

Effects of Long-Term Discharge of Acid Mine Drainage from Abandoned Coal Mines on Soil Microorganisms: Microbial Community Structure, Interaction Patterns and Metabolic Functions

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Abstract

More than twenty abandoned coal mines in the Yudong River basin of Guizhou Province have discharged acid mine drainage (AMD) for a long time. The revelation of microbial community composition, interaction patterns and metabolic functions can contribute to the ecological remediation of AMD pollution. In this study, reference and contaminated soils were collected along the AMD flow path for high-throughput sequencing. Results showed that the long-term AMD pollution promoted the evolution of *γ-Proteobacteria*, and the acidophilic iron-oxidizing bacteria *Ferrovum* (relative abundance of 15.50%) and iron-reducing bacteria *Metallibacterium* (9.87%) belonging to this class became the dominant genera. Co-occurrence analysis revealed that the proportion of positive correlations among bacteria increased from 51.02% (reference soil) to 75.16% (contaminated soil), suggesting that acidic pollution promotes the formation of mutualistic interaction networks of microorganisms. Metabolic function prediction (Tax4Fun) revealed that AMD contamination enhanced the microbial functions such as translation, repair, and biosynthesis of peptidoglycan and lipopolysaccharide etc., which may be an adaptive mechanism for microbial survival in extremely acidic environment. In addition, the acidic pollution promoted the high expression of nitrogen fixing genes in soil, and the discovery of autotrophic nitrogen fixing bacteria such as *Ferrovum* provided the possibility of bioremediation of AMD pollution.

Introduction

The total number of coal mines in China has been decreased from more than 80,000 (Feng et al. 2016; Xu et al. 2016) to around 4,700 (by the end of 2020) under the guidance of a series of national policies. Among all coal mines in China, 90% belong to underground coal mines. Large amounts of groundwater rebound after coal mine abandonment, making the mining area a collection of contaminants (Chen et al. 2019). For high sulfur abandoned coal mines, associated FeS_2 in coal seams and gangue was oxidized by microbial catalysis, producing large amounts of acid mine drainage (AMD) (Sharma et al. 2020). AMD is considered to be one of the major environmental issues in mining industry. AMD is typically characterized by low pH (usually < 3.0) and high levels of iron, sulfate and other toxic heavy metals (Akcil and Koldas 2005). Therefore, if not treated properly, AMD can contaminate soil, groundwater and surface water, etc., posing a major threat to humans and the environment (Jin et al. 2020; Rambabu et al. 2020). Due to the lack of effective management measures, AMD from abandoned coal mines was usually discharged directly into the environment without any treatment, posing a higher ecological risk compared to producing mines. China currently has a large number of abandoned coal mines, and the associated safety and environmental problems need more attention.

Microorganisms are an important part of the soil system and play a significant role in the transformation and cycling of elements. Soil microbial community structure and diversity are highly influenced by organic matter, temperature, salinity, pH, and heavy metal etc. (Zhao et al. 2014). AMD pollution can alter the structure of ecosystems by changing the physicochemical properties of the soil and the composition of the microbial community (Sun et al. 2016). AMD is typically characterized by excessive dissolved solids, high mineralization, strong acidity and extremely low pH, which can be a harsh environment for most living organisms. As a result, only acidophilic and metal-tolerant microorganisms can survive, including *Proteobacteria*, *Firmicutes*, *Acitinobacteria*, *Nitrospirae*, and *Acidobacteria* etc. (Baker and Banfield 2003). Microorganisms typically found in AMD environments have novel cellular mechanisms and specific elemental metabolism to better adapt to harsh conditions (Johnson and Hallberg 2005). For example, iron- and sulfur-metabolizing bacteria showed high abundance in samples contaminated by abandoned coal mine drainage (Weimin et al. 2015). These

microorganisms with special metabolic functions in the AMD environment can be candidates for bioremediation of contaminated sites (Johnson and Hallberg 2005). Therefore, an in-depth understanding of microbial communities, co-occurrence interaction patterns and metabolic functions in AMD-contaminated environments is beneficial for the screening of microorganisms with specific metabolic functions and provides a basis for better bioremediation of contaminated environments.

AMD pollution has been reported worldwide. However, due to the wide variation in geochemical conditions of different acid mine wastewaters, it is essential to select microorganisms suitable for bioremediation in AMD-contaminated environments. Multiple AMD overflow sites from abandoned coal mines were identified during our preliminary investigation. A large amount of yellow sediment has been deposited on the surface soil in the area of AMD runoff from abandoned coal mines, which severely limits the function of the soil and the development of biological communities. Microbial remediation provides a better way to carry out the restoration of ecological functions of contaminated soils, which requires a clear understanding of microorganisms in AMD-contaminated environments first. Therefore, we collected soils from contaminated areas of four abandoned coal mines, along with reference soils as controls, and analyzed the microbial communities, co-occurrence patterns and metabolic functions of the soils, respectively. The objectives of this study were to 1) reveal the typical microbial communities in long-term AMD-contaminated soils from abandoned coal mines; 2) reveal the effects of AMD contamination on the co-occurrence patterns and interaction relationships of soil microorganisms; and 3) explore the special metabolic functions of microorganisms in AMD-contaminated environments.

Materials And Methods

Sites Description and Sample collection

The study area is located in the Yudong River basin, Kaili City, southwestern Guizhou Province. Local coal mining started in the 1980s, and most of the mines have been closed now. Its coal-bearing rock system is the Middle Permian Liangshan Group, which is a high-sulfur, medium-high-ash fatty coal. The sulfur (st,d) content of the coal ranges from 0.90–16.65% (generally >3%), with sulfide content ranging from 0.80–4.60%, mostly in spherical particles, stellately distributed in the coal seam. For abandoned coal mines, acid mine water is formed when atmospheric precipitation seeps into the shaft or groundwater bounces into the mine, and sulfide minerals in the coal seams and surrounding rocks are oxidized. When the groundwater level exceeds the surface, AMD will overflow into the surface environment along the coal mining channel, causing serious environmental pollution (Liang et al. 2019).

We collected 13 topsoil samples (sampling depth 0–5 cm), of which eight contaminated soils (group M, M1-M8) were collected from the AMD runoff areas of four abandoned coal mines and five reference soils (group S, S1-S5) were collected from areas more than 500 m away from the AMD runoff areas (Fig. 1). Each sample was a mixture of four subsamples collected from adjacent points, homogenized and divided into two parts. Soil samples for physicochemical index testing were stored at 4°C until processing, and the fraction used for DNA extraction was stored at 4°C after field collection and –80°C after being sent to the laboratory until analysis.

Physicochemical parameter measurements

Soil samples were freeze-dried using a freeze-dryer (FD-1A-50, BioCool, Beijing, China) for 48 h. 10.0 g of dry samples sieved through a 2 mm mesh were mixed with 25 ml of distilled water (1:2.5 soil-water ratio) for testing

soil pH. After shaking for 2 min the mixture was left to stand for 30 min, and then measured using a calibrated pH meter (PXSJ-216F, Shanghai, China). For samples with pH < 7, KCl solution (1 mol/L) was used instead of distilled water for testing (ISO 10390:2005) (Asta et al. 2014). Total iron in the soil was measured according to the method reported (Sun et al. 2018a). And 0.2 g dry sample sieved through a 0.15 mm mesh was digested in a microwave digestion bath, and then heavy metals Zn, Mn, Cu, Cr, Pb, and Cd were determined by inductively coupled plasma mass spectrometry (ICP-MS, TFS, USA) (Lu et al. 2012). After sample digestion, the samples were analyzed for Hg and As by atomic fluorescence spectrometry (AFS8130, Titan Instruments, Beijing, China) (Jiang et al. 2020). All measurements were performed in triplicate.

Illumina MiSeq sequencing and processing

Total soil DNA was extracted from 1.0g sample using E.Z.N.A.® Soil DNA Kit (Omega Bio-tek, Norcross, GA, U.S.). The concentration and quality of the extracted DNA were assessed using a Nanodrop®ND-2000 UV-Vis Spectrophotometer (NanoDrop Technologies, USA) for evaluation. And bacterial universal primers 338F and 806R for the V3-V4 region of the 16S rRNA gene were used to generate an amplification library (Lee et al. 2012). PCR products were extracted using 2% agarose gel, further purified by AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA), and quantified using QuantiFluor™-ST (Promega, USA) (Wang et al. 2018). Sequencing of the purified amplicons (300 bp paired) was performed on the Illumina MiSeq platform (Illumina, San Diego, USA) from Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China).

Sequences from the Illumina MiSeq platform were processed using the QIIME (v 1.9.1) package (Campbell et al. 2010). Raw fastq files were demultiplexed, quality controlled with Trimmomatic filtering, and merged by FLASH (v 1.2.11) (Magoč and Salzberg 2011). Clustering of Operational taxonomic units (OTUs) was performed according to the RDP Classifier algorithm (v2.11) against the SILVA database with a 97% similarity control (Elmar et al. 2007).

Statistical analyses

The diversity of bacterial communities was assessed using the alpha diversity indices OTUs (Sobs), Chao (Chao 1984), Shannon (Shannon and Weaver 1950) and Simpson. The interactions between microbial populations in the samples were studied by co-occurrence network analysis with Networkx software. Interactions between “microbe-microbe” were calculated using Spearman correlation coefficients (Gao et al. 2019). Positive and negative correlations may indicate mutualistic and competition relationship between microorganisms, respectively. Phylogenetic tree was constructed based on the maximum likelihood method (ML) (Wim and Olivier 2005) and IQ-tree (v1.6.8) for the top OTUs. The above data was analyzed on the free online platform of Majorbio Cloud Platform (www.majorbio.com).

The potential functions of the soil microbial community were profiled using Tax4Fun (v 0.3.1) (Aßhauer et al. 2015). Differences in the level 2 predicted functions of contaminated and reference soils were compared and analyzed by Wilcoxon rank-sum test. The main level 3 predictive functions of contaminated and reference soils were compared and clustered using an R-language based heat map. Bubble plot and heat map were plotted in an online platform for data analysis and visualization (<http://www.bioinformatics.com.cn>).

Results And Discussion

Physicochemical properties of soil samples

The physicochemical parameters of the soil samples are shown in Table 1. The local soils, mainly from long-term weathering of limestone, are weakly alkaline with a pH range of 7.04 to 8.22. The pH of the soils decreased significantly after AMD contamination, changing from weakly alkaline to acidic with a pH range of 2.53 to 6.79. According to the classification criteria of soil pH (Xiong and Li, 1990), most of the samples (6 out of 8) in the contaminated group were extremely acidic soils (pH < 4.5). This indicates that long-term scouring and erosion of AMD from abandoned coal mines has led to severe acidification and contamination of soils in the runoff area (Zhang et al. 2013a). Statistical analysis showed that the pH of the contaminated soil was significantly lower ($p < 0.001$) compared to the reference group, while the total iron content (average 321.80 g/kg) was significantly higher, showing the physicochemical characteristics of typical AMD contamination (Gao et al. 2019). The high total iron content in the contaminated soil may be due to the high concentration of iron ions in AMD, which generates secondary minerals such as Jarosite, Schwertmannite, Goethite deposited on the surface soil under the combined action of oxygen and microorganisms (Bao 2018).

Table 1
Physicochemical properties of samples

Samples	pH	Fe	Zn	Mn	Cu	Cr	Pb	As	Cd	Hg
		g/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg
M1	2.75	257.60	23.80	69.30	5.35	68.30	9.96	36.00	0.10	0.02
M2	6.79	82.30	96.90	358.00	25.10	132.00	58.40	21.80	0.96	0.23
M3	4.03	105.00	84.00	388.00	23.10	99.60	55.40	25.10	0.59	0.20
M4	2.64	453.70	55.00	41.30	11.60	63.50	29.70	21.30	0.31	0.08
M5	3.48	457.90	47.90	57.10	10.20	57.20	24.30	19.20	0.31	0.06
M6	5.88	114.10	106.00	105.00	20.50	74.80	37.00	18.90	1.72	0.24
M7	2.53	540.80	17.70	18.90	5.36	56.20	17.00	42.60	0.10	0.01
M8	2.64	563.00	19.20	16.00	4.96	53.10	13.60	40.80	0.09	0.02
S1	7.04	34.09	112.00	1586.00	30.10	290.00	30.20	16.50	1.80	0.28
S2	8.08	21.63	97.80	280.00	29.00	154.00	18.00	8.68	0.89	0.38
S3	7.74	37.66	70.20	274.00	18.20	139.00	32.00	12.50	1.17	0.16
S4	7.55	36.33	55.70	415.00	16.90	158.00	28.20	13.40	1.00	0.20
S5	8.22	26.67	104.00	510.00	27.10	134.00	21.20	12.60	2.16	0.28

Sequencing results and microbial diversity

A total of 547,649 effective reads were identified in 13 sequencing libraries with an average length of 430 bp. To make the samples comparable and avoid bias due to differences in sequencing depth, we drew the sequences flat to the same sequencing depth based on the sample with the minimum sequence reads (18,528). The number of OTUs obtained for each sample and the alpha diversity indices are shown in Table 2. Based on the 97% similarity, all sequences were finally clustered into 4,224 Operational Taxonomic Units (OTUs), ranging from 153 to 1961. The abundance of soil microorganisms decreased significantly ($p < 0.05$) after AMD contamination, with the

mean number of OTUs obtained for groups M and S being 781 and 1708, respectively. As shown in Table 2, the Alpha diversity indices Chao1, Shannon and Simpson showed similar trends, i.e., the richness and diversity of bacteria in contaminated soils were significantly reduced. Statistical analysis showed that the alpha diversity indices of the contaminated soil was significantly lower than that of the reference group ($p < 0.05$). This may be due to the fact that AMD contamination altered the physicochemical properties of the soil (Table 1), where the survival of most microorganisms was inhibited in extremely harsh environments and only some acid-tolerant microorganisms were able to grow, showing an overall reduction in bacterial richness and diversity (Zhang et al. 2019).

Table 2
Alpha diversity indices of samples

Group	Sample	Number of OTUs	Chao1	Shannon	Simpson	Coverage
Group M	M1	701	932.78	4.31	0.046	98.93%
	M2	1961	2667.55	5.94	0.013	96.36%
	M3	1487	1994.08	5.18	0.021	97.23%
	M4	362	519.76	4.15	0.030	99.41%
	M5	360	529.45	4.00	0.041	99.44%
	M6	1047	1495.82	5.32	0.015	98.08%
	M7	178	213.65	2.98	0.098	99.78%
	M8	153	205.50	2.83	0.122	99.81%
	Average	781	1069.80	4.34	0.048	98.63%
	Variance	663	900.94	1.10	0.040	1.28%
Group S	S1	1845	2274.17	6.45	0.005	97.45%
	S2	1724	2100.16	6.34	0.004	97.53%
	S3	1623	2021.72	6.16	0.007	97.59%
	S4	1823	2352.00	6.47	0.004	97.14%
	S5	1528	1988.11	6.10	0.007	97.64%
	Average	1708	2147.20	6.30	0.005	97.47%
	Variance	134	159.16	0.17	0.002	0.19%

Comparison of microbial community composition

Figure 2 shows the relative abundance of the main bacterial phyla in the samples. It can be seen that the dominant phylum in all samples was *Proteobacteria*, which accounted for 54.22% of the total bacterial reads in group M (31.91–69.66% per sample), while in the group S, it was only 29.26%. The percentage of *Proteobacteria* in the soil increased significantly after AMD contamination, which is consistent with the findings of other scholars (Gao et al. 2019). In addition, we found that although *Proteobacteria* were the most abundant phylum in both groups M and S, there were significant differences in composition at the lower taxonomic levels. At the class level,

γ-Proteobacteria were predominant in group M with an average of 47.56%, while *α-Proteobacteria* dominated in group S (19.55%). *Actinobacteriota* ranked as the second abundant phylum, accounting for 10.54% and 23.24% in Group M and Group S samples, respectively. *Actinobacteriota* are suitable for survival in neutral or slightly alkaline environments (Yang 2017), which may explain their low abundance in AMD-contaminated soil. *Chloroflexi* and *Acidobacteriota* showed the same characteristics as *Actinobacteriota*, with an average of 20.29% and 13.69% in Group S compared to only 6.25% and 4.58% in Group M, suggesting that AMD pollution may inhibit the growth of bacteria belonging to *Chloroflexi*, *Acidobacteriota* and *Actinobacteriota*.

Figure 3 shows detailed information on the relative abundance of the main genera (at least 2% in one sample). It is evident that *Ferroplasma* spp. and *Metallibacterium* spp. dominate in the group M, especially in soil samples with extremely low pH (Table 1). *Ferroplasma* can use the energy generated by ferrous iron oxidation to fix carbon through the Calvin-Benson-Bassham cycle (Hua et al. 2015), and is often considered the main carbon sequestrator within AMD microbial communities (Moya Beltran et al. 2014). *Metallibacterium* is widely distributed in environments with high concentrations of metals or sulfur (Sylvan et al. 2012), and certain species, such as *Metallibacterium scheffleri*, have the ability to reduce Fe(III) but not oxidize Fe(II) (Ziegler et al. 2013). In addition, studies have shown that some strains of *Metallibacterium* have carbon fixation and sulfur oxidation genes, suggesting their potential for chemoautotrophic growth (Sibylle et al. 2017). The other genera with relatively high abundance in contaminated soil samples also include *Acidithiobacillus*, *Acidibacter*, *Ferrithrix*, *Leptospirillum*, etc. Among them, *Acidithiobacillus* is the most studied chemoautotrophic microorganism in acidic environments, and one of the species, *Acidithiobacillus ferrooxidans*, can rapidly catalyze the oxidation of Fe^{2+} to Fe^{3+} and the dissolution of sulfide minerals, etc. (Williams and Kelly 2013). *Acidibacter* is considered acidophilic, exclusively heterotrophic bacteria with the ability to reduce Fe(III) (Falagán and Johnson 2014). *Ferrithrix* is a Gram-negative, thermotolerant heterotrophic acidophilic bacterium capable of oxidizing ferrous iron (Johnson et al. 2009). *Leptospirillum* has been reported to be responsible for AMD production (Schrenk 1998), and this group can be involved in metal and sulfur metabolism (Goltsman 2009). The species *Leptospirillum Ferriphilum* is an obligate aerobic microorganism that can live in extremely acidic environment (pH 1.5–1.8), and can only obtain energy through ferrous oxide, and some studies have shown that it is highly tolerant to arsenic (Tuffin et al. 2006). In summary, it is clear that the dominant genera in AMD-contaminated soil are acidophilic bacteria, which have the ability to metabolize iron and sulfur, especially the oxidation of ferrous iron.

AMD contamination can alter the microbial community structure of the soil by promoting the development of some acidophilic bacteria as well as inhibiting the growth of other bacteria. As shown in Fig. 3, the relative abundance of *Sphingomonas*, *Gaiella*, *Nocardioideae*, *Ramlibacter*, *Pedomicrobium* were significantly higher in the uncontaminated reference soil than in the contaminated soil. These bacteria are suitable to live in neutral environments, for example, *Gaiella* grows at an optimum pH between 6.5 and 7.5 (Albuquerque et al. 2011), and *Nocardioideae* is aerobic, pH-neutral actinomycetes in soil (Du et al., 2012). *Sphingomonas* are strictly aerobic heterotrophic bacteria widely distributed in various habitats such as aqueous and terrestrial, and plant roots (Chen et al. 2018). *Pedomicrobium* belongs to heterotrophic and aerobic bacteria with Mn oxidation ability (Larsen et al. 1999). *Ramlibacter* (Chaudhary et al. 2017), *Arthrobacter* (Jones and Keddie 2006) and *Lysobacter* (Weon et al. 2007) are all aerobic, mesophilic and neutrophilic bacteria in soil. These neutrophilic aerobic bacteria are an important part of the soil microbial system and play an important role in soil material cycling and energy metabolism.

Phylogenetic tree analysis of the main bacteria in contaminated soil

To clarify the metabolic capacity of the major bacteria in contaminated soil, we selected the top OTUs (> 0.5% relative abundance in all samples) and constructed phylogenetic trees between the known bacteria and top OTUs by comparison with the NCBI database (Fig. 4). Number in the box following each OTUs indicates their average relative abundance in the contaminated soil. Name of the known bacteria in the NCBI database were represented in italicized and underlined font. Clearly, the top OTUs in contaminated soil are similar to several known iron-oxidizing and iron-reducing bacteria.

Most of the top OTUs (17 out of 31) are more closely related to iron-oxidizing bacteria, such as *Ferroplasma myxofaciens*, *Acidithiobacillus ferrooxidans*, *Gallionella* sp., *Sideroxydans lithotrophicus*, *Leptospirillum* sp. and *Ferrithrix thermotolerans*. The relative abundance of these top OTUs with Fe oxidation capacity totaled account for 29.71% of the total library of contaminated soil. *Ferroplasma myxofaciens* was an extremely acidophilic, obligate autotroph that can use the energy of ferrous iron oxidation for endogenous nitrogen fixation as well as CO₂ fixation via the Calvin-Benson-Bassham pathway. In addition, it can produce large amounts of extracellular polymers that lead to intercellular attachment (Johnson et al. 2014). In our study, four top OTUs were related to *Ferroplasma myxofaciens*, including OTU833 (9.76%), OTU839 (4.27%), OTU796 (0.76%), and OTU450 (0.71%), with a total relative abundance of up to 15.50%, indicating the presence of considerable amounts of autotrophic bacteria with iron oxidation and nitrogen fixation abilities in contaminated soil. This can be attributed to a survival strategy of bacteria in a strongly acidic and oligonitrogenous environment, which provides carbon and nitrogen sources for cell survival through autotrophic carbon fixation and carbon sequestration (Sun et al. 2020b). OTU835 and OTU322 were identified to be similar to *Acidithiobacillus ferrooxidans*, an acidophilic chemolithoautotrophic bacterium that can grow by oxidizing Fe(II) and reducing sulfur compounds (Jin et al. 2020). Phylogenetic evolutionary tree analysis revealed that five OTUs, OTU479, OTU342, OTU542, OTU475 and OTU829, are more similar to *Gallionella* sp. and *Sideroxydans lithotrophicus*, both of which are Fe-oxidizing bacteria (Kipry et al. 2013; Anke et al. 2019). The other OTUs that can be identified to the known Fe-oxidizing bacteria included OTU287 (*Leptospirillum* spp.), OTU522 and OTU546 (*Thiobacillus thioparus*), OTU760, OTU284 and OTU141 (*Ferrithrix thermotolerans*). Among them, *Leptospirillum* spp. is considered to be an iron-oxidizing bacterium and carries genes for dissimilatory reduction of sulfate (Goltsman 2009); *Ferrithrix thermotolerans* was an extremely acidophilic, heterotrophic, and iron-oxidizing actinobacteria (Johnson et al. 2009).

Six of the 31 top OTUs were more similar to Fe-reducing bacteria, with a total relative abundance of 15.23%. Most of these OTUs with iron-reducing ability were more similar to *Metallibacterium Scheffleri*, including OTU834 (6.26%) and OTU143(3.61%), with a total relative abundance of up to 9.87%. *Metallibacterium Scheffleri* is considered to be a facultatively anaerobic and acid-tolerant microorganism with the ability to reduce Fe(III) (Ziegler et al. 2013). *Metallibacterium Scheffleri* can thrive on casitone as an electron donor and carbon source, possibly due to the production of NH₃ during the cellular metabolism of amino acids, which may be the survival strategy for *Metallibacterium Scheffleri* to be able to tolerate the extremely acidic environments (Ziegler et al. 2013). Additionally, genes for sulfur and hydrogen oxidation were found in this bacteria, suggesting a possible potential for sulfur oxidation (Sibylle et al. 2017). The other three OTUs are thought to be more similar to *Acidibacter ferrireducens*, an acidophilic, obligate heterotroph that can reduce Fe(III) (Falagán and Johnson 2014).

Changes of microbial interactions after AMD pollution

In addition to soil microbes being influenced by environmental conditions, microbial interactions, such as mutualism, commensalism and parasitism, may also play a role in the structure and composition of microbial communities (Nemergut et al. 2013). Co-occurrence networks among microorganisms can provide considerable information and have been successfully used to identify microbial interactions (Sun et al. 2016). In this study, co-occurrence networks between microorganisms were constructed separately for the main microorganisms in contaminated soil (Fig. 5(a)) and reference soil (Fig. 5(b)). Only strong relationship ($|r| > 0.8$) and significant Spearman correlation ($p < 0.05$) between main OTUs (relative abundance $> 0.2\%$ in the corresponding group samples) were visualized. The size of each key node in the figure is proportional to the relative abundance of that OTU, and the assignment of OTUs at the phylum level is indicated by different colors. The positive and negative correlations between OTUs are indicated by the red and green edges, respectively.

The number of links in the co-occurrence network can be used to assess the inter-microbial interactions, and the higher the number of links, the stronger the microbial interactions (Banerjee et al. 2018). The numbers of strong links in the reference soil and contaminated soil were 833 and 318, respectively (Fig. 5(d)), indicating that the strength of microbial interactions in the reference soil was stronger than that in the contaminated soil. This may be due to the fact that AMD contamination decreased the richness and diversity of soil microorganisms, which in turn leads to a weakening of the overall interactions between microorganisms. The interaction between microorganisms can be divided into positive and negative effects, which represent mutualistic and competition interactions, respectively. In this study, the proportion of positive and negative links in the reference soil was 51.02% and 48.98%, respectively (Fig. 5(d)), indicating that the positive and negative interactions between microorganisms were basically in balance. However, we observed a significant increase in the proportion of positive links in contaminated soils, although the overall intensity of the interaction between microorganisms decreased, with 75.16% and 24.84% of positive and negative links in contaminated soils, respectively (Fig. 5(d)). Positive correlations among microorganisms may indicate symbiosis, parasitic relationships or collective synergistic degradation of organic matter, while negative correlations may be attributed to competition among microorganisms or different living environments (Liu et al. 2020). The predominance of positive correlations in contaminated soils indicates an enhanced reciprocal symbiosis among microorganisms, which may be a survival strategy for microorganisms in extreme environments by forming mutually beneficial interaction networks for better survival in the harsh environment of strong acidity.

There was a significant difference in the assignment of key nodes in contaminated and reference soils when considering the taxonomic attribution of each node (Fig. 5(c)). The key nodes in the reference soil samples were mainly attributed to the phylum *Proteobacteria*, *Actinobacteriota*, *Acidobacteriota*, and *Chloroflexi*, accounting for 38.38%, 28.28%, 17.17% and 12.12% of the total number of nodes, respectively. Most of the key nodes in the contaminated soil also belonged to these four phyla, however, the proportion changed significantly. The percentage of key nodes belonging to *Proteobacteria* increased from 38.38–46.75%, while the proportion of the other three phyla all decreased, accounting for 12.98% (*Actinobacteriota*), 9.09% (*Acidobacteriota*) and 3.89% (*Chloroflexi*), respectively. In addition, some new nodes were displayed in the contaminated soil compared to the reference soil, mainly attributed to *Firmicutes*, *Nitrospirota* and *Patescibacteria*, accounting for 5.19%, 3.90% and 3.90% of the total number of nodes, respectively. Bacteria of the *Firmicutes* usually show a higher resistance to harsh environments, which is attributed to the high peptidoglycan content of the bacterial cell wall that can protect the cells from the damage of external environment; in addition, most bacteria of this phylum can produce

spores to preserve cellular activity (Dworkin et al. 2006). The presence of *Leptospirillum* makes *Nitrospirota* become the key nodes in the microbial network of contaminated soil. *Leptospirillum* was almost absent in the reference soil, but it showed highly abundance in AMD contaminated soil. *Leptospirillum* has been reported as an acidophilic iron-oxidizing bacterium with carbon fixation genes, as well as with multiple pathways for osmotic protection that allow it to thrive in the oligotrophic and extremely acidic environments (Goltsman 2009; Tuffin et al. 2006). *Patescibacteria* is commonly found in groundwater systems (Herrmann et al. 2019; Tian et al. 2020), and its emergence as a new critical node in contaminated soils may be related to the source of AMD in abandoned coal mines. After the abandonment of coal mines, groundwater rebounded into the mining area to form AMD, and some of the microorganisms in the groundwater will be carried into the surface environment along with the overflowing of AMD.

Co-occurrence pattern of microorganisms in contaminated soil

The previous analysis showed that the dominant bacteria in contaminated soil were mainly iron-metabolizing bacteria, and positive interactions between these microorganisms were significantly enhanced after AMD contamination. To determine the specific interactions between these microorganisms, we constructed a co-occurrence network of the main iron-metabolizing bacteria in contaminated soil at the genus level (Fig. 6). Here, yellow nodes are used to indicate iron-oxidizing bacteria, while blue nodes for iron-reducing bacteria. The size of the nodes is proportional to the number of links (degree).

As illustrated in Fig. 6, the main bacteria in the contaminated soil have different interactions with each other. The main iron-metabolizing bacteria in contaminated soil can be divided into three groups according to the interactions between microorganisms. The first group included *Acidicapsa*, *Metallibacterium*, *Acidibacter* and *Acidiphilium*, and these iron-reducing bacteria have a positive correlation with each other. Among them, *Metallibacterium* is concurrently autotrophic bacteria, and the other three genera are all heterotrophic bacteria. In addition, we also found iron-oxidizing bacteria *Ferrithrix*, *Acidithiobacillus*, *Acidibacillus* and *Ferroplasma* were positively correlated with each other. All of these four bacteria can oxidize Fe(II), but their nutritional patterns were quite different. *Ferroplasma* and *Acidithiobacillus* belong to autotrophic bacteria (Holanda et al. 2015; Schopf et al. 2017), while *Ferrithrix* and *Acidibacillus* were heterotrophic. Autotrophic and heterotrophic bacteria have obvious synergistic relationship in carbon source utilization, mutual elimination of growth and inhibition of metabolism (Baker and Banfield 2003), which may account for the positive correlation between these bacteria. *Gallionella*, *Thiobacillus*, *Sideroxydans*, *Curvibacter*, *Ferritrophicum*, *Geothrix*, and *Geobacter* formed another group of bacterial networks that are positively correlated with each other. Among them, *Geothrix* and *Geobacter* belong to iron-reducing bacteria, and the other bacteria were all iron-oxidizing bacteria.

The distribution of the above three groups of bacteria in the contaminated soil samples also showed significant differences (Fig. 3). The bacteria in the first and second groups showed higher relative abundance in the contaminated soil, and these bacteria were mainly distributed in samples M1, M3, M4, M5, M7 and M8 with low pH value (pH < 4). However, bacteria in the third group such as *Sideroxydans*, *Thiobacillus*, *Geothrix* and *Geobacter* were mainly distributed in samples M2 and M6 with pH values of 6.79 and 5.88, respectively. This indicates that microorganisms formed different interaction networks in moderately (pH range 4 to 7) and severely (pH < 4) acidic contaminated soils. Iron-oxidizing and iron-reducing bacteria formed separate networks of independent interactions in soils with pH < 4. In contrast, iron-oxidizing bacteria and iron-reducing bacteria suitable for living in a moderately acidic environment (pH value 4 ~ 7) together formed a collaborative network with a positive correlation with each other.

Predictive functional profiling of microbial community

The microbial community composition of environmental samples is closely related to environmental conditions. However, studies have demonstrated that environmental conditions are more associated with microbial community function, i.e., microbial communities in similar environments may differ significantly, while their community functions may be similar (Gibbons 2017). Therefore, in addition to revealing the composition and interaction of microbial communities in soils, it is particularly important to reveal the differences in metabolic functions of microbial communities in contaminated and reference soils. The application of metagenome is limited due to the inappropriate pretreatment of samples or prohibitive costs, and the functional prediction based on 16S rRNA sequence have become a beneficial complement (Aßhauer et al. 2015). As one of the best functional prediction methods, Tax4fun has shown high correlation with metagenomic sequencing on functional profiles (Aßhauer et al. 2015). It has been successfully applied in various habitats such as the pond sediment (Silveira et al. 2020), soil (Sun et al. 2020a), hot spring (Mehetre et al. 2018), etc. In this study, we used Tax4fun, a metabolic prediction method with SILVA as reference database (Aßhauer et al. 2015), to explore the changes of metabolic potential in soils after AMD contamination. In this method, metabolic pathways based on KEGG predictions were compared between contaminated and reference soils.

The prediction results (Table 3) showed that the predicted pathways at level 1 for both contaminated and reference soil samples were most dominant by Metabolism (61.12% and 61.80%, respectively), followed by Environmental Information Processing (18.08% and 19.88%) and Genetic Information Processing (13.88% and 10.64%). Significant differences were discovered between contaminated and reference soil samples for most of the predicted KOs (32 of 40 KOs) at the level 2 (Wilcoxon rank sum test, $p < 0.05$; Fig. 7). The proportions of Amino acid metabolism, Membrane transport, Xenobiotics biodegradation and metabolism all decreased significantly after AMD contamination, from 12.74–11.01%, 12.72–9.24%, 4.70–3.28%, respectively. However, we also discovered that some metabolic functions were enhanced after contamination, such as some Metabolism functions (Energy metabolism, Glycan biosynthesis and metabolism, Nucleotide metabolism etc.), and most of the Genetic Information Processing functions (Translation, Folding, sorting and degradation, Replication and repair) and part of the Environmental Information Processing functions (Signal transduction) (Fig. 7). The high expression of these functions may be related to the survival mechanism and adaptations of microorganisms in harsh environment. Studies have demonstrated that microorganisms in AMD environment have novel cellular mechanisms for adaptation to harsh conditions (Johnson and Hallberg 2003). Genes associated with adaptation are over expressed in AMD systems compared to normal metabolism (Ram et al. 2005).

Table 3
Predicted pathways by Tax4fun of Group M and Group S samples (level 1)

Pathway level1	Group M(%)	Group S(%)
Cellular Processes	3.99	4.91
Environmental Information Processing	18.08	19.88
Genetic Information Processing	13.88	10.64
Human Diseases	1.89	1.85
Metabolism	61.12	61.80
Organismal Systems	0.98	0.81

The main predictive functions (relative abundance > 1.0%) and hierarchical clustering in soil samples are shown in Fig. 8. The contaminated and reference soil were marked in yellow and blue, respectively. The results of the clustering analysis showed that the soil samples could be divided into two groups according to the community function. Studies have confirmed that pH has an important effect on the distribution of microbial community structure and function (Sharma et al. 2020; Villegas-Plazas et al. 2019). In our study, the microbial community functions of M2 and M6 with pH value of 6.79 and 5.88, respectively, were close to those of the reference soil, while the community functions of other contaminated soil samples with lower pH value were similar. As shown in Fig. 8, most of the main microbial functions of level 3 (20 out of 27 KOs) are higher in cluster II than those in cluster I, especially in severely acid contaminated samples M1, M7 and M8. Metabolic functions overexpressed in Cluster II samples Translation (Ribosome, and Aminoacyl-tRNA biosynthesis), Replication and repair (Homologous recombination, Nucleotide excision repair, and Mismatch repair), Nucleotide metabolism (Purine metabolism, and Pyrimidine metabolism), RNA degradation, Two-component system and Bacterial secretion system, which were regarded as the essential factors for the survival of microorganisms in harsh environmental conditions. For example, the Bacterial secretion system is thought to regulate the secretion of protective molecules that allow bacteria to withstand the harsh conditions (Lukhele et al. 2019). Moreover, since the extreme conditions of AMD systems can cause significant damage to biomolecules (Lukhele et al. 2019), microorganisms in AMD have evolved an efficient repair system usually exhibiting the overexpression of genes involved in replication, repair, recombination, and translation. In our study, in addition to the high expression of genes related to heredity and repair functions, we also observed that the synthetic functions of peptidoglycan and lipopolysaccharide were overexpressed in the samples of cluster II. Peptidoglycan is the main component of the cell wall of Gram-positive bacteria, and the solid cell wall plays an important role in maintaining the bacterial cell morphology and resisting osmotic damage (Reith and Mayer 2011). And lipopolysaccharide is a unique component of the cell wall of Gram-negative bacteria, which enables the outer membrane of bacteria to act as a tight barrier to most substances harmful to bacteria (Zhang et al. 2013). Therefore, we hypothesize that the high expression of the synthetic functions of peptidoglycan and lipopolysaccharide in the AMD environment is also an adaptation mechanism of microorganisms to the acidic environment.

Nitrogen is an essential element for biological life activities, and since AMD environments are usually oligotrophic, this limits the possibility of bioremediation using microorganisms or plants. In this study, we found that the nitrogen metabolism of bacteria was significantly enhanced after AMD pollution, and the average

abundance of nitrogen metabolism function in cluster II (2.33%) was higher than that in cluster I (2.10%). The distribution of some metabolic genes associated with nitrogen fixation in soil samples is shown in Fig. 9. Results showed that the total relative abundance of nitrogen-fixing genes in cluster II (0.30% on average) was significantly higher than that in cluster I (0.09%), especially in the extremely acidic samples M1, M7 and M8 samples with total relative abundance of 0.34%, 0.37% and 0.37%, respectively, while their total abundance in the reference soil samples were all below 0.1%. The relative abundance of nitrogen fixation genes can reveal their potential nitrogen fixation capacity, and we found that the nitrogen fixation capacity of microorganisms in AMD-contaminated soils was significantly enhanced, especially in extremely acidic soils. Studies have shown that some microorganisms in AMD environments can grow autotrophically using inorganic compounds to provide the necessary nitrogen source for the environment through the carbon fixation process (Sun et al. 2018), which provides an idea for using the native microorganisms for ecological remediation. In this study, *Ferrovum*, as the most abundant genus in AMD-contaminated soil, was considered to endogenously fix nitrogen and CO₂ using the energy from ferrous iron oxidation (Johnson et al. 2014). Therefore, *Ferrovum* can be used in the bioremediation of AMD environments to achieve the provision of nitrogen sources in the initial stage of remediation.

Conclusions

In this study, we found that the long-term AMD pollution significantly affected the microbial community structure, interaction patterns and metabolic functions of soil microorganisms, mainly included:

- (1) The AMD contamination inhibited the survival of *Chloroflexi*, *Acidobacteriota*, *Actinobacteriota* etc. of soil, significantly reducing the richness and diversity of soil microorganisms while promoting the evolution of γ -*Proteobacteria*. Acidophilic iron-metabolizing bacteria became the dominant microbial community in the the long-term acidic contaminated soil, including iron-oxidizing bacteria such as *Ferrovum*, *Acidithiobacillus*, *Gallionella*, *Sideroxydans* and *Leptospirillum*, and iron-reducing bacteria, *Metallibacterium* and *Acidibacter* etc.
- (2) Co-occurrence network analysis revealed that the percentage of positive correlations among bacteria in soil increased from 51.02% (reference soil) to 75.16% (contaminated soil), which may be a survival strategy for microorganisms in extreme environments by forming mutually beneficial interaction networks for better survival in the harsh environment of strong acidity. Some new nodes were displayed in co-occurrence network of soil microorganisms after AMD contamination, mainly attributed to *Firmicutes*, *Nitrospirota* and *Patescibacteria*.
- (3) The microbial interaction patterns varied in soils with different levels of AMD contamination. Iron-oxidizing and iron-reducing bacteria formed separate networks of independent interactions in soils with pH < 4. In contrast, iron-oxidizing bacteria and iron-reducing bacteria suitable for living in a moderately acidic environment (pH value 4 ~ 7) together formed a collaborative network with a positive correlation with each other.
- (4) Metabolic function prediction (Tax4fun) was used to explore the changes of metabolic potential in soils after AMD contamination. Results showed that most of the main microbial functions of level 3 (20 out of 27 KOs) are higher in contaminated soil. And the AMD contamination enhanced the microbial functions such as translation, remediation, and biosynthesis of peptidoglycan and lipopolysaccharide etc., which may be an adaptive mechanism for microbial survival in extremely acidic environment.

(5) In addition, we found that the AMD contamination of soil promoted the enhancement of nitrogen metabolic functions and the highly expression of nitrogen fixation genes, which may be responsible for the high development of autotrophic nitrogen fixing bacteria such as *Ferrovum*. Therefore, we suggested that *Ferrovum* can be used in the bioremediation of AMD environments to achieve the provision of nitrogen sources in the initial stage of remediation.

Declarations

Author contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Chen Di, Liang Haoqian and Feng Qiyan. The first draft of the manuscript was written by Chen Di and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Figures

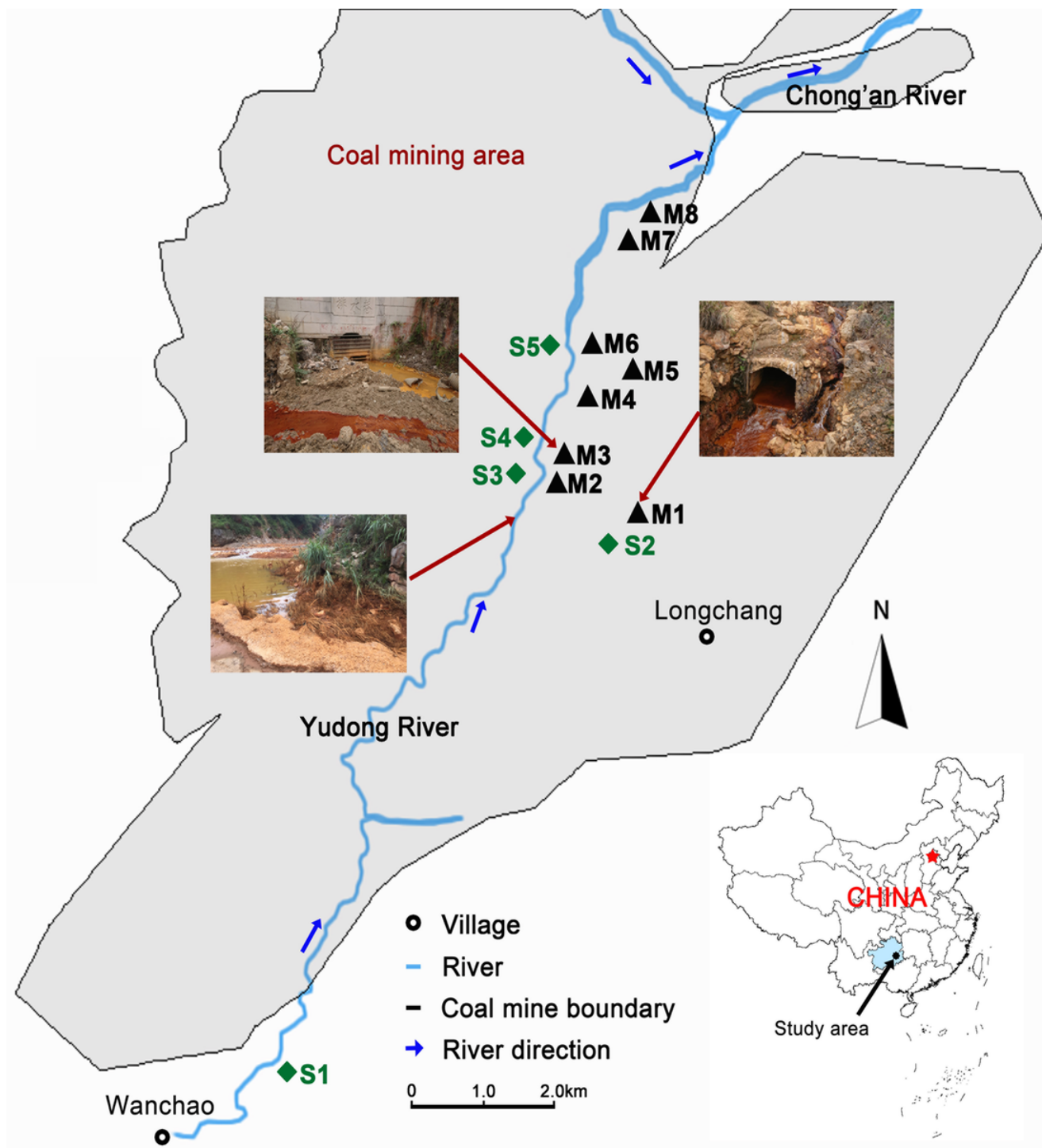


Figure 1

Study area and sampling sites Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

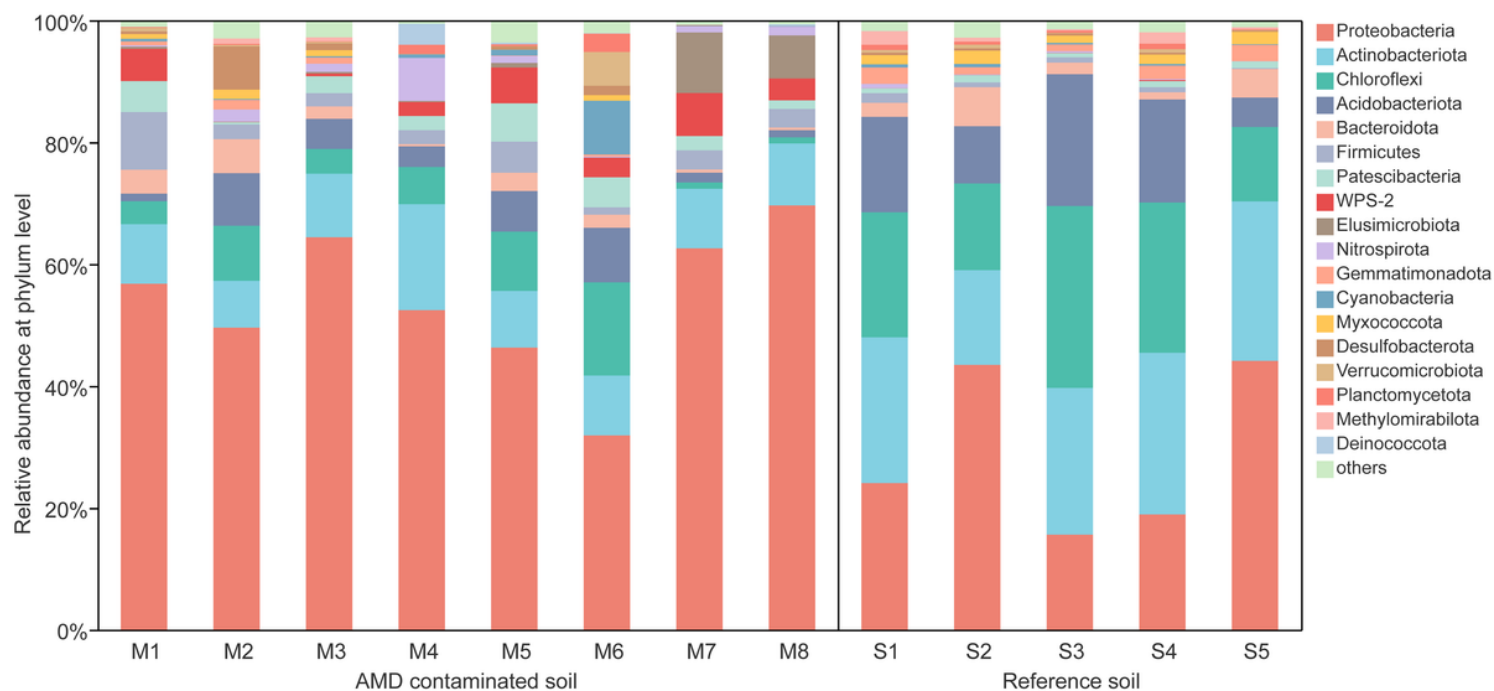


Figure 2

Distribution of dominant bacteria groups in AMD contaminated soil and reference soil samples at phylum level. Relative abundance below 2% was classified as others

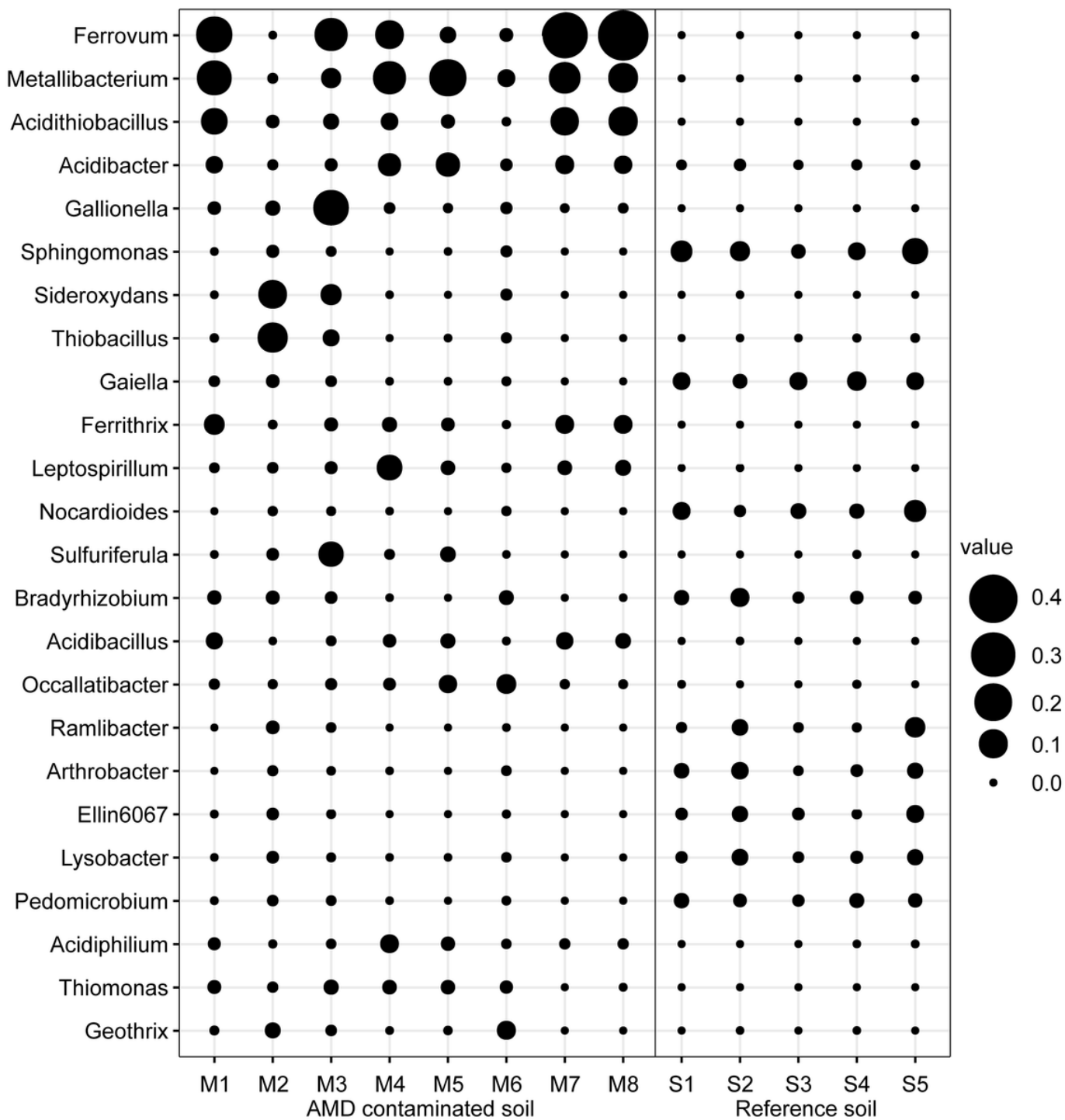


Figure 3

Bubble plot demonstrated the relative abundance of the main genera in soil samples. The size of the circle represents the relative abundance of genera in samples

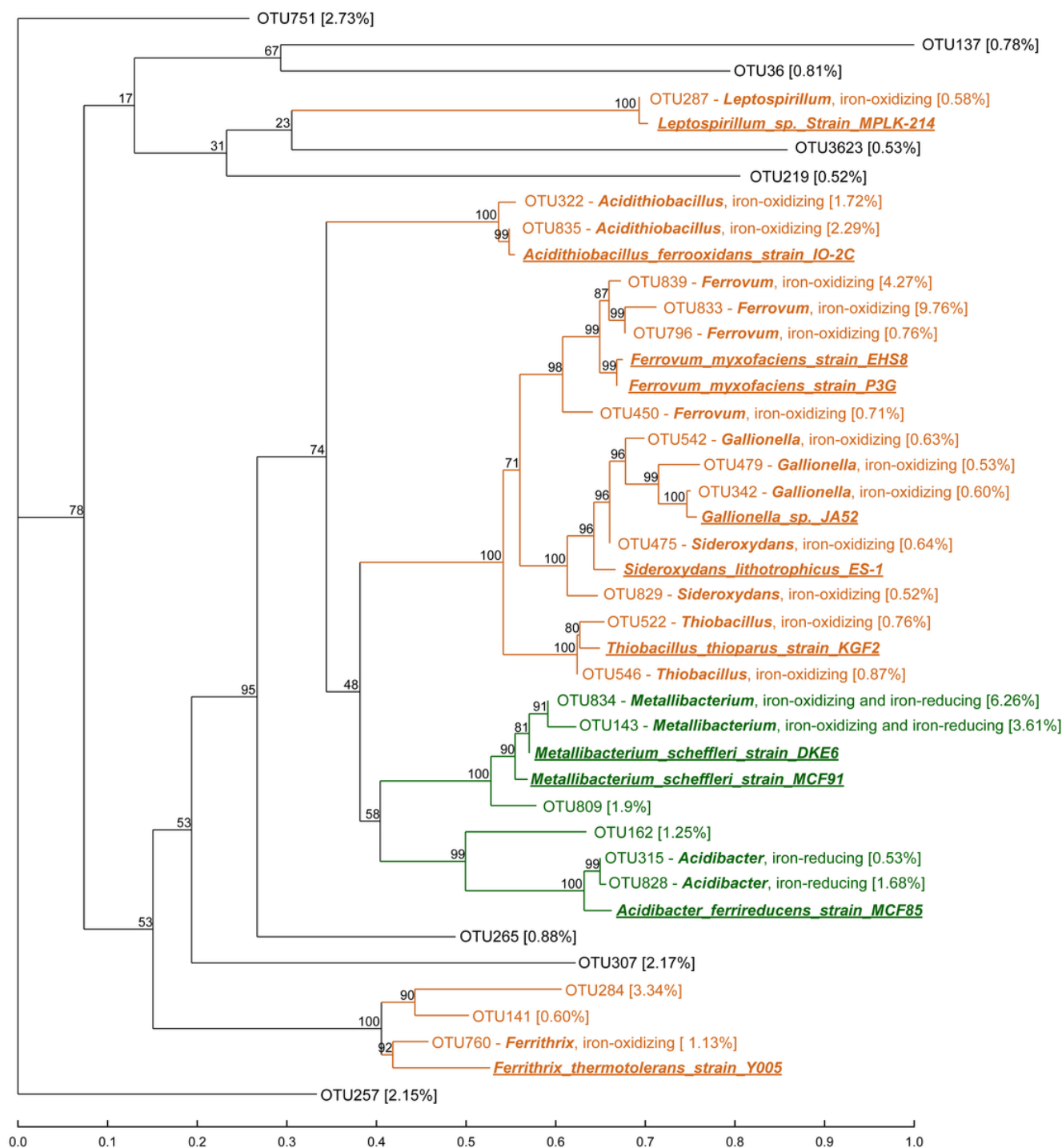


Figure 4

Phylogenetic tree of the top OTUs (relative abundance > 0.5% in all samples) in contaminated soil. Number in the box indicates the relative abundance of each OTU in all samples. Bold and underlined font indicates the closest known species. Fonts in Yellow and Green indicated iron-oxidizing bacteria and iron-reducing bacteria, respectively

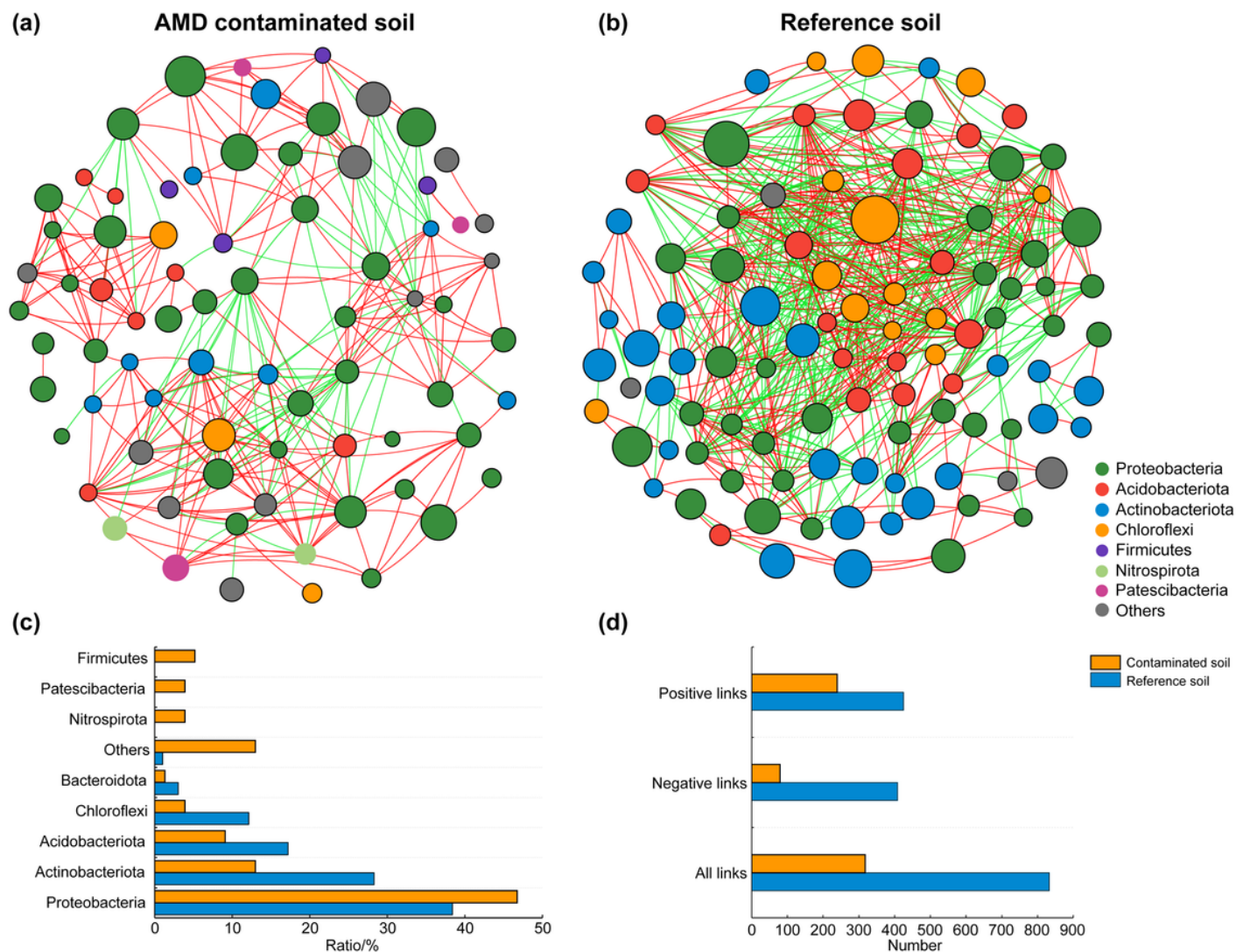


Figure 5

Network of co-occurring based on top OTUs (relative abundance $> 0.2\%$ in the corresponding group samples) in the contaminated soil (a) and reference soil (b). A connection stands for the significant Spearman correlation with $0.8 < |r| < 1$ ($p < 0.05$). The color of a node represents the assignment at phylum level. The red and green edges stand for positive and negative correlations, respectively. Number of keystone OTUs within two networks were counted at phylum level (c). Number of the strong links ($0.8 < |r| < 1$), including positive and negative links in two groups (d)

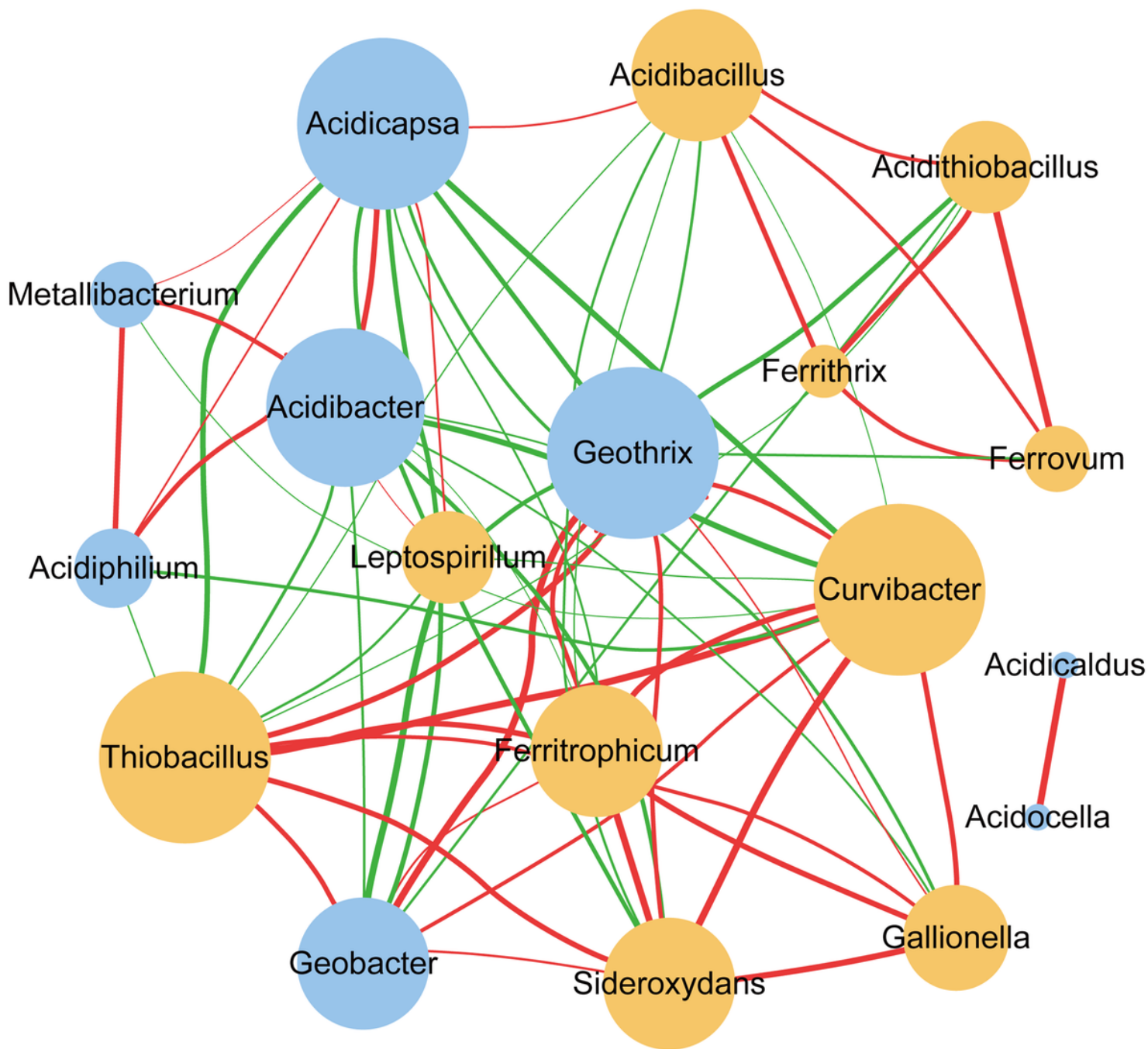


Figure 6

Co-occurrence network showing correlations among the main iron-metabolizing genera in contaminated soil. A connection indicates a strong (Spearman's $0.7 < |r| < 1$) and significant ($p < 0.05$) correlation. The size of a node is proportional to the number of connections (degree). The nodes colored in yellow and blue represent for iron-oxidizing and iron-reducing bacteria, respectively. The width of an edge is proportional to the Spearman correlation coefficient. The red and green edges stand for positive and negative correlations, respectively

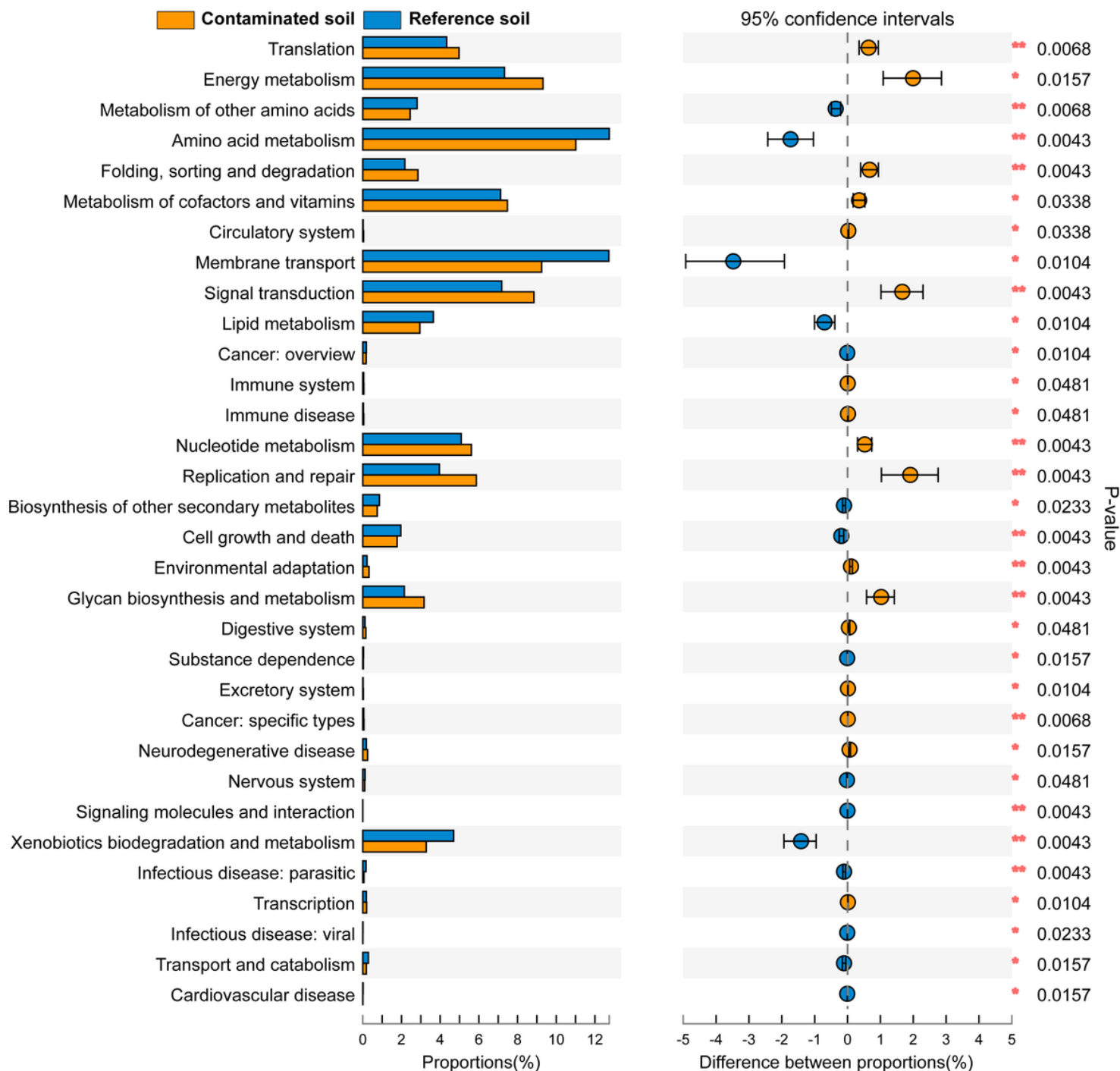


Figure 7

Statistical differences of the predicted functional profiles of significant variations between contaminated soil and reference soil (level 2). Corrected P-values were calculated using the Wilcoxon test ($p < 0.05$)

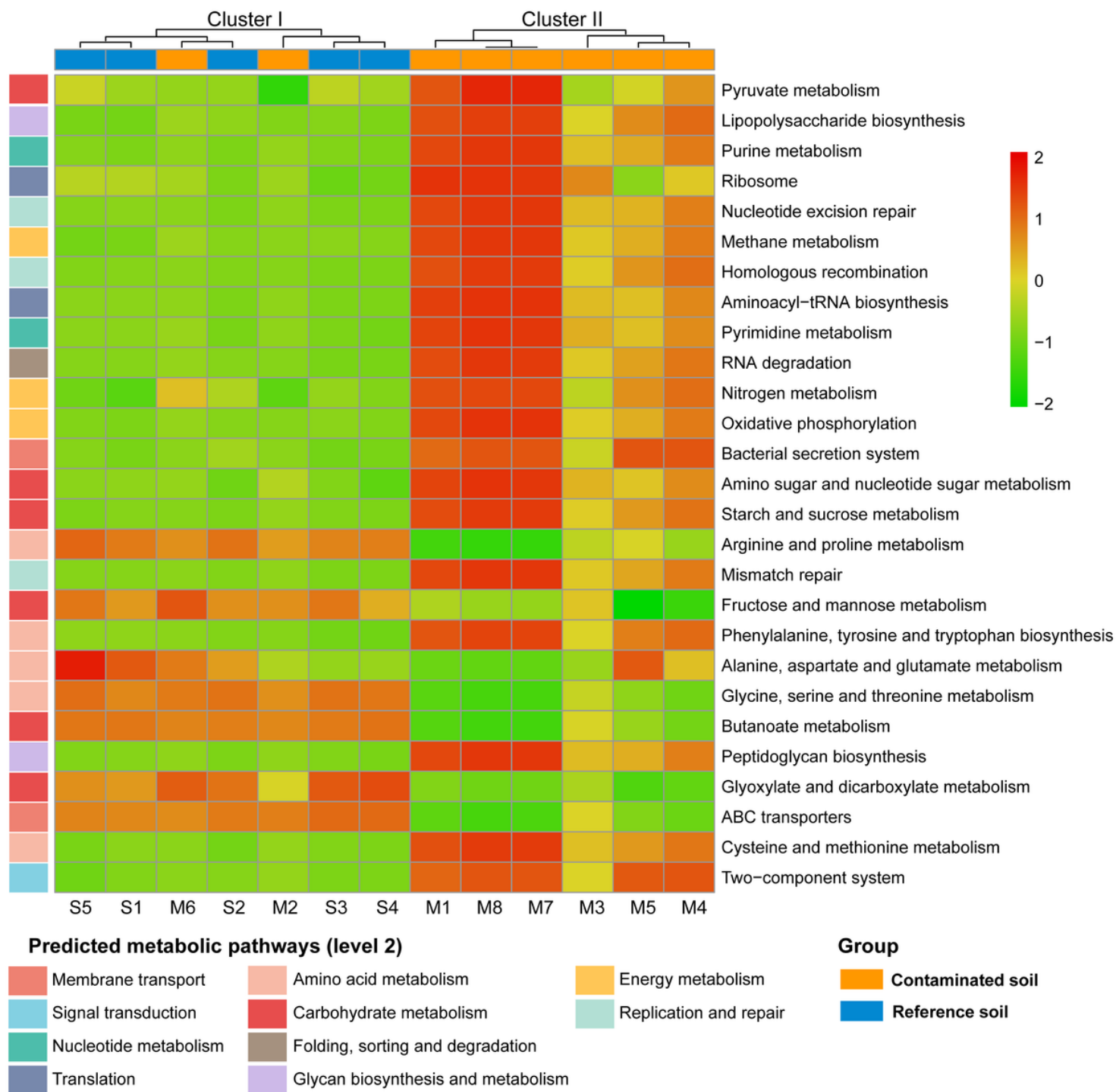


Figure 8

Tax4fun-derived heatmap and hierarchical clustering comparing the main predicted functions on level 3 (relative abundance >1.0%) of contaminated soil and reference soil

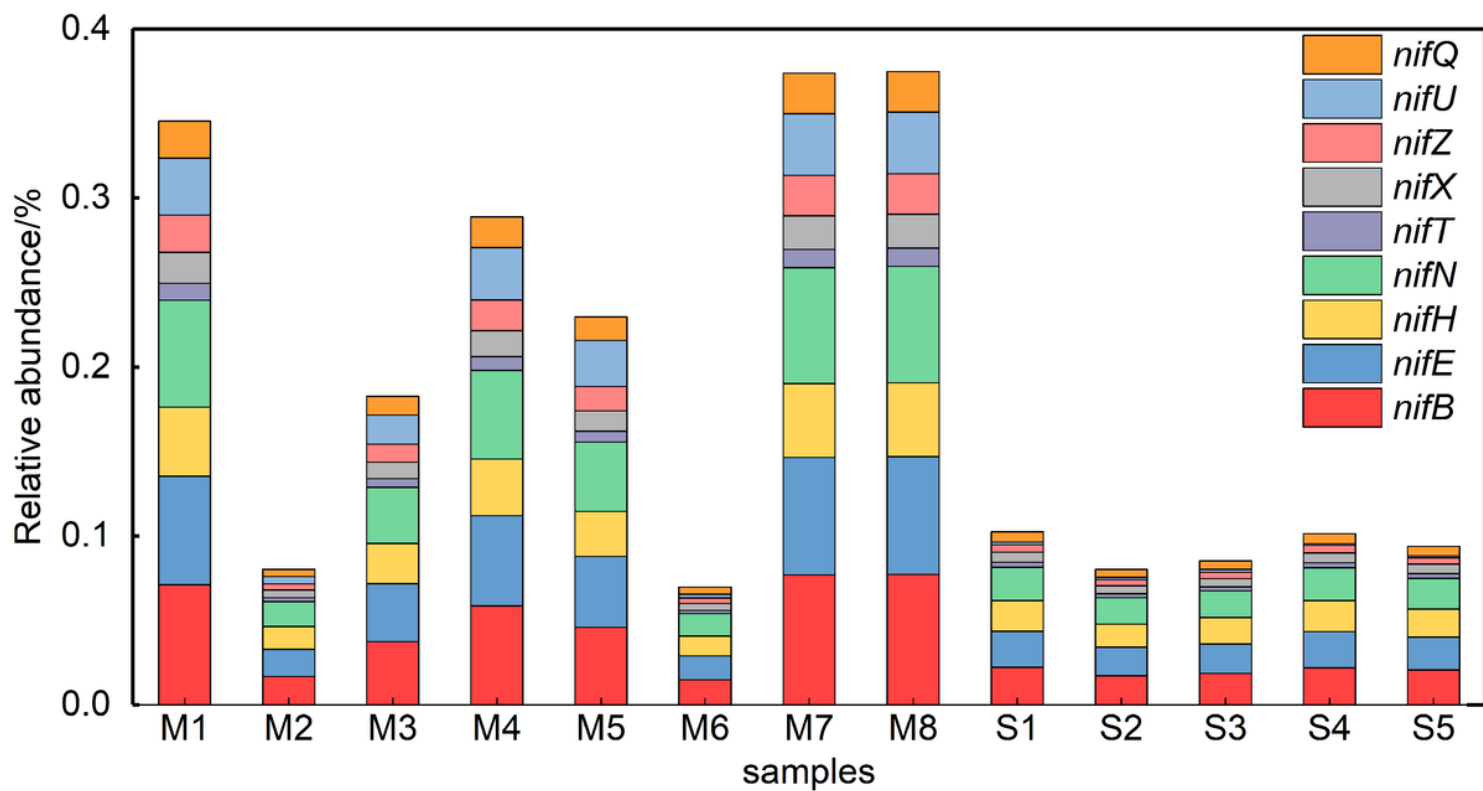


Figure 9

Distribution of predicted genes related to nitrogen fixation in soil samples