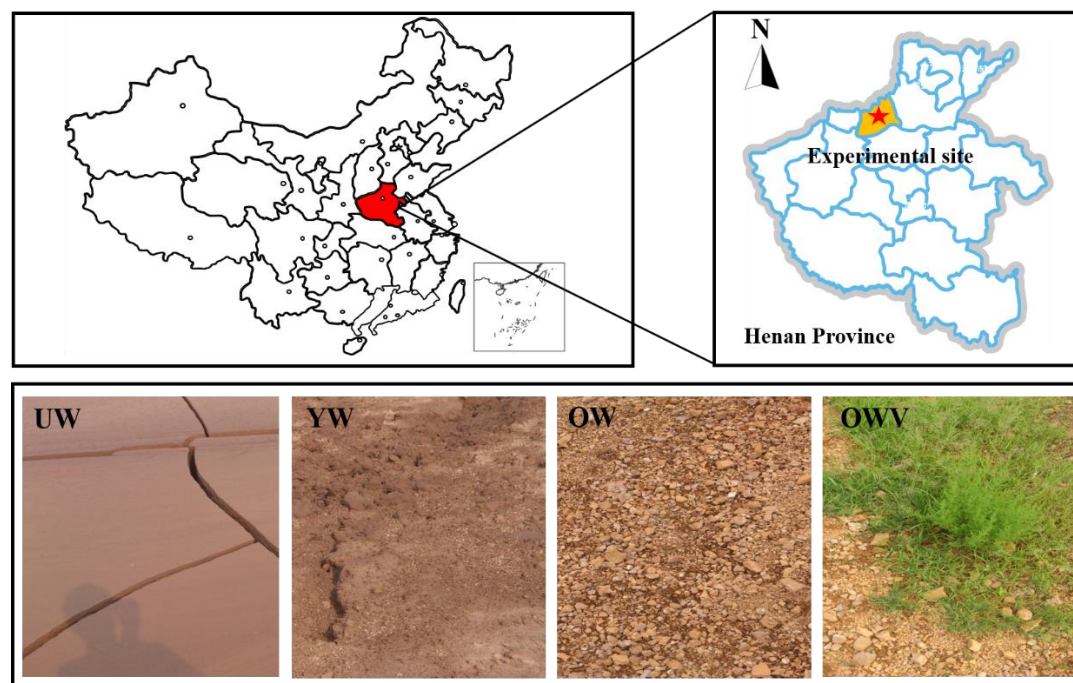


Fig. 1. The effect of weathering on bauxite residue at different temporal stages following disposal. UW: unweathered residue; YW: young weathered residue; OW: old weathered residue; OWV: old weathered residue covered with vegetation.

## 2.2 Determination of residue properties

pH and EC were analyzed by water extraction with a ratio of 1:5 (w/w, solid/liquid) using a pH detector and a conductivity meter, respectively (Rayment and Higginson, 1992). Exchangeable Ca, Mg, K, and Na were extracted with 1 M ammonium acetate and analyzed by ICP-AES (Rayment and Higginson, 1992). Exchangeable sodium percentage (ESP) was calculated by the proportion of exchangeable sodium in the total exchangeable bases. Total organic carbon (TOC) was determined by low-temperature external-heat potassium dichromate oxidation colorimetric method. Total nitrogen (TN), ammonium and nitrate concentrations were determined by an automated flow injection analyzer after extraction of the soil samples with 2 M KCl. Available phosphorus was extracted with 0.5 M NaHCO<sub>3</sub> (pH 8.5) (1:100 w/v ratio for 16h) and measured using UV spectrophotometry by the molybdenum blue method.

## 2.3 DNA extraction and PCR amplification

DNA extraction was carried out using the Ultra Clean Soil DNA extraction kit from MoBio (Carlsbad, CA) according to the manufacturer's instructions. Extracted DNA was quantified using a Nano Drop ND-1000 (Thermo Scientific, Waltham, MA). The V4 region of the bacterial 16S rRNA gene was amplified with the primers 338F and 806R.

All PCRs were conducted in a G-Storm GS2 thermo-cycler (Somerset, UK) with primers obtained from Metabion (Munich, Germany). DNA was amplified via PCR using established protocols with 0.5 U of Dreamtaq polymerase, 1× buffer with 2 mM Mg, 0.2 mM dNTP each (all Fermentas, Germany) and 0.4 μM primer each in a total volume of 25 μL. The PCR was performed under the following conditions: 94 °C (180s); 28 cycles of 94°C (30 s), 53 °C (40 s), and 72°C (60 s); and 72 °C (300 s). Amplicon products were purified using the Agencourt AMPure PCR Purification system (Beckman Coulter, Indianapolis, IN). Briefly, 180 mg of residue was incubated with 1 M CaCO<sub>3</sub> solution for 1 hr and subsequently extracted by the phenol-chloroform method. DNA extracts were purified using a GeneCleanTurbo Kit (Biogenic), following the manufacturer's instructions, and the replicates of each sample were pooled together and stored in -20 °C before further use.

## 2.4 Data analysis and statistical procedures

Sequence reads were first analyzed using the Qiime pipeline. Briefly, operational taxonomical units were clustered with a similarity cut off at 97% and diversity analysis was calculated, resulting in alpha and beta diversity analysis based on sequences that exceeded 54,000 reads in total.

The estimation of microbial diversities of the bauxite residue sites were conducted via QIIME platform. Taxonomic analysis of sequences was implemented with a combination of BLAST 30 against the 16S-specific SILVA database (version 100) and MEGAN 431 with a bit-score cutoff of 86. Sequences of selected families were exported into Mega 532 for alignment and import of related sequences using the BLAST tool. Realigned sequences were used for maximum likelihood tree generation (Jukes-Cantor).

Statistical analysis of fungal community profiles was performed using the STAMP software (Parks et al., 2014) following the Two-sided Welch's t-test. Alpha-diversity and beta-diversity of fungal communities were calculated on the R statistical platform, using the 'vegan' package. Alpha-diversity included observed OTU number, Chao1, Shannon diversity index (H), Simpson index of diversity (1/D) and Pielou evenness index (J). Beta diversity of comparing fungal community structure in different treatments including correspondence analysis (CA), Nonmetric Multidimensional

Scaling (NMDs) and analysis of similarity (ANOSIM) were carried out based on the Bray-Curtis distance matrix. Canonical correspondence analysis (McCarthy and Williams) was employed to reveal the relationship between soil properties and the fungal community. LEfSe (Linear discriminant analysis Effect Size) for detecting biomarkers was performed on the online Galaxy platform. Student t-test was performed to compare the significant difference between two groups using Minitab software. A *p* value of less than 0.05 was considered as significant.

### 3 Results

#### 3.1 Residue properties

Residue sample properties significantly differed during the natural weathering processes (Table 1). The unweathered residue (UW) presented high alkalinity and salinity, as well as a lack of nutrients. Values of pH, EC, and ESP significantly decreased as weathering time increased ( $P < 0.05$ ), whereas the contents of TOC, TN, and AP significantly increased as weathering time increased ( $P < 0.05$ , Table 1).

**Table 1** Characteristics of residue samples from the different stages of weathering

Samples		UW	YW	OW	OWV
pH		11.03±0.11D	10.6±0.07C	10.1±0.09B	9.4±0.10A
EC <sup>b</sup>	mS·cm <sup>-1</sup>	3.65±0.57D	2.28±0.49C	0.92±0.16B	0.34±0.02A
ESP <sup>c</sup>	%	72.51±0.32D	49.65±1.98C	34.72±1.36B	28.99±1.19A
TOC <sup>d</sup>	g·kg <sup>-1</sup>	5.71±0.26A	8.00±0.30B	9.24±0.25C	10.81±1.15D
TN <sup>e</sup>	g·kg <sup>-1</sup>	0.039±0.008A	0.150±0.06B	0.729±0.07C	1.532±0.28D
AP <sup>f</sup>	mg kg <sup>-1</sup>	5.32±0.25A	10.48±0.25B	22.74±5.44C	34.94±5.44D

UW: unweathered site; YW: young weathered site; OW: old weathered site; OWV: old weathered with vegetation covert;

EC = electrical conductivity; ESP = exchangeable sodium percentage; TOC= total organic carbon; TN= total nitrogen;

AP= available phosphorus; mS = milli Siemens; abc= significantly different ( $P < 0.05$ ); ±= standard deviation.

With the same weathering time, the values of pH, EC, and ESP were significantly higher at unvegetated sites (OW) compared to vegetated areas (OWV) ( $P < 0.05$ ). At the same time, the concentrations of TOC, TN, and AP were significantly lower in unvegetated sites (OW) than those in vegetated areas (OWV) ( $P < 0.05$ ).

#### 3.2 Diversity of bacterial community

In order to investigate the diversity and structure of the microbial communities, Illumina high-throughput sequencing technology was used to sequence the 16S rRNA. In total, 509,008 effective sequences were obtained from all residue samples. At the 97% similarity level, all effective sequences were assigned to 5,490 OTUs and each sample contained 642 to 1733 OTUs. Furthermore, the coverage of effective sequences in each sample was higher than 98%, and all rarefaction curves tended to approach the saturation plateau. The rarefaction curve showed that a deeper sequencing depth would not cause an obvious increase in observed OTU number, which indicated that the sequencing depth was adequate for further analysis (Fig.2a).

Natural weathering processes promoted bacterial diversity in bauxite residue. The number of OTUs and parameter indices (Chao1, Shannon and Shannoneven) of microbial communities increased significantly from unweathered to old weathered sites ( $P < 0.05$ ), but the indices were not significantly different between those of unweathered and young weathered locations (except Chao) (Fig.2).

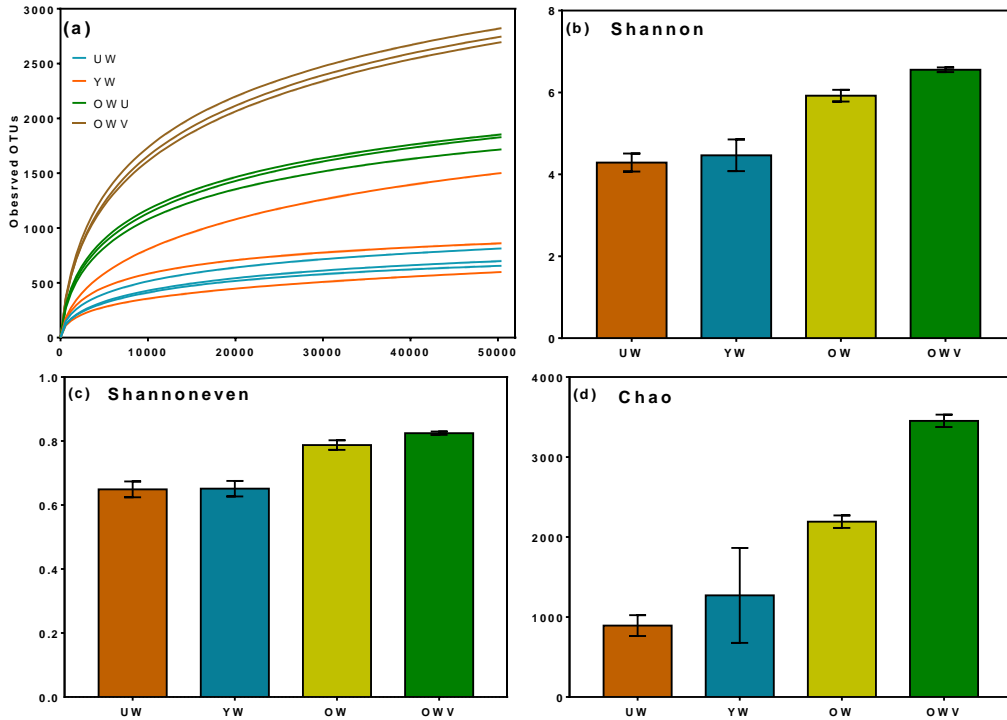


Fig. 2. Alpha diversity of bauxite residue during weathering process. (a) Observed OTUs, (b) Shannon diversity (c) Shannoneven evenness diversity, (d) Chao richness.

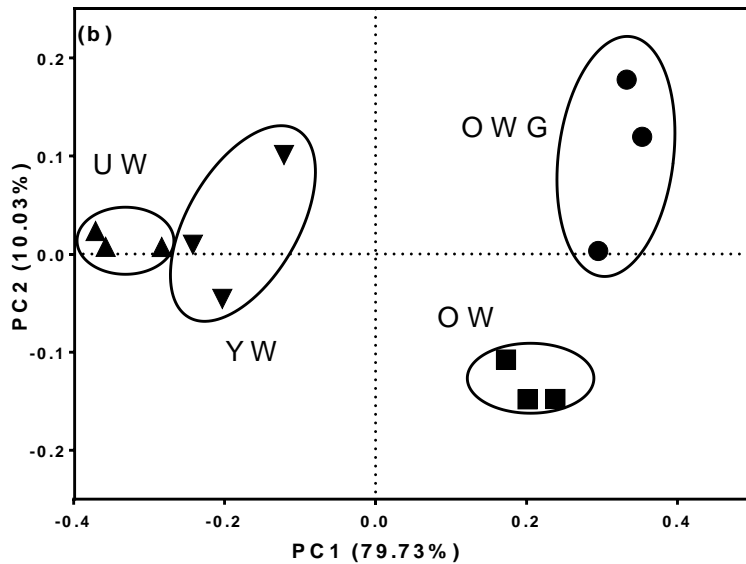


Fig. 3. Principal coordinate analysis (PCoA) of bacteria community composition for bauxite residue from different stages of weathering.

Principal coordinate analysis (PCoA) (Fig. 3) revealed that the bacterial communities in UW and YW clustered closely and grouped separately from those in OW and OWV. Coordinate axes 1, 2, and 3 (PC1, PC2, and PC3) can explain 79.73%, 10.03% and 3.98% of the variation. The high explanation (PC1) in the first principal-coordinate axis revealed that the bacterial structure of residue samples was significantly changed.

### 3.3 Composition of bacterial community

Microbial composition at the phylum level (relative abundance > 0.1%) is shown in Fig. 4. A total of 31 phyla were identified across all residue samples. *Firmicutes* (6.18%-33.8%), *Actinobacteria* (8.9%-32.3%), *Chloroflexi* (6.8%-25.3%), *Proteobacteria* (10.9%-19.5%), *Acidobacteria* (<1%-18.8%), *Planctomycetes* (1.0%-13.7%) and *Deinococcus-Thermus* (2.3%-5.6%). These seven phyla accounted for 89.9–92.9% of the effective sequences. Bacterial community distribution pattern also differed between residue samples from the different locations (Fig. 4). In unweathered residue (UW) samples, the most abundant phyla were *Firmicutes* and *Actinobacteria*, accounting for 29.5% - 53.7% and 26.2%-40.5% of all the sequences. Following *Actinobacteria*, the phyla were dominated by *Chloroflexi* and *Proteobacteria*, accounting for a low abundance with a prevalence of 8.1% and 9.2%, respectively. Other phyla that existed in UW residue samples were *Deinococcus-Thermus* (1.8-5.3%), *Gemmatimonadetes* (1.1%-2.8%) and *Bacteroidetes* (0.7%-1.5%). Natural weathering processes significantly changed the structure of bacterial communities in bauxite residue. Natural weathering processes significantly decreased the abundance of *Firmicutes* and *Actinobacteria*, whereas they increased the abundance of *Chloroflexi* (YW 17.4%, OW 27.9%). Furthermore, several new taxonomic groups including *Acidobacteria* (OW 9.5 %) and *Planctomycetes* (YW 1.3%, OW 9.8%), which are typical bacterial populations in soils, were found to be enriched. During long-term weathering processes, spontaneous vegetation encroachment occurred randomly at some sites. The vegetation colonization further increased the abundance of *Acidobacteria* (OWV 13.7 %) and *Planctomycetes* (OWV 18.8 %) and *Proteobacteria* (OWV).

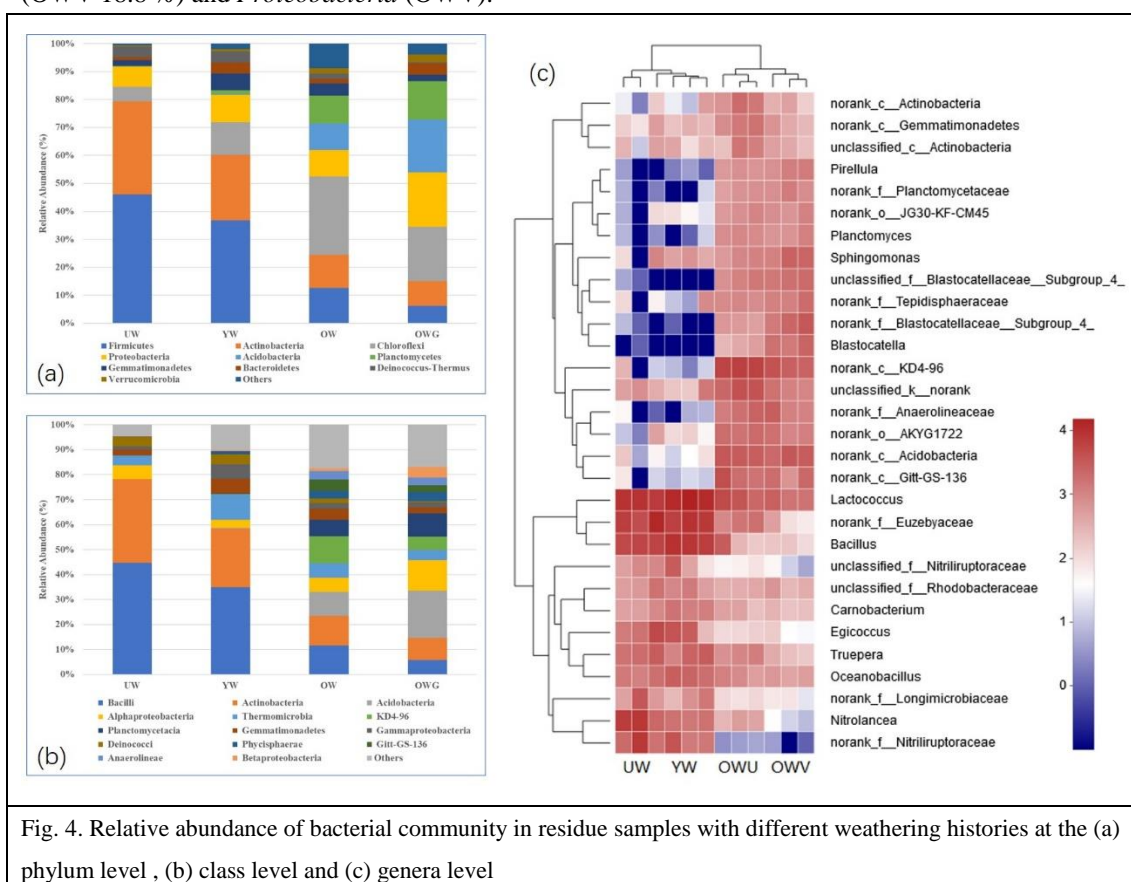


Fig. 4. Relative abundance of bacterial community in residue samples with different weathering histories at the (a) phylum level, (b) class level and (c) genera level

At the class level, a total of 61 classes were obtained across all residue samples (Table S4). 14 classes including *Bacilli*, *Actinobacteria*, *Acidobacteria*, *Alphaproteobacteria*, *Thermomicrobia*,



*KD4-96*, *Planctomycetacia*, *Gemmatimonadetes*, *Gammaproteobacteria*, *Deinococci*, *Phycisphaerae*, *Gitt-GS-136*, *Anaerolineae* and *Betaproteobacteria* (defined as >2% of the total effective sequences), accounted for 82.5 to 95.4 % in each sample. The relative abundances of *Actinobacteria*, *Bacilli* and *Deinococci* were significantly higher at the unweathered (UW) and young weathered (YW) sites compared to the old weathered (OW and OWV) sites ( $P < 0.05$ ; Table S5). The relative abundances of *Acidobacteria*, *Alphaproteobacteria*, *KD4-96*, *Planctomycetacia*, *Phycisphaerae*, *Gitt-GS-136*, *Anaerolineae*, and *Betaproteobacteri* were however significantly higher in the two old weathered sites (OW and OWV) compared to the unweathered (UW) and young weathered (YW) sites ( $P < 0.05$ ). Bacterial composition at the class level revealed significant differences between OW and OWV sites (Table S4). The relative abundances of *Actinobacteria*, *Bacilli*, *KD4-96*, *Thermomicrobia*, *Gitt-GS-136*, and *Gemmatimonadetes* were significantly higher in OW sites than in OWV sites, whereas the relative abundances of *Acidobacteria*, *Alphaproteobacteria*, *Planctomycetacia*, *Phycisphaerae*, and *Betaproteobacteria* were significantly higher in OWV sites than those at OW sites.

For a more detailed analysis, a hierarchically clustered heat map based on the Bray-Curtis similarity index was generated to exhibit the hierarchical relationships of the top 30 genera among the 12 residue samples (Fig. 4c). The heat map revealed that the most often detected bacterial genera and the distribution characteristics of the higher abundance genera were different in residue samples with different weathering histories. In UW and YW residue, the most abundant genera were *Lactococcus* followed by *norank\_f\_Euzebyaceae*, *Bacillus*, *Nitrolancea*, *norank\_f\_Nitriliruptoraceae*, *Truepera*, *Egicoccus*, *Oceanobacillus*, *unclassified\_f\_Rhodobacteraceae*, and *Carnobacterium*. The abundance of these groups was significantly higher than those in OW and OWV sites ( $P < 0.05$ ). However, the abundant genera changed following long-term weathering. In OW and OWV sites, the most abundant genera were *norank\_c\_KD4-96*, followed by *norank\_c\_Acidobacteria*, *Lactococcus*, *norank\_c\_Gitt-GS-136*, *Sphingomonas*, *norank\_f\_Anaerolineaceae*, *norank\_o\_AKYG1722*, *norank\_f\_Blastocatellaceae* and *norank\_f\_Tepidisphaeraceae*. The abundance of these groups was significantly higher than those in UW and YW sites ( $P < 0.05$ ).

Linear discriminant analysis (LDA) effect size (LEfSe) was used to detect groups or species causing significant differences in bauxite residue with different weathering histories. As shown in Fig. 5(a), 23 bacteria classes exhibited statistically significant differences among the different weathered residue sites with an LDA threshold of 4.0 (Fig. 5b). Specifically, *Bacilli* (class), *Firmicutes* (phyla), *Actinobacteria* (class), *Actinobacteria* (phyla), *Lactobacillales* (order) were enriched in UW. *Acidobacteria* (phyla), *Acidobacteria* (class), *Proteobacteria* (phyla), *Planctomycetes* (phyla), *Blastocatellaceae\_\_Subgroup\_4\_* (family) were enriched in OW (Fig. 5c).

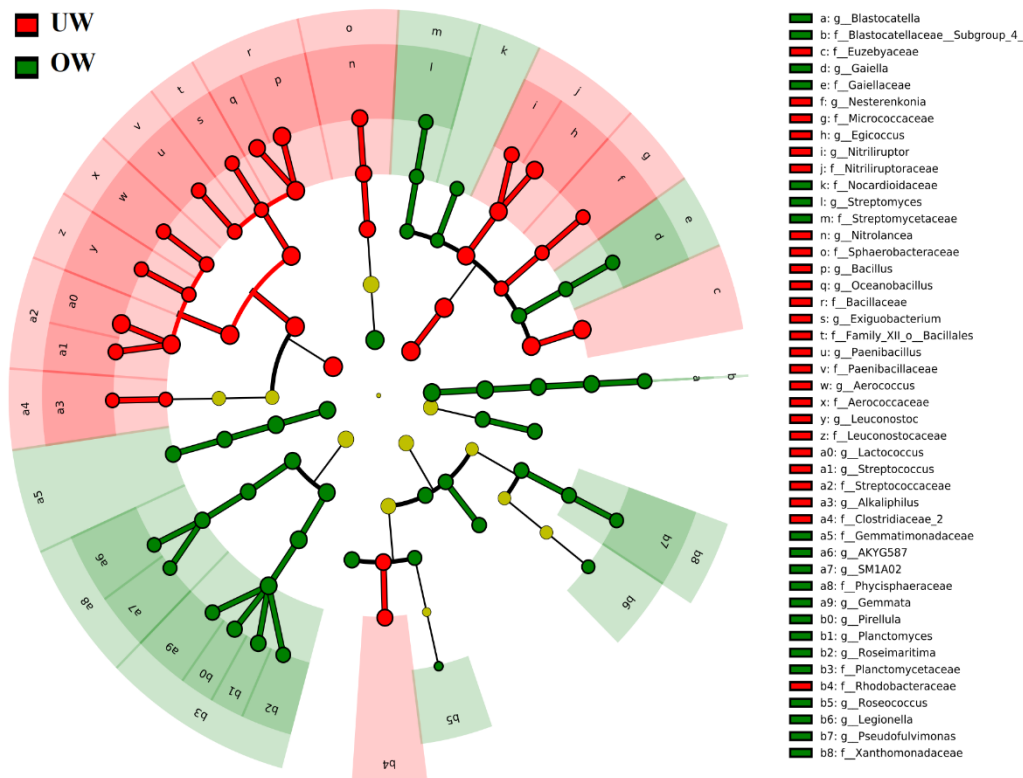


Fig. 5. LEFSe analysis of bacterial community in UW and OW residue sites. Yellow circles represent non-significant differences in abundance between UW and OW soils. Taxa enriched in UW samples with a positive LDA score (red), and taxa enriched in OW samples have a negative score (green).

### 3.4 Correlation between bacterial community and residue property

In this study, six parameters, including pH, EC, ESP, TOC, TN and AP were taken into consideration to investigate the relative contributions to bacterial communities. The Shannon index was significantly positively correlated with the contents of TOC, TN and AP, whilst negatively correlated to residue pH, EC and ESP (Fig. 6).

The RDA results showed that physico-chemical properties of bauxite residue had significant effects on the bacterial community. Overall, the first two axes explained 88.85% of the variation of microbial composition, and the correlation of species-environment of both axes was  $> 95\%$  (pseudo-canonical correlation). It suggested that there were remarkable correlations between microbial community composition and environmental properties.

For a more detailed analysis, linear-regression analysis was conducted to reveal the relationships between residue properties and bacterial taxa. High alkalinity and salinity showed significant influence on bacterial communities. The relative abundance of *Fimicutes* and *Actinobacteria* were positively correlated with pH, EC, and ESP ( $P < 0.001$ ), while *Acidobacteria*, and *Planctomycetes* were negatively correlated with residue pH ( $P < 0.001$ ). The species from *Chloroflexi* and *Proteobacteria* showed a weak correlation with pH, EC, and ESP. The relative abundance of *Chloroflexi* were positively correlated with pH, EC, and ESP ( $P = 0.03$ ,  $P = 0.02$ ,  $P = 0.03$ , respectively). The *Proteobacteria* showed a weaker correlation with EC compared to pH and ESP.

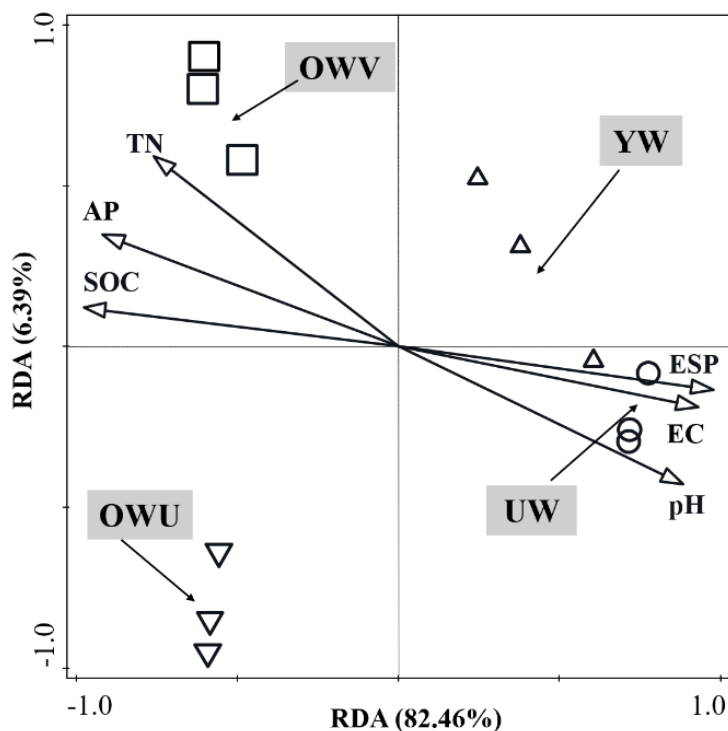


Fig .6. Redundancy analysis (RDA) of the microbial community and environmental parameters across all residue samples with different weathering histories.

Except for the high alkalinity and salinity, the variation in nutrient concentrations in bauxite residue also significantly influenced its bacterial communities. The relative abundances of *Acidobacteria* and *Planctomycetes* revealed significant positive correlations with the concentrations of TOC, TN and AP (all  $P < 0.05$ ), whilst those of *Firmicutes* and *Actinobacteria* were significantly negatively correlated with the concentrations of TOC, TN and AP ( $P < 0.05$ ).

## 4. Discussion

### 4.1. Effect of natural process on residue properties

The long-term weathering process caused significant changes to the properties of bauxite residue. pH and EC of residues at the weathered sites were significantly lower than at the unweathered sites, which may be caused by wind erosion and water leaching. Kong et al. (2017a) reported that long-term natural weathering processes decreased the alkalinity and salinity of bauxite residue through the leaching of free hydroxides, carbonates and aluminates, and dissolution of alkalinity solids including sodalite, hydrogarnet and calcite. In addition, some bacterial communities may secrete organic acids, which benefit the reduction of alkalinity in bauxite residue (Hamdy and Williams, 2001). The contents of TOC, TN, and AP at the weathered sites were predominantly higher than those at the unweathered sites (Table 2); indicating that natural weathering processes enhanced the concentrations of nutrients in bauxite residue. Zhu et al. (2016) found that long-term weathering processes could promote the accumulation of organic carbon and aggregate formation in bauxite residue. This may be caused by the shift from haloalkaliphile-dominated assemblages to diverse soil species with diverse functions such as C/N fixation (Santini et al., 2015).

## 4.2. Effect of natural process on bacterial community

The abundance (16S gene abundance) of bacterial communities was higher in weathered sites than those in unweathered sites. Similar results were also observed in bacterial diversity indexes, indicating that natural weathering processes increased the bacterial diversity in bauxite residue. This has been demonstrated previously in the restoration of mine tailings (Li et al., 2015; Wu et al., 2018). At the weathered site, the establishment of vegetation generally promoted the development of bacterial communities by creating a nutrient enriched environment, with root exudates and organic acids. On the other hand, microorganisms were involved in the degradation of organic substances, C/N fixation and the mobilization of mineral nutrition (Bao et al., 2019; Courty et al., 2010; Uroz et al., 2011), which were all beneficial to plant growth. Plant-microbe interactions at the restored site were conducive to the establishment of plants and improvement of soil quality, and finally promoted ecosystem reconstruction (Wu et al., 2018).

The composition of bacterial communities significantly changed during natural weathering processes (Figure 3). Bacterial communities in unweathered sites were dominated by Firmicutes and Actinobacteria. This finding contradicted a previous study with fresh bauxite residue which showed that *Proteobacteria* was the predominant bacterial phylum (Krishna et al., 2014; Santini et al., 2015). *Firmicutes* and *Actinobacteria* are considered to have strong metabolic capacities and are frequently found in alkaline lakes and hypersaline mats. However, long-term weathering dramatically changed the bacterial communities, which were dominated by *Chloroflexi*, *Acidobacteria*, *Planctomycetes* and *Proteobacteria* in old weathered sites. This partially coincides with the results from Schmalenberger (Schmalenberger et al., 2013), in which restoration resulted in the accumulation of *Acidobacteria*. *Acidobacteria* are generally acidophilic, and ubiquitous in various ecosystems, especially soils. The abundance of *Acidobacteria* in soils is correlated with soil pH (Jones et al., 2009). *Acidobacteria* can make up 20% of all bacteria in soils with a pH ranged from 7–8 (Lauber et al., 2009). *Acidobacteria* is divided into 26 subgroups (Barns et al., 2007), and the subgroups of Gp1, Gp2, Gp3, Gp4 and Gp6 are abundant in soil environments (Barns et al., 1999). In this study, the high abundances of Gp4 and *norank\_c\_Acidobacteria* dominated the weathered sites (Table S5). However, Wei et al. (Wei et al., 2019) reported that Gp1, Gp2 and Gp3 were abundant in the restored tailings (pH=4.5 - 4.8). Griffiths et al. (Griffiths et al., 2011) found that low pH (pH= 4.3), medium pH (pH= 6.1) and high pH (pH=8.2) soils were dominated by Gp1, Gp1 + Gp6 and Gp6, respectively.

*Proteobacteria* is another abundant bacterial phylum at weathered residue sites (Fig. 2 and Table S2). These results were consistent with many previous studies. For example, Santini et al. (Santini et al., 2015) found that the relative abundance of *Proteobacteria* ranged from 40-80% of all the sequences in bauxite residue. *Proteobacteria* often plays an important role in nitrogen cycling (Rick and Thomas, 2001). *Proteobacteria* may play a key role in phylogenetic, ecological and pathogenic values and participate in energy metabolism, such as the oxidation of organic and inorganic compounds and obtaining energy from light (Bryant and Frigaard, 2006). *Proteobacteria* often consist of four classes including Alpha-, Beta-, Gamma- and Deltaproteobacteria. In this study, *Alphaproteobacteria* and *Betaproteobacteria* increased whereas *Gammaproteobacteria* decreased during the natural weathering process. These results corresponded to the report of Liu et al (Liu et al., 2014). The relative abundance of *Gammaproteobacteria* increased with increasing soil pH, while *Alphaproteobacteria* showed the opposite pattern. Shen et al. (Shen et al., 2013) also found a similar negative relationship between *Alphaproteobacteria* and soil pH in Changbai Mountain soils, while Lauber et al. (2009) and Chu et al. (2010) reported that the population of *Alphaproteobacteria* was positively correlated to soil pH.

### 4.3. Relationship among bacterial community and residue properties

Natural weathering processes improved residue properties and changed bacterial community structure at the disposal area. RDA analysis showed that residue properties including pH, organic carbon, total nitrogen and available phosphorus were the major drivers of microbial community diversity (Fig. 3).

Many studies have demonstrated pH is the primary factor affecting soil microbiota in various types of soils (Fierer and Jackson, 2006; Shen et al., 2013; Griffiths et al., 2011), and have proposed that pH is a universal factor determining soil microbiota (Chu et al., 2010; Liu et al., 2014; Nacke et al., 2011). A decrease in pH, such as soil acidification, may enhance the release of mineral nutrients for microbial growth and subsequently influence microbial community composition (Carson et al., 2007). In addition, high concentrations of  $H^+$  or  $OH^-$  in soil may restrict microbial community diversity by imposing stress on microbial colonization, and regulating the availability of nutrient elements (Cookson et al. 2007). Xiong et al. (2012) found that pH was an effective indicator to predict bacterial community structure in alkaline sediments. The results in our study also showed that residue pH could drive the development of bacterial communities. For instance, with the decrease of residue pH, the relative abundance of *Acidobacteria* increased. This was similar to the distribution patterns of *Acidobacteria* across the related pH gradient (Jones et al., 2009; Dimitriu and Grayston, 2010; Shen et al., 2013). Furthermore, the relative abundance of *Alphaproteobacteria* increased at a lower pH, which contrasted with the results in other studies (Shen et al., 2013). It was worth noting that these soils were weakly acidic or nearly neutral (3.5-6.5), whilst the pH in residue samples ranged from 9.4 to 11.3. The different pH environments may result in different variations in abundance of *Alphaproteobacteria*.

Besides pH, other environmental variances are also important for the geographic distribution of microbial communities in different environments (Freedman and Zak, 2015). Huang et al. (2013) demonstrated that bacterial communities in hot springs were predominantly correlated with temperature, sulfur, total nitrogen and calcium content. Azarbad et al., (2013) observed that the bacterial communities in heavy metal contaminated soils was driven by soil organic matter. In this study, long-term natural process accumulated nutrients in bauxite residue, including TOC, TN and AP (Table 1). The relative abundances of six major phyla, including *Proteobacteria*, *Acidobacteria*, *Firmicutes*, *Actinobacteria*, *Chloroflexi* and *Planctomycetes*, had significant correlations with the contents of TOC, TN and AP. Among these groups, *Actinobacteria* and *Firmicutes* showed negative correlation with TOC, TN and AP in bauxite residue, whilst *Acidobacteria*, *Proteobacteria*, *Chloroflexi* and *Planctomycetes* showed positive correlation with these indexes (Fig. S5-S7). This was consistent with the findings from Schmalenberger et al. (2013). At the BRDA restored for 12 years, Schmalenberger et al. (2013) found that *Verrucomicrobia*, *Acidobacteria*, and *Proteobacteria* were closely related to the contents of TC and TN. In addition, the evolution of bacterial communities from haloalkaliphile-dominated assemblages may respond from the accumulation of *Planctomycetes* in bauxite residue (Santini et al., 2015). However, the significant relationship between *Chloroflexi* and nutrients here have not been found in other research (Schmalenberger et al., 2013). This may be caused by the unique bacterial communities in this study, from which *Chloroflexi* made a large contribution. In well-remediated bauxite residue, its abundance was quite low and therefore showed no significant correlation with residue properties.

## 5. Conclusion

This study has revealed the dynamic development of diversity and structure in microbial communities following natural weathering processes at bauxite residue disposal areas. Alkalinity and salinity decreased, whilst nutrient elements improved at the old weathered site. Both microbial diversity index and microbial community structure differed significantly following long-term residue weathering. The dominant phyla were *Firmicutes* and *Actinobacteria* at the unweathered site, whilst *Proteobacteria*, *Chloroflexi*, *Acidobacteria* and *Planctomycetes* dominated in the old weathered residues. Twenty-one biomarkers were found in the bauxite residue through a linear discriminant analysis (LDA) effect size (LEfSe) analysis. LEfSe analysis revealed that the biomarker changed significantly from *Firmicutes* (phyla) and *Actinobacteria* (class) in unweathered residues to *Acidobacteria* (phyla) and *Planctomycetes* (phyla) in old weathered residues. Soil microbial community composition and diversity were mainly regulated by soil nutrients (TOC, TN and AP) and pH, whilst soil nutrients were the major factors. This study has improved our understanding of microbial diversity in bauxite residue disposal areas and further studies should focus on functional gene prediction to reveal possible mechanisms of metabolic pathways of microorganisms on soil formation in bauxite residue disposal areas.

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