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454 Pyrosequencing Analysis of Bacterial Diversity Revealed by a Comparative Study of Soils from Mining Subsidence and Reclamation Areas

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Significant alteration in the microbial community can occur across reclamation areas suffering subsidence from mining. A reclamation site undergoing fertilization practices and an adjacent coal-excavated subsidence site (sites A and B, respectively) were examined to characterize the bacterial diversity using 454 high-throughput 16S rDNA sequencing. The dominant taxonomic groups in both the sites were Proteobacteria, Acidobacteria, Bacteroidetes, Betaproteobacteria, Actinobacteria, Gammaproteobacteria, Alphaproteobacteria, Deltaproteobacteria, Chloroflexi, and *Firmicutes*. However, the bacterial communities' abundance, diversity, and composition differed significantly between the sites. Site A presented higher bacterial diversity and more complex community structures than site B. The majority of sequences related to Proteobacteria, Gemmatimonadetes, Chloroflexi, Nitrospirae, Firmicutes, Betaproteobacteria, Deltaproteobacteria, and Anaerolineae were from site A; whereas those related to Actinobacteria, Planctomycetes, Bacteroidetes, Verrucomicrobia, Gammaproteobacteria, Nitriliruptoria, Alphaproteobacteria, and Phycisphaerae originated from site B. The distribution of some bacterial groups and subgroups in the two sites correlated with soil properties and vegetation due to reclamation practice. Site A exhibited enriched bacterial community, soil organic matter (SOM), and total nitrogen (TN), suggesting the presence of relatively diverse microorganisms. SOM and TN were important factors shaping the underlying microbial communities. Furthermore, the specific plant functional group (legumes) was also an important factor influencing soil microbial community composition. Thus, the effectiveness of 454 pyrosequencing in analyzing soil bacterial diversity was validated and an association between land ecological system restoration, mostly mediated by microbial communities, and an improvement in soil properties in coal-mining reclamation areas was suggested.

Keywords: 454 Pyrosequencing, bacterial diversity, mining soil reclamation, soil organic matter, total nitrogen

Introduction

A sustainable ecosystem depends on functional soil microbial communities, which act as biofertilizers and degraders of toxic substances and play an important role in nutrient cycling, plant health, and growth [26, 39]. The extent of soil microbial diversity in agricultural soils is critical for the maintenance of soil health and quality [20, 53]. Bacteria are the most abundant and diverse group of soil organisms [18]. Thus, the diversity of bacteria in agro-ecosystems is immense and critical to maintain soil quality in order to sustain productivity and ecological balance in mining areas. However, exploitation of coal resources has inevitably affected, at various degrees, land resources and

the equilibrium of the ecological environment in mining areas. Coal mining has resulted in subsided lands, destroying the ecological environment. In particular, land subsidence in a coal-mining area potentially induces remarkable variation in soil microbial communities, resulting in soil degradation and a decrease in crop productivity. Reclamation of the coal-mining subsidence area is one of the hot research topics in the field of Restoration Ecology [35, 54]. Understanding the soil bacterial diversity following surface subsidence resulting from underground coal mining is an important component in selecting reclamation practices to improve ecosystem services and soil functions [8, 10, 23, 34, 54]. The variability in reclaimed soil properties, when it can be measured, may represent indicators of choice for monitoring the impact of coal-mining subsidence on soil ecosystems. Moreover, this variability may offer data support for scientific guidance to supervise land reclamation and ecological constructions in mining areas. Despite the key role of soil biodiversity in ecological restoration, there is still a lack of information about bacterial diversity and its relationship with land reclamation following coal-mining subsidence.

In recent years, many studies have focused on the influence of fertilizer applications on soil physical properties, soil fertility, soil organic matter (SOM), and crop yield [5, 7, 13, 36, 45]. Some researchers have examined the soil microbial community changes during the land reclamation process, particularly focusing on the distributions of microbial population, biomass, enzyme activities, and soil functional diversity [24, 32, 65]. Whereas previous studies on the microbial communities in reclamation soils have greatly aided in our knowledge of the biological processes [10, 16, 46], to the best of our knowledge, they failed to consider what influences on ecological function of microbial communities are exerted by mine soil reclamation. Our understanding of soil microbial ecological functions can be broadened by further efforts to identify the bacterial community diversity associated with species abundance and structure.

Molecular microbial ecology tools, such as terminal restriction fragment length polymorphism (TRFLP), denaturing gradient gel electrophoresis (DGGE) fingerprinting, and clone library analysis, have made it possible to obtain relatively deep insights into the ecological processes and identity of microbial populations in various environments independent of cultivation [11, 17, 19, 41, 58]. With the introduction of molecular methods, it is possible to determine the characteristics of uncultured microorganisms, also known as the majority population, in environments. Nevertheless, these tools usually underestimate the overall diversity of a microbial community and are unable to detect rare species in complicated environmental samples. However, such limitations are being overcome with the ongoing advances in metagenomics and the development of new approaches for DNA sequencing. These advances can provide comprehensive insights into the biogeography of bacterial soil communities and taxa [47, 52, 64]. Pyrosequencing, a next-generation sequencing technology, has gained increasing attention as a novel tool for studying microbial diversity [29, 31, 44, 49, 50]. However, to the best of our knowledge, there has been no report on the pyrosequencing analysis of soil bacterial communities' species richness in mine reclamation areas. Furthermore, the impact of SOM or nitrogen on the overall diversity of the bacterial community has not been addressed in any study to date.

In the present study, 454 pyrosequencing analysis was employed to compare the bacterial diversity in reclamation soils with that in subsidence soils from a Chinese mining area. We hypothesized that the changes in management practices and other anthropogenic activities in the process of reclamation could impose distinct impacts on the soil microenvironments in which microorganisms exist, and that the variation in bacterial community structures could be associated with the changes in the physicochemical properties or plant species. Furthermore, we aimed to explore key edaphic factors affecting the taxonomic and metabolic diversities of soil microbial communities in the reclaimed soil ecosystem. Our study might add some new insights into the bacterial and microbial communities in Chinese mining reclamation areas.

Materials and Methods

Experimental Design and Soil Sampling

The experiment was conducted at the Liuxin mining field study area, comprising subsidence areas and nearby reclamation areas, located in the north of Xuzhou City, Jiangsu Province, East China. A north temperate zone monsoon climate dominates this area, with a mean annual rainfall of 868.6 mm, sunshine hours of 2,390 h, average frost-free period of 216 days, and mean annual air temperature of 13.8°C. The coldest month is January (lowest mean monthly temperature of −13°C) and the hottest month is July (highest mean monthly temperature of 39°C).

Soil sampling was performed at two selected sites in the Liuxin mining area representing two treatments: a mining reclamation site (A) and an adjacent subsidence site (B), from December 2011 to February 2012. Treatment of soil on the reclaimed site (A) followed fertilization reclamation practices or 12 years using organic mixed manure. The vegetation in this site was mostly leguminous, with common native plants such as soybean, vetch,

and bean. The inputs of organic amendments varied between 3 and 5 mg/ha before planting the crop (one or two times per year). Most of the organic amendments applied were fresh vegetable residues with high carbon/nitrogen ratio, composted vegetables, and animal waste. Site B, the control site, is a mining disturbed area and situated adjacent to site A. This mining subsidence land was not cultivated with any restoration project for at least the last 20 years.

Six plots were established on both the reclamation and subsidence sites. The experimental design was a completely randomized block with six replicates for each treatment, with an area of 12 × 10 m for each plot. Thirty-six soil samples were collected at a depth of 0–20 cm along an S-shaped transect of each site, using a soil corer of 3 cm diameter. After being sieved through a 2 mm mesh, samples were mixed and homogenized thoroughly, and were placed in polyethylene bags in triplicate for each treatment. Some of the samples were stored at 4°C prior to microbial analyses, while others were dried at 40°C and sieved to <2 mm for the determination of soil chemical properties.

Edaphic Properties of the Soil Samples

The SOM and soil total nitrogen (TN) were determined according to the method reported by Wilson and Sanders [61]. Total phosphorus (TP) and cation exchange capacity (CEC) were measured by using ammonium molybdate spectrophotometry [32] and the ammonium acetate method at pH 7 [33], respectively. Soil pH was determined on air-dried subsamples (sieved to <5 mm) using a glass combination electrode with a soil: water ratio of 1:2.5.

DNA Extraction and Purification

Community DNA was extracted from approximately 0.5 g of soil per sample by employing the E.Z.N.A Soil DNA Kit for soil (OMEGA, Biotek, Inc., Norcross, GA, USA), according to the manufacturer's instructions. The concentration and purification of the extracted DNA (2 μl) were determined by using agarose gel electrophoresis (1%) and microspectrophotometry (NanoDrop-2000; NanDrop Technologies, Wilmington, DE, USA). Finally, extracted DNAs were diluted to 2 ng/μl concentration for samples of site A and 2.2 ng/μl for site B prior to PCR amplification. All DNA samples were diluted to equivalent 0.5 ng/μl concentrations for a 20 μl PCR.

PCR Amplification and Pyrosequencing

Bacterial 16S rRNA genes were amplified using the forward primer 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and the reverse primer 533R (5′-TTACCGCGGCTGCTGGCAC-3′) [6]. Products were confirmed by subjecting 2 μl of each sample to electrophoresis on a 2% agarose gel. The PCRs were carried out in triplicate with 20 μl of the reaction mixture comprising 4 μl of 5-fold FastPfu buffer, 2 μl of 2.5 mM dNTPs, 5 μM of each primer, 0.4 μl of diluted DNA sample, 0.4 μl of TransStart FastPfu DNA Polymerase, and approximately 10 ng of DNA template by using the PCR Gene Amp 9700 (Applied Biosystems, Foster City, CA, USA). The amplification program consisted of an initial denaturation step at 95°C for 2 min, followed by 25 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, and elongation at 72°C for 30 sec, with a final extension step at 72°C for 5 min. The replicate PCR products of the same soil treating group were assembled within a PCR tube. The PCR products were visualized on agarose gels (2% in TBE buffer) containing ethidium bromide, and purified using an Axy Prep DNA gel extraction kit (Axygen, Biotechnology, Hangzhou, China). Prior to sequencing, the DNA concentration of each PCR product was determined using a PicoGreen dsDNA Quantitation Reagent (Molecular Probes, Eugene, OR, USA), and its quality was controlled by employing a QuantiFluor-ST Real-time PCR System (Promega, Madison, WI, USA), according to the manufacturer's protocol. Following quantitation, the amplicons from each reaction mixture were pooled in equimolar ratios based on concentration and subjected to emulsion PCR to generate amplicon libraries, as recommended by 454 Life Sciences. Amplicon pyrosequencing was performed from the A-end using a 454 Roche GS FLX+ Sequencing Method Manual_XLR70 kit on a Roche Genome Sequencer GS FLX Titanium platform (Roche, NJ, USA) at Majorbio Bio-pharm Technology Co., Ltd, Shanghai, China.

Statistical and Bioinformatics Analyses

Before bioinformatics analysis, the valid reads were processed according to the following rules: Tags that did not have 100% homology to the original sample tag designation were not considered, because their quality might be questionable; short reads <200 bp in length or of low quality were removed from the pyrosequencing-derived data sets; and the primer and one of the barcode sequences used were matched, and at least a 95% match to a previously determined 16S rRNA gene sequence was considered. The quality-trimmed sequencing reads obtained from the above-mentioned procedure were confirmed using the comprehensive bioinformatics software package, seqcln and mothur (http://sourceforge.net/projects/seqclean/&http://www.mothur.org/wiki/Main_Page). The detection and removal of chimeras were processed using a new program/algorithm, UCHIME (http://drive5.com/uchime) [15]. The sequences from each operational taxonomic unit (OTU) were taxonomically assigned to a bacterial 16S rRNA Silva reference alignment using a naïve Bayesian classifier. A consensus threshold of 80% was applied with a distance of 0.03. To acquire the consensus taxonomy of these OTUs, the sequences were aligned to a 16S rRNA Silva reference alignment. The OTUs were used to calculate community diversity (Shannon and Simpson diversity indices), evenness (Shannon equitability index), and richness (abundance-based coverage estimator (ACE), bias-corrected Chao1) to a cutoff value of 0.03. Completeness of the sampling effort was evaluated using Good's coverage and rarefaction curves. All the analyses were performed by employing the mothur program (http://www.mothur.org). The taxonomic identities of the sequences were assigned by using the Classifier program of the RDP-II at a confidence level of 97%. The sequences obtained in this study from pyrosequencing have been

deposited in the NCBI short-read archive under Accession No. SRA091276.

SOM, TN, TP, CEC, and pH were tested for differences between soil sites and samples with the one-way analysis of variance (ANOVA). Owing to a lack of the normal distribution in the data, the nonparametric Mann-Whitney test was used to evaluate differences in abundances of phyla at a confidence level of 95%. All statistical analyses were performed with SPSS BASE ver. 11.5 statistical software (SPSS, Chicago, IL, USA).

Results and Discussion

General Characteristics of the Soil Samples

In this study, the compositions of soil bacterial communities present in two different soil systems derived from a reclamation site (site A) and a coal-mining subsidence site (site B) were assessed and compared using large-scale pyrosequencing-based analysis of 16S rRNA gene sequences. The two analyzed soil groups showed obvious differences with respect to edaphic properties such as SOM, TN, TP, and CEC (Table 1). Most of the soil properties, listed in Table 1, were generally higher in site A compared with site B. In general, the SOM content of the soils of site A was 2-fold higher (0.03 g/kg) than that of the soils of site B (0.01 g/kg). Similarly, the TP concentration and CEC of the soils of site A were, on average, greater than those of the soils of site B, and the amount of TN was higher by 0.7 g/kg in the soils of site A (mean 1.15 g/kg in site A vs. 0.45 g/kg in site B). However, no significant differences were found in the soil pH between both the sites.

Long-term application of organic manure can significantly increase soil TN, SOM, and TP contents, and thus promote soil nutrient status by providing sufficient carbon and nitrogen sources. Reclamation practices, such as organic manure treatments and application of organic amendments, have been found to help modify the soil physicochemical and biochemical properties in the long term [66]. It has also been reported that legumes enhance soil quality through the significant effect of nitrogen fixation by nitrogen-fixing bacteria and, consequently, alter the soil fertility and ecological functions [22]. The results obtained showed that organic amendment treatment and vegetation are effective in improving the quality of coal reject reclaimed farmland.

Pyrosequencing-Derived Data Set

Across all the samples, 24,512 quality sequences were recovered with a read length of ≥200 bp. After applying quality control and trimming, a total of 10,450 and 10,103 high-quality and effective bacterial 16S rRNA gene sequences were obtained from the samples collected from sites A and B, respectively, accounting for 83.8% of the total reads. The average read length of the trimmed sequences was ~474 bp. The Good's Coverage estimator revealed that 83.4% (site A) and 92.8% (site B) of the estimated taxonomic richness were covered by the sequencing effort, respectively.

Pyrosequencing is a promising new tool that will expand our understanding of the microbial community structure of soils quickly and more comprehensively than other molecular approaches. However, to the best of our knowledge, this was the first study using pyrosequencing analysis to characterize the microbial communities in land reclamation areas. Although the identification of bacterial taxa at the finest level of taxonomic resolution by applying large-scale pyrosequencing is currently not feasible, technological advancement will reduce this limitation in the near future. Thus, pyrosequencing could be a powerful tool to elucidate the microbial diversity in reclaimed soil ecosystems.

Bacterial Richness and Diversity Indices

Libraries of sites A and B were composed of 2,997 and 1433 OTUs, respectively. Rarefaction curves were generated at 3% cutoff to make a comparison of species richness between the two soil groups. None of the curves approached a plateau, indicating that further sequencing would have resulted in more OTUs in each sample group.

As shown in Fig. 1, samples from site A displayed relatively higher species richness than those from site B. The nonparametric analysis showed similar comparative values with regard to the number of OTUs for the two sample groups, with higher level of diversity observed for samples from site A (Fig. 2). When the genetic distances

Table 1. Mean values (±standard deviation) of soil parameters for soil samples from the mining reclamation site (A) and subsidence site (B).

Sample sites	SOM (g/kg)	TP (g/kg)	TN (g/kg)	pH	CEC (cmol/kg)
A	0.03 ± 0.39	0.91 ± 0.06	1.15 ± 0.47	7.90 ± 0.13	0.24 ± 0.04
B	0.01 ± 0.63	0.70 ± 0.17	0.45 ± 0.34	8.00 ± 0.08	0.19 ± 0.02

SOM, soil organic matter; TP, total phosphorus; TN, total nitrogen ;AP, available phosphorus; CEC, cation exchange capacity.

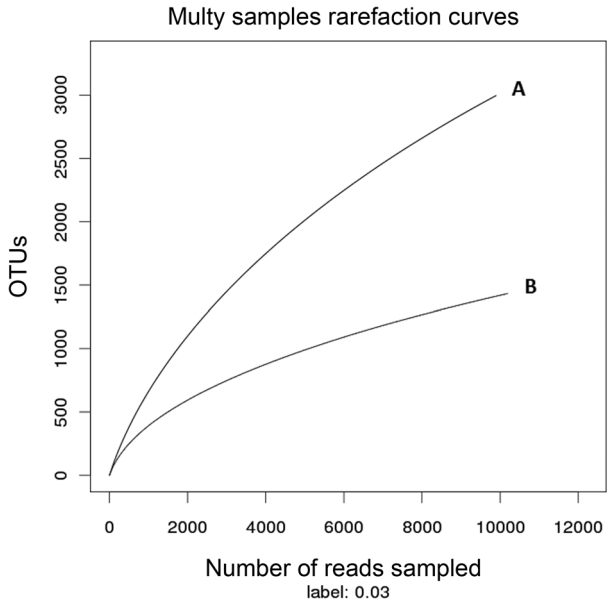

Fig. 1. Rarefaction curves showing the observed number of operational taxonomic units (OTUs) at 3% dissimilarity for samples from sites A and B.

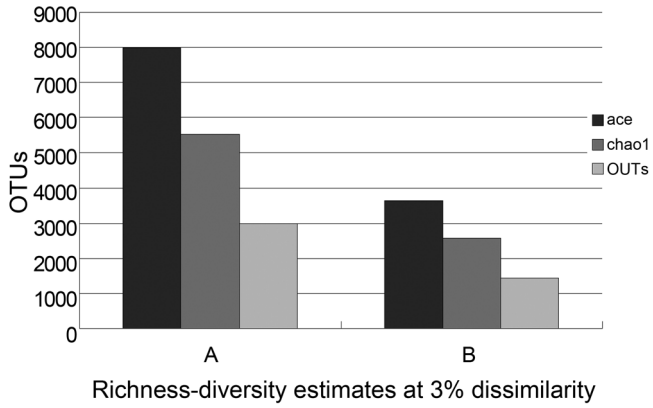

Fig. 2. Bacterial richness indices of aggregates and whole soil at a genetic distance of 3%, expressed as the number of observed unique OTUs, ACE, and Chao1.

were 0.03, the average value of ACE for samples from site B was 3,643, whereas that for samples from site A was 7,992. Both the Chao1 and Shannon-Wiener diversity indices revealed similar trends, with higher values observed for samples from site A, when compared with those from site B, confirming the higher microbial richness and biodiversity in site A.

Several studies [38, 49, 61, 63] have shown that the number of analyzed sequences per sample has an effect on the predicted number of OTUs. In general, fewer sequences of samples from site B resulted in a lower curve progression and a lower number of predicted OTUs. Pyrosequencing analysis of environmental samples can obtain much more sequences and OTUs than conventional cloning and sequencing methods [1]. Thus, the higher OTUs and Shannon indices observed in this study demonstrated the usefulness of pyrosequencing analysis in elucidating the bacterial community structure and, thus, observing high soil bacterial diversity in samples from the mining reclamation soils.

Effect of Soil Properties on Bacterial Diversity

A difference in the bacterial diversity was observed between the two sites, A and B, suggesting that these two soil systems are quite different with diverse soil properties. The data obtained clearly demonstrated that the population of bacteria isolated from soils of site B had lower biodiversity, when compared with that from soils of site A. The differences in the soil bacterial diversity might be explained, to a large extent, by the differences in the concentration of SOM and TN. Previous studies have shown that SOM, TN, vegetation, and tillage are important regulators of soil microbial community composition and activities [9, 30, 56].

Most of the microbiological processes take place in SOM, because it is a reservoir of carbon and nitrogen sources, and acts as a water absorbent and binding agent for microbial biofilms and soil aggregates [59]. Successive decomposition of SOM produces diverse substrates for microbiota, thus helping to generate high species richness. The results of our present study showed that soil reclamation under long-term fertilization management increased SOM and TN (see Table 1), and that site A with higher SOM and TN, in contrast to site B, exhibited higher predicted diversity, illustrating that the bacterial diversity at a genetic distance of 3% was strongly related to the SOM and nitrogen contents as well as CEC. Hence, the application of organic manure may improve soil structure and function, enhance the water and nutrient supplying capacity, and thus promote the microbial population, favoring microbial richness and abundance. Similar to our findings, several studies have documented that organic mixed fertilizers increased the microbial biomass and microbial diversity, affirming that the organic amendment application resulted in significant increases ($p < 0.05$) in richness and Shannon-Weaver index, when compared with the unamended plots [5, 12, 21]. It is therefore reasonable to surmise that the high soil microorganism diversity observed in site A might be

driven by its high content of SOM or TN, which could be attributed to the effect of land rehabilitation with fertilizers and organic materials to restore soil fertility and modify soil physicochemical properties.

High Bacterial Diversity of Reclamation Soils Is Affected by Vegetation

Previous studies have indicated that the type of vegetation is another factor that may affect soil microbial communities associated with carbon and nitrogen cycling in the soils [51, 55]. Hence, the presence of a particular functional group, that is, legumes in site A examined in the present study, could have probably been responsible for the stimulation of microbial community size and function. As shown earlier, the bacterial diversity was higher in site A beneath the legumes than in site B, which may be associated with improvement in SOM or nutrient resources for soil microbial communities by aboveground plants such as soybean, vetch, and bean. It has been demonstrated that legumes produce highly diverse litter and, consequently, cause the formation of a species-rich microbial community by increasing the SOM and decreasing the carbon/nitrogen ratio, which lead to uniform utilization of the substrates [4]. Similarly, earlier studies have found that microbial biomass, respiration, and catabolic activity could be stimulated in the presence of legumes [27, 55, 60]. In the mining area north of China, where there is a threat from ecosystem degradation, further research is required to clarify the relationship between vegetation and soil microorganisms, which may give insight into the process of restoration and ecosystem management.

Differences in Bacterial Community Composition Between Mining Reclamation and Subsidence Soils

The 20,084 classifiable sequences were affiliated with 23 phyla across the entire data set. The major phylum groups were those with a relative abundance of >2%. Fig. 3 shows the phylum compositions of the two soil groups. Site A was mainly composed of Proteobacteria (24.57%), Gemmatimonadetes (8.49%), Chloroflexi (7.59%), Actinobacteria (7.08%), Planctomycetes (5.83%), Nitrospirae (5.17%), and Firmicutes (3.82%). Site B mostly comprised Proteobacteria (48.23%), Actinobacteria (13.52%), Planctomycetes (9.9%), Bacteroidetes (5.32%), and Verrucomicrobia (1.24%). Notably, Proteobacteria contributed to the majority of the community composition of both soil groups, indicating that sequences affiliated with Proteobacteria contributed to a higher

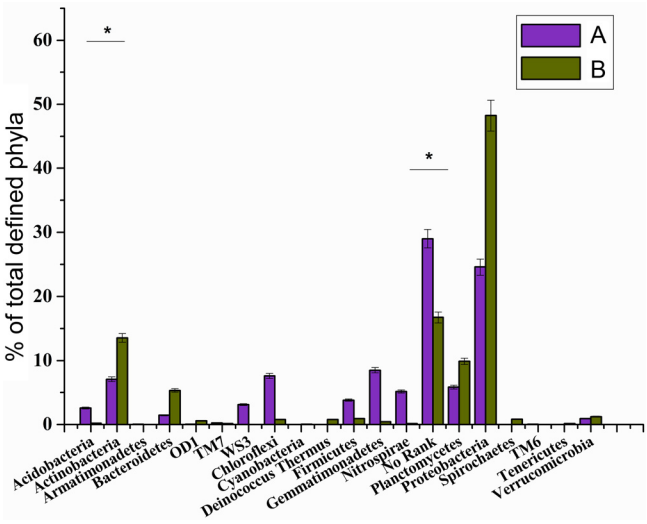

Fig. 3. Relative abundance of major bacterial phyla for each soil library, identified by pyrosequencing analysis of 16S rRNA gene amplicons.

Sequences not classified to any known phylum are included as No Rank. Bars show taxonomic assignments of 16S rRNA sequences that could be classified to the phylum level using the RDP-II Classifier tool with an 80% confidence level. Relative fractions of the most abundant phyla are indicated. Asterisks indicate significant differences in the relative abundance of groups in the A versus B sites using the Mann-Whitney test at a confidence level of 95% (*).

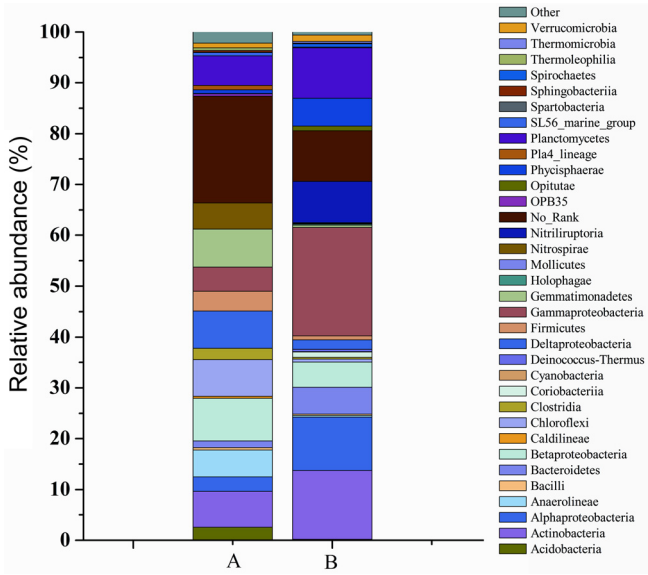

Fig. 4. 16S rRNA gene surveys revealed a clear distinction between the two bacterial class populations investigated.

Comparison of the percentage of sequences (relative abundances) affiliated with different classes from sites A and B. Phylogenetic groups accounting for ≤0.1% of all classified sequences are summarized in the artificial group 'Other'. Sequences not classified to any known class are included as 'No Rank'.

percentage of community DNA.

Furthermore, the differences among the samples at the class level of taxonomic classifications were evident (Fig. 4). Gemmatimonadetes, Betaproteobacteria, Deltaproteobacteria, Anaerolineae, and Nitrospira were the most dominant classes in site A, accounting for 8.49%, 8.36%, 7.3%, 5.28%, and 5.17% of the total effective bacterial sequences, respectively. In site B, Gammaproteobacteria, Alphaproteobacteria, Nitriliruptoria, and Phycisphaerae were predominant, accounting for 26.28%, 13.54%, 8.1%, and 5.63% of the total effective bacterial sequences, respectively. Moreover, the two sites exhibited marked difference in the abundances of the classes Alphaproteobacteria, Gammaproteobacteria, and Betaproteobacteria of the most dominant phylum, Proteobacteria.

A thorough investigation at the genus level showed an enrichment trend of beneficial bacterial groups in the soil. The relative abundance of any genus was <6% in each sample, implying high bacterial diversity in the two sample groups. Among the top 16 predominant genera in the soils evaluated, the following preponderant bacterial genera were common in the two sites (Fig. 5): Anaerolineaceae, *Chloracidobacterium*, SM1A02, Planctomycetaceae, *Nitrospira*, *Lysobacter*, *Gemmatimonas*, Gemmatimonadaceae, and *Gemmata*. However, the compositions of the bacterial community and the distributions of the common nine genera varied between samples from sites A and B.

Subsequently, the respective abundances of the 16 most-represented genera in the samples from the two sites were examined (Fig. 5). The majority of the sequences affiliated with *Nitrospira* (5.15%), *Gemmatimonas* (4.63%), *Chloracidobacterium* (1.83%), Planctomycetaceae (1.53%), *Haliangium* (1.7%), Gemmatimonadaceae (1.51%), *Geobacter* (1.09%), *Gemmata* (0.94%), *Flexibacter* (0.68%), and *Roseiflexus* (0.47%) were derived from site A, whereas those related to *Nitriliruptor* (7.94%), SM1A02 (2.77%), Planctomycetaceae (1.99%), *Lysobacter* (1.33%), Coriobacteriaceae (1.02%), and *Truepera* (0.78%) were obtained from site B.

Impact of Soil Properties on the Relative Abundances of Bacterial Taxa

The fertilization management-induced shifts in soil microbial communities that were linked to soil functioning changes between the two sites were also explored. Our results suggested that the significant difference in the abundances of the 16 most-represented genera between the two sites might also be due to the dissimilar concentrations of SOM and TN. He *et al*. [23] reported a significant impact from 8 years of reclamation and cultivation on the structure and taxonomic composition, and TN and CEC were noted to be potentially important factors for soil microbial composition and function. Furthermore, other studies indicated that the long-term effect of reclamation was essential for improving soil TN content and soil fertility, which are the major factors responsible for shifts in soil community structures [57]. In general, the shifts in bacterial community structure under different environments may not only be ascribed to some significant changes in the soil properties, but also to soil fertility and TN change caused by reclamation measure amendment.

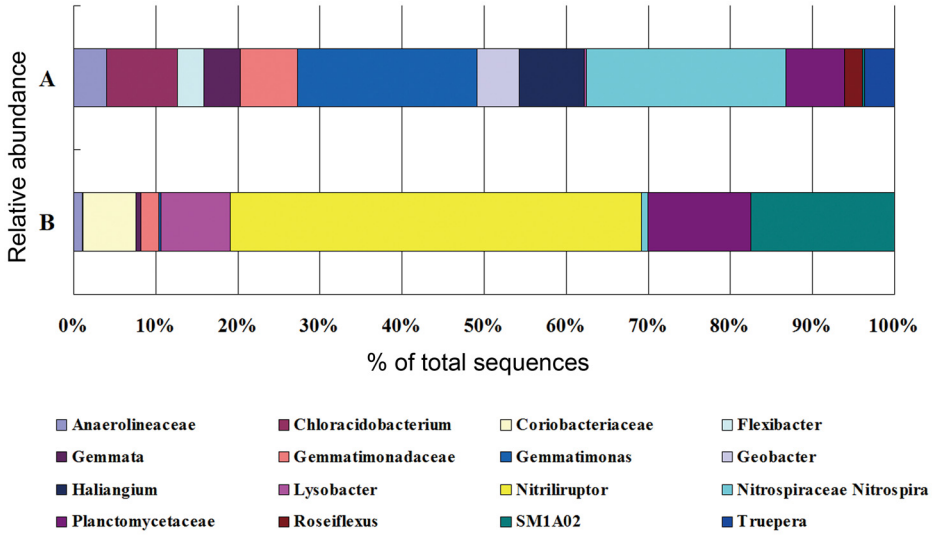

Fig. 5. Comparison of the overall distribution of the selected 16 predominant genera within sites A and B.

However, information about the effects of SOM and TN on bacterial community at the genus level in mining areas is still limited. In the present study, the composition of selected genera was found to significantly differ between the two sites. For example, microorganisms of the genus *Nitrospira* were commonly observed in the samples from site A, and they might have roles in soil nitrogen cycling and soil ecological function. As nitrifying bacteria play an important role in the soil nitrogen cycle, a positive correlation between *Nitrospira* and microbial biomass nitrogen may support their ecological relevance to soil nitrogen cycling [40]. Therefore, the higher abundance of *Nitrospira* spp. in site A may be associated with higher nutrient turnover and SOM content found in legume-covered reclamation soils, when compared with those in subsidence soils.

Remarkably, the distribution of the genera *Nitriliruptor*, Coriobacteriaceae, *Haliangium*, *Flexibacter*, *Truepera*, *Roseiflexus*, and *Geobacter* occurred almost exclusively in site A or B. This may be due to the alteration in edaphic factors following different management practices, which made the soil environment unsuitable for some species to survive and grow. The bacterial genera unique in site A were *Nitriliruptor*, Coriobacteriaceae, *Roseiflexus*, and *Truepera*; whereas those unique in site B were *Haliangium*, *Flexibacter*, and *Geobacter*. Moreover, the occurrence of *Nitriliruptor* and *Truepera* exclusively in site B may be of ecological significance. This trend emphasizes the ability of these types of bacteria to survive under conditions of very low substrate availability and a lack of plant rhizosphere. Furthermore, it is also possible that these bacteria have the ability to survive under extreme environmental changes as a result of mining. Thus, the soil bacterial communities under the influence of environmental changes might gradually be replaced by another community composed of different species that can survive better in the new conditions. However, not much information could be obtained about *Nitriliruptor* and *Truepera* from a literature review.

The present study revealed that soil properties are linked to the structures of bacterial communities that are known to mediate a number of biogeochemical processes in mining soils. The interaction between plants, soil, and microorganisms is the driver of ecosystem functions, and any modification of this relationship might affect the microbial structure, which, in turn, may influence the ecological processes. Despite its limitation, the current study might provide a better understanding of the importance of these critical factors in promoting soil microbial activity and thus boost the reclamation of mining areas.

Changes in the process of reclamation were found to induce variation in edaphic environmental conditions, with distinct impacts on the composition and diversity of soil bacterial communities. The results obtained in the present study suggest that the reclamation of mining subsidence soils enhances the level and variability of the soil community and diversity, when compared with subsided soils without management. The increases in SOM and TN concentration were indicative of the positive effects of fertilizer application on the soil structure, soil diversity, and microenvironment. The specific plant functional group such as legumes was also an important factor influencing the soil microbial diversity. Thus, it can be concluded that the study of soil attributes in mining areas is important as a part of post-construction monitoring of mining reclamation to track their functional development better, to achieve not only structural, but functional success of land-use sustainability. More efforts are necessary to elucidate the effect of nutrients and carbon substrates on soil bacterial community evolution in mining areas.

Ecological Function of the Dominant Phylum, Proteobacteria

In this study, Proteobacteria was the most abundant division, comprising approximately 24.57% community DNA of reclaimed soils and 48.23% of subsided soils, respectively. This finding is in accordance with other studies on bacterial communities in soils [25, 28, 49], in which the most dominant community was noted to be Proteobacteria. These authors concluded that Proteobacteria can represent 25–40% of total sequences by clone library studies or 42–50% abundance from shorter fragments (~100 bp) obtained by pyrosequencing. Liebner *et al*. [33] summarized that, based on more than 30 clone libraries and other studies [14,25], α-, β-, and γ-Proteobacteria, Actinobacteria, Acidobacteria, and to a lesser extent Firmicutes, Bacteroidetes, and Plantcomycetes have been identified as major soil phyla, although recognizing that their relative abundances vary with the study site. Thus, based on their abundance and presence in various soil types, Proteobacteria appear to play an important role in the ecosystem function of soils.

Proteobacteria was largely composed of subphyla Gammaproteobacteria (31.04%), Alphaproteobacteria (16.39%), Betaproteobacteria (13.34%), and Deltaproteobacteria (9.16%). Our results showed that most proteobacterial clones belong to members of photosynthetic and nitrogen-fixing bacteria. The bacterial community of those functional species played an important role in C, N cycles and in maintaining integrity of the ecosystem. In this study, photosynthetic bacteria, such as *Rhodobacter* or Rhodospirillaceae, are

mostly distributed in Alpha- and Betaproteobacteria. They play an irreplaceable role in the carbon cycle and material transformation, and their diversities directly affect the processes of the nitrogen cycle and agricultural production. We also discovered the specific bacteria Rhizobia (Rhizobiales) below the α-subclass of Proteobacteria, a major contributor to the global nitrogen cycle. Rhizobia can form a symbiotic nitrogen fixation with many plants in the soil system, improve soil fertility, promote the benign circulation of soil materials, and increase soil microbial activities [3]. It has been pointed out that native nitrogen-fixing plants management combined with nitrogen-fixing microbes vaccination could be an effective measure for the recovery of a degraded ecosystem [37]. During the process of reclamation practices and re-vegetation in the Liuxin mining area, the success of legumes can thus largely be attributed to their ability to form a nitrogen-fixing symbiosis with Rhizobia. Futhermore, heavy metal contaminations existed in reclaimed lands by filling mining wastes and fly ash into subsided lands. Because of the action of absorbing, oxidizing, decomposing, and reducing pollutants, microbes had significant contribution on ecological health and soil purification [67]. For example, Pseudomonas/Pseudomonadaceae (γ-subclass of *Proteobacteria*) observed here played an important role in purifying the contaminated mine soil because of its heavy metals detoxification mechanism on arsenic, ferrum, and manganese [2, 67].

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