

# Bacterial communities in the polluted area of pyrite tailings: From the upstream, pollutant source, and to the downstream

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## Research Article

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# Abstract

Pyrite tailings can cause serious pollution to the surface water as the strong acidity, high iron and sulfate concentration in the leachate. The bacterial communities of pyrite tailings polluted area were still unclear which could restrict the recognition of the pyrite tailings pollution effect and further impede the development of microbial or ecology treatment technologies. In this study, the bacterial communities in the polluted area of pyrite tailings, from the upstream, pollutant source, and to the downstream, were analyzed with Illumina HiSeq sequencing. Results showed that *Acinetobacter* and *Flavobacterium* were abundant in the water and sediment of upstream and downstream while *Bacteroides*, *Lactobacillus*, and *Akkermansia* were abundant in the pollutant source. Sulfur-metabolizing or iron-metabolizing bacteria extensively existed in the polluted area in which *Acidiferrobacter*, *Ferrithrix*, and *Desulfovibrio* played crucial roles on the whole communities. Sulfur-metabolizing bacteria (e.g. *Thiomonas*, *Sulfurospirillum*, and *Desulfobulbus*) and iron-metabolizing bacteria (e.g. *Ferrimicrobium*, *Ferrithrix*, and *Ferrovum*) were introduced to the river polluted by pyrite tailings. Pyrite tailings can remarkably change the physicochemical characteristics and bacterial communities of river water and sediment.

## 1 Introduction

Pyrites ( $\text{FeS}_2$ ) were widespread and important minerals, mainly used in the manufacture of sulfuric acid, and were heavily mined in the last few decades globally which generated huge numbers of pyrite tailings. In many developing countries and low income areas, pyrite tailings were discarded in the valley near the pyrite mine without further reasonable disposal as the reuse value of pyrite tailings was low. The  $\text{FeS}_2$  in pyrite tailings can be oxidized to soluble iron ( $\text{Fe}^{2+}$  and  $\text{Fe}^{3+}$ ), sulfate, and hydrogen ion ( $\text{H}^+$ ), present in the leachate, with the participation of oxygen, water, and certain microbes when the pyrite tailings are directly exposed to the air (Lowson, 1982; Edwards et al., 1998). Therefore, the pyrite tailings leachate, one kind of acid mine drainage (AMD), has strong acidity and contains high concentration of iron (Fe), sulfate ( $\text{SO}_4^{2-}$ ), and other heavy metals which can cause serious pollution to the downstream water (Li et al., 2019). The ecological system of the river polluted by pyrite is usually seriously destroyed by low pH, high heavy metal concentration, and high turbidity caused by hydrolysis of iron ion (Liu et al., 2015). To better understand the pollutant generating process and further reduce the production of pollution, plenty of research had revealed the special bacteria such as *Thiobacillus*, *Leptospirillum*, *Sulfobacillus*, and *Thiomonas* which could fasten the pyrite oxidization (Crundwell, 1996; Edwards et al., 1998; Yahya and Johnson, 2002; Han et al., 2013). However, the holistic bacterial communities in pyrite tailings polluted areas and the modification of the downstream bacterial communities by pyrite tailings are still not clear which further impede the cognition of the potential effect of pyrite tailings pollution.

To increase pH and precipitate iron ion, alkali neutralization process was used to AMD treatment and gradually became a common method (Santomartino and Webb, 2007; Park et al., 2019). With the development of treatment technology, sodium hydroxide (NaOH) or calcium hydroxide ( $\text{Ca}(\text{OH})_2$ ) coupled with polyacrylamide (PAM) was recognized as the rapid and effective approach to treating pyrite tailings

leachate. However, this method consumed large amounts of potions and further increased the cost of pollutant control. The generation of leachate in pyrite tailing ponds or heaps usually continues for decades and alkali neutralization process will cause large amounts of cost. More importantly, a great deal of sludge generated from neutralization process needs further treatment and management which are high priced (Naidu et al., 2019). The government or relevant company will be difficult to cover the cost of potions for decades which need to develop a series of new technologies based on microbial or ecology methods to reduce the overall treatment cost partial like the municipal or industrial wastewater treatment process. In recent years, many biological remediation methods such as bio-reactors, permeable reactive barriers, and wetlands had been established mainly utilized the sulfate reducing bacteria (SRB) to remove sulfate and heavy metals (Giordani et al., 2019; Rambabu et al., 2020; Singh and Chakraborty, 2020). However, the limitation of existing biological technology is the dependence on carbon sources and hard adaptation to high pollutant concentrations which is very common in contaminated sites, since the characteristics of SRB (Rambabu et al., 2020; Zhang et al., 2021). Therefore, the bacterial communities and their interaction of real pyrite tailings leachate and its pollutant areas need to be deeply researched to find more adaptable bacteria in future and provide the fundamental theories of bio-ecology pyrite tailings leachate treatment process.

To explore the bacterial communities in the polluted area of pyrite tailings, an actual river flowing through the pyrite tailings polluted areas was selected and the bacteria in the water and sediment of upstream, pollutant source, and downstream were analyzed by Illumina Hiseq sequencing in this study. The findings innovatively revealed the bacterial communities of pyrite tailings polluted area and further confirmed the effect of pyrite tailings pollution on natural water bodies.

## **2 Materials And Methods**

### **2.1 Research spot and sampling method**

The research spot (110°2'44" E, 32°38'48" N), polluted by open stacked pyrite tailings, was located on one river in a county of China (Fig. 1). From 1950s to 2000s, millions cubic meter of pyrite tailings generated and were discarded in the county and over 0.3 million cubic meter of pyrite tailings were stacked in the studied river region. One pyrite tailings leachate treatment project, mainly taking lime and PAM as the treatment agent, was established in 2021 and the iron ion and acidity of leachate were partially removed. The position of treatment project was the pollutant source of this region. The water and sediment in pollutant source, upstream (not contaminated) and downstream (contaminated) were sampled (Table 1). The water and sediment were sampled thrice (named XX-1, XX-2, and XX-3) with sterile bottles and sterile medicine spoon, respectively, and stored at 4°C (for conventional analysis) and -20°C (for bacterial communities detection). In detailed, water samples used to bacterial communities detection were filtered with 0.22µm sterile microfiltration membrane after sampling immediately and stored at -20°C.

Table 1  
Samples information.

Type	Name	Spot	Description
Upstream (not contaminated)	WU-1/2/3	Water in upstream	Liquid, clear, colorless
	SU-1/2/3	Sediment in upstream	Solid, small pebble, sand
Pollutant source	PT-1/2/3	Pyrite tailings	Solid, gravel, black brown
	PL-1/2/3	Pyrite tailings leachate	Liquid, clear, reddish brown
	ST-1/2/3	Sediment after treatment	Solid, mud, yellowish-brown
Downstream (contaminated)	WD-1/2/3	Water in downstream	Liquid, turbid, yellowish-brown
	SD-1/2/3	Sediment in downstream	Solid, fine sand, yellowish-brown

## 2.2 Water characteristic analysis

The concentration of chemical oxygen demand (COD), ammonia nitrogen (AN), total nitrogen (TN), total phosphorus (TP), turbidity (TU), total iron ion ( $\text{Fe}^{2+/3+}$ ), sulfate ( $\text{SO}_4^{2-}$ ), and sulfide ( $\text{S}^{2-}$ ) were analyzed by the standard methods (APHA, 2005). The pH, dissolved oxygen (DO), and temperature (T) were measured by the portable pH meter (PHB-4, INESA, China) and DO meter (JPB-706A, INESA, China) on site. The concentration of manganese ion ( $\text{Mn}^{2+}$ ), copper ion ( $\text{Cu}^{2+}$ ), zinc ion ( $\text{Zn}^{2+}$ ), and nickel ion ( $\text{Ni}^{2+}$ ) was detected with inductively coupled plasma mass spectrometry (ICP RQ ICP-MS, Thermo Fisher Scientific, USA). The total bacteria count (TBC) was detected by standard nutritional agar plates (HuanKai Microbial, China) with 100 $\mu\text{L}$  diluted samples and cultured at 30°C for 48 h which was expressed as colony forming unit (CFU).

## 2.3 Illumina sequencing and data processing

The DNA from the water samples and sediment samples was extracted with the PowerSoil® DNA Isolation Kit (MOBIO, USA) for Illumina sequencing. Universal PCR bacterial primer sets 338F (5'-ACTCCTACGGGAGGCAGCA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') were used to amplify the 16S rRNA gene sequences and the Illumina HiSeq sequencing (HiSeq 2500, Illumina, USA) was conducted by Beijing Biomarker Technologies Co. Ltd., Beijing, China. The PCR procedure and sequencing data processing were described by previous study (Li et al., 2020a). Operational taxonomic units (OTUs) were regarded with 97% similarity threshold. Taxa were assigned to all OTUs by comparing them to SILVA databases using QIIME2. The network of total bacterial communities was operated by the CoNet module in Cytoscape (3.6.1) with five methods (Pearson correlation, Spearman correlation, mutual information similarity, Bray Curtis dissimilarity distance, and Kullback–Leibler dissimilarity distance) (Li et al., 2020b) and the relationship of sulfur-related and iron-related bacteria was calculated with Spearman method (correlation index > 0.5 and  $P < 0.05$ ) in SPSS (25.0). The network was finally visualized with Gephi (0.9.1).

## 3 Results And Discussion

### 3.1 Water characteristics in research spot

Water characteristics in this research were shown in Table 2. Pyrite tailings leachate in this study showed a strong acidity (pH = 1.94) and contained high concentration of Fe (2789.80 mg/L) and  $\text{SO}_4^{2-}$  (3197.40 mg/L) which were similar with other research (Sánchez España et al., 2005; Naidu et al., 2019). Water in downstream (contaminated) was obviously influenced by pyrite tailings leachate (pollutant source) on the parameters, e.g. pH (from 8.06 in upstream to 7.22 in downstream), Fe (from 0.01 mg/L in upstream to 2.66 mg/L in downstream), and  $\text{SO}_4^{2-}$  (from 7.15 mg/L in upstream to 73.94 mg/L in downstream). With the increasing of  $\text{SO}_4^{2-}$ , the concentration of  $\text{S}^{2-}$  in the downstream water (from 0.01 mg/L in upstream to 0.04 mg/L in downstream) also increased which might be caused by SRB metabolism. As the acidity of leachate, many heavy metals in pyrite tailings such as Mn, Cu, Zn, and Ni dissolved in the leachate leading to high heavy metal concentration and further increased the heavy metal concentration in downstream water. Strong acidity and high heavy metal concentration caused huge bacteriostasis (Ogbughalu et al., 2020) and resulted the culturable bacteria number decreased dramatically in downstream water (from 4100 CFU/mL in upstream to 2010 CFU/mL in downstream) even though TU in downstream water was high (from 0.50 NTU in upstream to 29.40 NTU in downstream) since the hydrolysis of Fe ions. From the perspective of culturable bacteria number, it might be difficult to treat undiluted or unpretreated pyrite tailings leachate with conventional bacteria based methods since the culturable bacteria number in pyrite tailings leachate was 8 times lower than that in natural river water in the upstream.

Table 2  
The mean value of water characteristics.

Parameter	WU	PL	WD	Parameter	WU	PL	WD
pH	8.06	1.94	7.72	Fe (mg/L)	0.01	2789.80	2.66
T (°C)	19.0	19.2	20.5	Mn (mg/L)	0.00	36.77	0.19
TU (NTU)	0.50	7.07	29.40	Cu (mg/L)	0.00	6.08	0.01
DO (mg/L)	9.80	6.40	9.10	Zn (mg/L)	0.00	14.45	0.01
COD (mg/L)	1.15	10.98	1.22	Ni (mg/L)	0.00	3.61	0.02
AN (mg/L)	0.00	0.20	0.12	$\text{SO}_4^{2-}$ (mg/L)	7.15	3197.40	73.94
TN (mg/L)	1.32	6.73	1.96	$\text{S}^{2-}$ (mg/L)	0.01	0.01	0.04
TP (mg/L)	0.01	2.96	0.10	TBC (CFU/mL)	4100	510	2010

### 3.2 Bacterial communities in pyrite tailings polluted area

Based on Illumina HiSeq sequencing, 1,174,592 sequences were obtained from 21 samples in this study, assigned to 2,261 OTUs. The alpha diversity index of samples is shown in **Fig. S1**. The solid in pollutant source (i.e. PT and ST) had low bacteria richness but high bacteria diversity. The bacteria diversity of water and sediment samples in downstream (contaminated by pyrite tailings leachate) was much lower than that in upstream, indicating pyrite tailings leachate significantly changed bacterial communities of the river. Samples from different positions showed obvious distinction especially in upstream (WU and SU) and downstream (WD and SD) based on principal component analysis (**Fig. S2**). Samples obtained from the pollutant source (PT, PL, and ST) were relatively similar compared with other positions. *Proteobacteria* (mean at 47.0%) was the most abundant bacterial phylum in this study, followed by *Firmicutes* (mean at 16.4%) and *Bacteroidetes* (mean at 16.2%), shown in Fig. 2a. In pollutant source, the relative abundance of *Cyanobacteria*, *Verrucomicrobia*, and *Nitrospirae* was higher than upstream and downstream. *Acinetobacter* (mean at 17.4%) and *Flavobacterium* (mean at 11.1%) was the most abundant bacterial genus in upstream water and sediment, respectively, while the relative abundance of *Novosphingobium* (mean at 18.1%) increased in the downstream (Fig. 2b). The bacterial communities of pollutant source in the genus level had visible difference with the river where *Bacteroides* (mean at 3.2%), *Lactobacillus* (mean at 2.6%), and *Akkermansia* (mean at 2.4%) were abundant in the pollutant source. The bacterial communities in samples from the liquid phase or sediment phase showed distinction analyzed by ternary method (**Fig. S3**). Some genus belong to *Chloroflexi* (such as *Kouleothrix* and *Leptolinea*) tended to inhabit in pyrite tailings leachate compared with water in the upstream or downstream and many iron-related genus such as *Ferrimicrobium*, *Leptospirillum*, and *Ferrovum* also tended to exist in pyrite tailings leachate. In sediment phases, many genus belong to *Firmicutes* (*Butyrivibrio* and *Megasphaera*, for instance) were more likely to live in leachate treatment sediment and sulfur-related genus, e.g. *Thiobacillus*, *Sulfuricurvum*, *Desulfoprunum*, were also likely to exist in leachate treatment sediment compared with sediment in the upstream or downstream.

Bacteria network was generated with 736 nodes and 1628 edges which copresence relationship was 58.1% and mutual exclusion relationship was 41.9% (Fig. 3). The average degree, average path length, network diameter, average clustering coefficient, and modularity index of this network was 4.57, 2.69, 9, 0.06, and 0.72, respectively. Bacterial relationship in the polluted area of pyrite tailings showed obvious distinction and aggregation which was divided into three main modules since the role of keystone species. *Acidiferrobacter*, an extreme acidophilic iron oxidizer (Issotta et al., 2017), played an important role in the bacterial communities whose species was the center of mutual exclusion with other species. *Ferrithrix*, an acidophilic iron oxidizer or reductor (Johnson et al., 2009), played similar mutual exclusion center roles with *Acidiferrobacter*. *Bacteriovorax*, a biphasic life cycle (predacious form or saprophytic form) bacteria (Baer et al., 2000), was another mutual exclusion center in the bacterial communities. Different with the two mutual exclusion center, copresence relationship showed relatively uniform distribution. *Alloprevotella*, *Desulfovibrio*, and *Lactobacillus* had the highest degree in the copresence relationship and all these three genus were anaerobic bacteria (Devereux et al., 1990; Downes et al., 2013; Zheng et al., 2020). The bacterial communities showed great characteristics which could reflect the pyrite

polluting area as the key effect of sulfate-reducing bacteria (i.e. *Desulfovibrio*) and iron-metabolizing bacteria (i.e. *Acidiferrobacter* and *Ferrithrix*).

### 3.3 Sulfur-metabolizing bacteria and iron-metabolizing bacteria distribution

Abundant sulfur-metabolizing bacteria and iron-metabolizing bacteria existed in pyrite tailings polluting area as the sufficient sulfate and iron of the polluted area. The pollutant source (i.e. PT, PL, and ST) contained high amount of sulfur-metabolizing bacteria and iron-metabolizing bacteria and further influenced the detailed distribution in the downstream (Fig. 4). *Thiobacillus*, *Acidiferrobacter*, and *Cloacibacterium* were the predominant sulfur-oxidizing bacteria and showed obvious different habitat characteristics (Fig. 4a). *Thiobacillus* (2.6%) was mainly existed in leachate treatment sediment while *Acidiferrobacter* (1.3%) and *Cloacibacterium* (1.1%) tended to live in pyrite tailings and downstream sediments, respectively. *Desulfovibrio* (0.3%) was the most abundant sulfate-reducing bacteria in this study and widely distributed in all samples. The total abundance of sulfur-metabolizing bacteria in the downstream (water and sediment) was higher than that in the upstream, caused by the input of pollutant sources. *Flavobacterium* had the highest relative abundance (2.9%) in sulfur-metabolizing bacteria but mainly existing in the upstream and downstream, indicating that *Flavobacterium* was the natural and background bacteria but not the bacteria introduced by pollutant. Compared with the upstream water, the pyrite tailings leachate markedly increased the relative abundance of many sulfur-metabolizing bacteria, such as *Thiomonas* (403 times), *Sulfurospirillum* (19 times), and *Desulfobulbus* (4 times), in the downstream water and the leachate was the source of *Acidiferrobacter* and *Sulfurisoma* in the downstream water (Fig. S4a).

*Pseudomonas* (1.3%), *Sphingomonas* (1.0%), and *Thiobacillus* (0.5%) were the predominant iron-metabolizing bacteria and mainly existed in pollutant sources (Fig. 4b). *Thiobacillus*, has the ability to form biofilms on pyrite and further oxide ferrous and sulfide (Crundwell, 1996), mainly existed in solids (i.e. PT and ST) which had the potential to reinforce the dissolve of iron and sulfur from pyrite tailings and leachate treatment sediment. Therefore, the leachate treatment sediment (ST) should be disposed timely to avoid the redissolution of pollutant. Different with sulfur-metabolizing bacteria, the total abundance of iron-metabolizing bacteria in the downstream was lower than that in the upstream mainly resulted from the reduction of *Pseudomonas*. Previous research had demonstrated that *Pseudomonas* can be suppressed rapidly by pyrite leachate within five days (Han et al., 2013). The pyrite tailings leachate obviously increased iron-metabolizing bacteria abundance in the downstream water, e.g. *Ferribacterium* (increased by 61%), *Sediminibacterium* (increased by 52%), and *Hyphomicrobium* (increased by 11%), and the leachate was the source of *Ferrimicrobium*, *Ferrithrix*, *Ferrovum*, and *Curvibacter* (Fig. S4b). Overall, the pyrite tailings leachate introduced abundant sulfur-metabolizing bacteria and iron-metabolizing bacteria into the river passing through the pollutant source and further changed the distribution of related bacteria.

Based on Spearman correlation analysis, sulfur-metabolizing bacteria and iron-metabolizing bacteria showed close connection (Fig. 4c). The genus of sulfur-metabolizing bacteria had an intimate connection with each other especially in the high positive relationship of *Sulfurospirillum*, *Desulfobulbus*, *Paludibacter*, *Desulfomicrobium*, *Sulfurovum*, and *Arcobacter*. However, the internal correlation of iron-metabolizing bacteria was relatively weak. *Sphingomonas* (1.0%) played an important role in the relationship of sulfur-metabolizing bacteria and iron-metabolizing bacteria which connected nine genus of sulfur-metabolizing bacteria mainly with negative relationship. The most strong negative relationship (Spearman correlation index = -0.826) in Fig. 4c occurred in the genus of *Bosea* and *Desulfoprimum* while the most strong positive relationship (Spearman correlation index = +0.811) occurred in *Arcobacter* and *Sulfurospirillum*. On the whole, positive relationship was the mainstream (65.4%) in the network of sulfur-metabolizing bacteria and iron-metabolizing bacteria. Regardless of the upstream, pollutant sources or downstream, sulfur metabolism functions was widespread based on PICRUST2 (Fig. 4d). Iron oxidation functions mainly existed in pyrite tailings leachate (PL) while sulfide oxidation functions mainly occurred in leachate treatment sediment (ST) analyzed by FAPROTAX (Fig. 4d). Therefore, the leachate treatment sediment should be transported from the scene timely to avoid the sulfide oxidization process to generate more amount of sulfate.

## 4 Conclusions

The bacterial communities in the polluted area of pyrite tailings, including the water and sediment of upstream, pollutant source, and downstream were analyzed by in this study. Pyrite tailings leachate had strong acidity, high concentration of Fe and  $\text{SO}_4^{2-}$ , and low culturable bacteria number which further increased the Fe and  $\text{SO}_4^{2-}$  concentration and influenced the bacterial communities in the downstream. *Acinetobacter* (~17.4%) and *Flavobacterium* (~11.1%) were abundant in the upstream and downstream while *Bacteroides* (~3.2%), *Lactobacillus* (~2.6%), and *Akkermansia* (~2.4%) were abundant in the pollutant source. Sulfur-metabolizing bacteria and iron-metabolizing bacteria were abundant in the polluted area and played important roles (e.g. *Acidiferrobacter*, *Ferrithrix*, and *Desulfovibrio*) on the bacterial communities. Pyrite tailings pollution introduced sulfur-metabolizing bacteria (*Thiomonas*, *Sulfurospirillum*, and *Desulfobulbus*, etc.) and iron-metabolizing bacteria (*Ferrimicrobium*, *Ferrithrix*, and *Ferrovum*, etc.) in the polluted river. Both the physicochemical characteristics and bacterial communities of river water and sediment were remarkably changed by pyrite tailings.

## Declarations

### Ethics approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.



## Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

## Competing interests

The authors declare that they have no competing interests.

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## Authors' contributions

Wenkai Li designed and performed the experiment, and was a major contributor in writing the manuscript. Yun Han provided the resources and conception. Hongwei Lei was one of the major contributors in the sampling work. Tianlong Zheng made the formal analysis and provided the resources. Yiming Lian participated in the sampling work and data analysis. Po Gao participated in the data analysis and manuscript writing. All authors read and approved the final manuscript.

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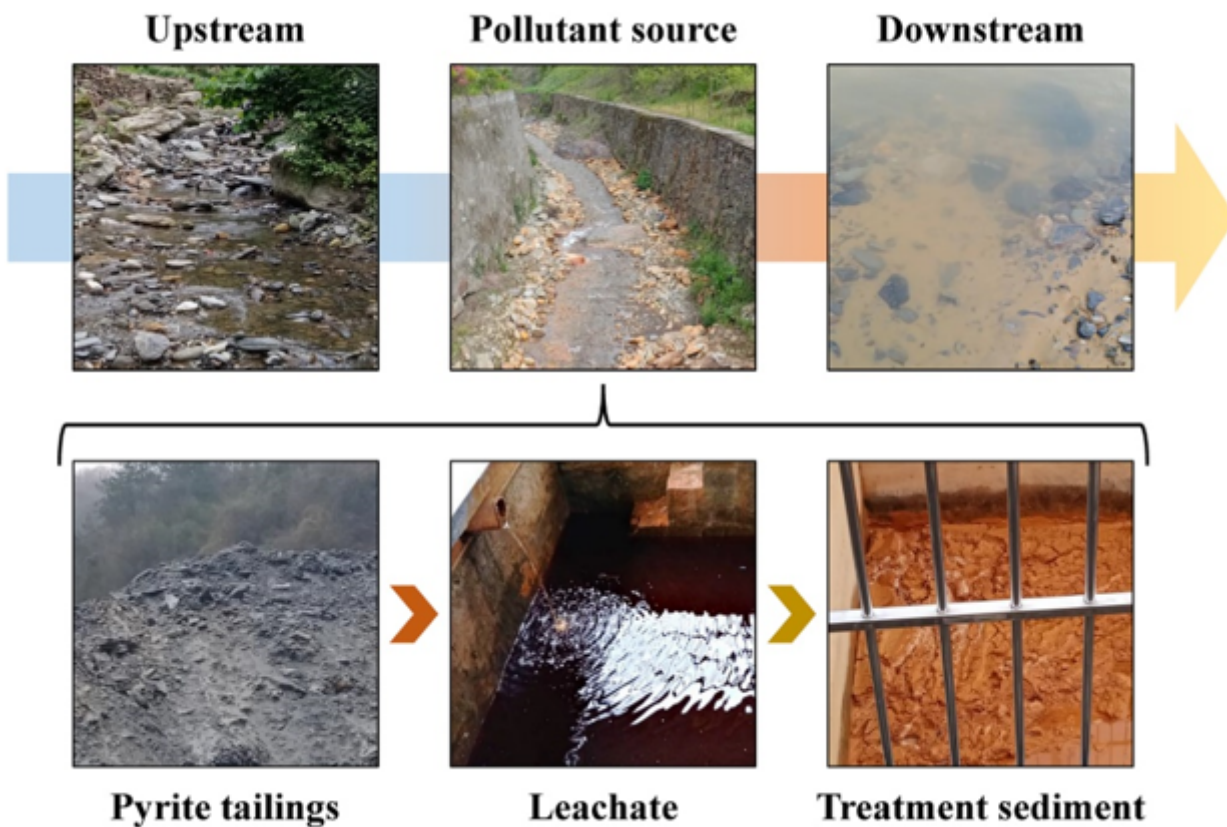
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## Figures



**Figure 1**

Photos of the research spot.

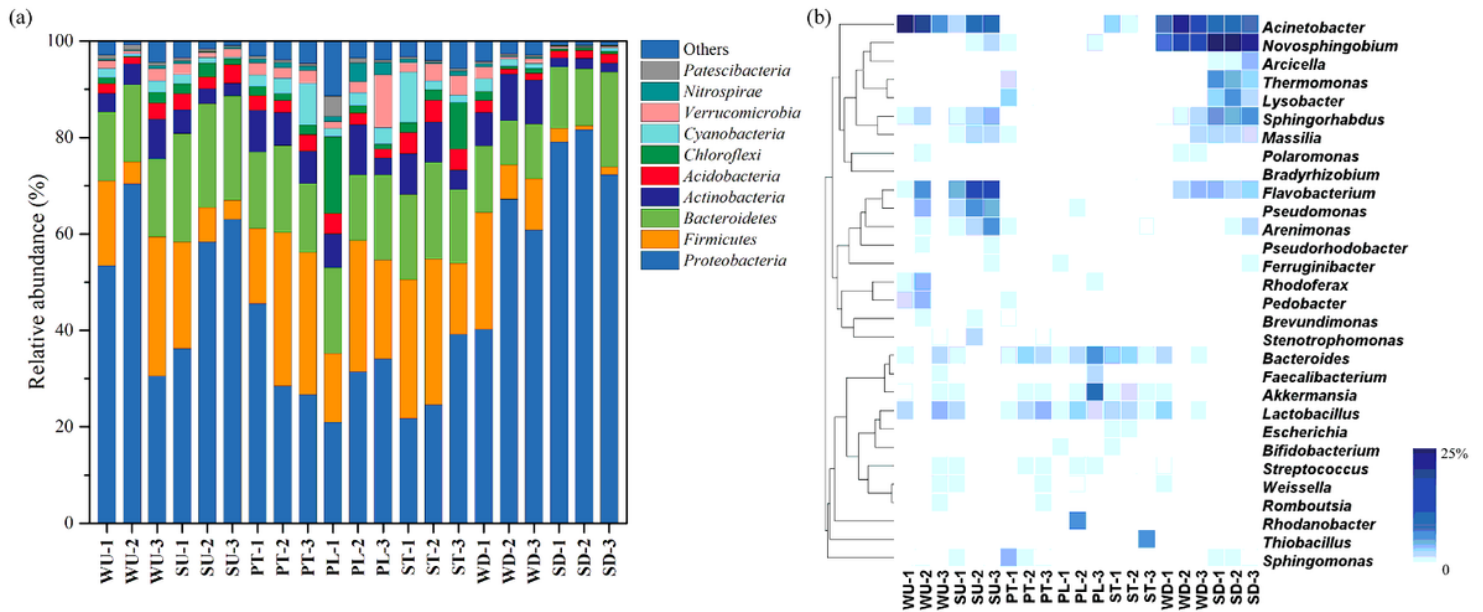


Figure 2

Bacteria characteristics at the phylum (a) and genus (b) level.

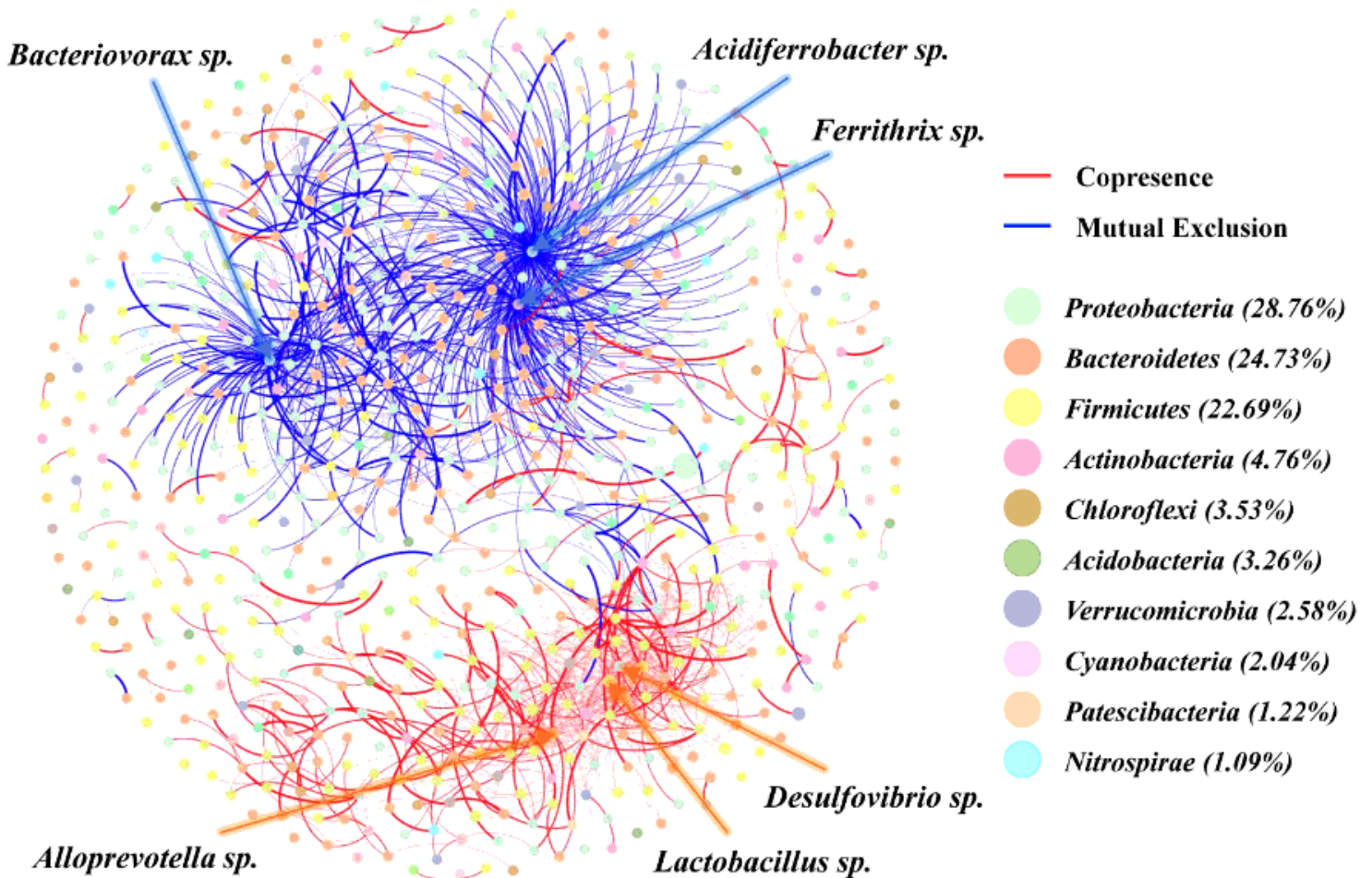


Figure 3

Bacteria network of the studied samples. The diameter of nodes means the relative abundance of the species.

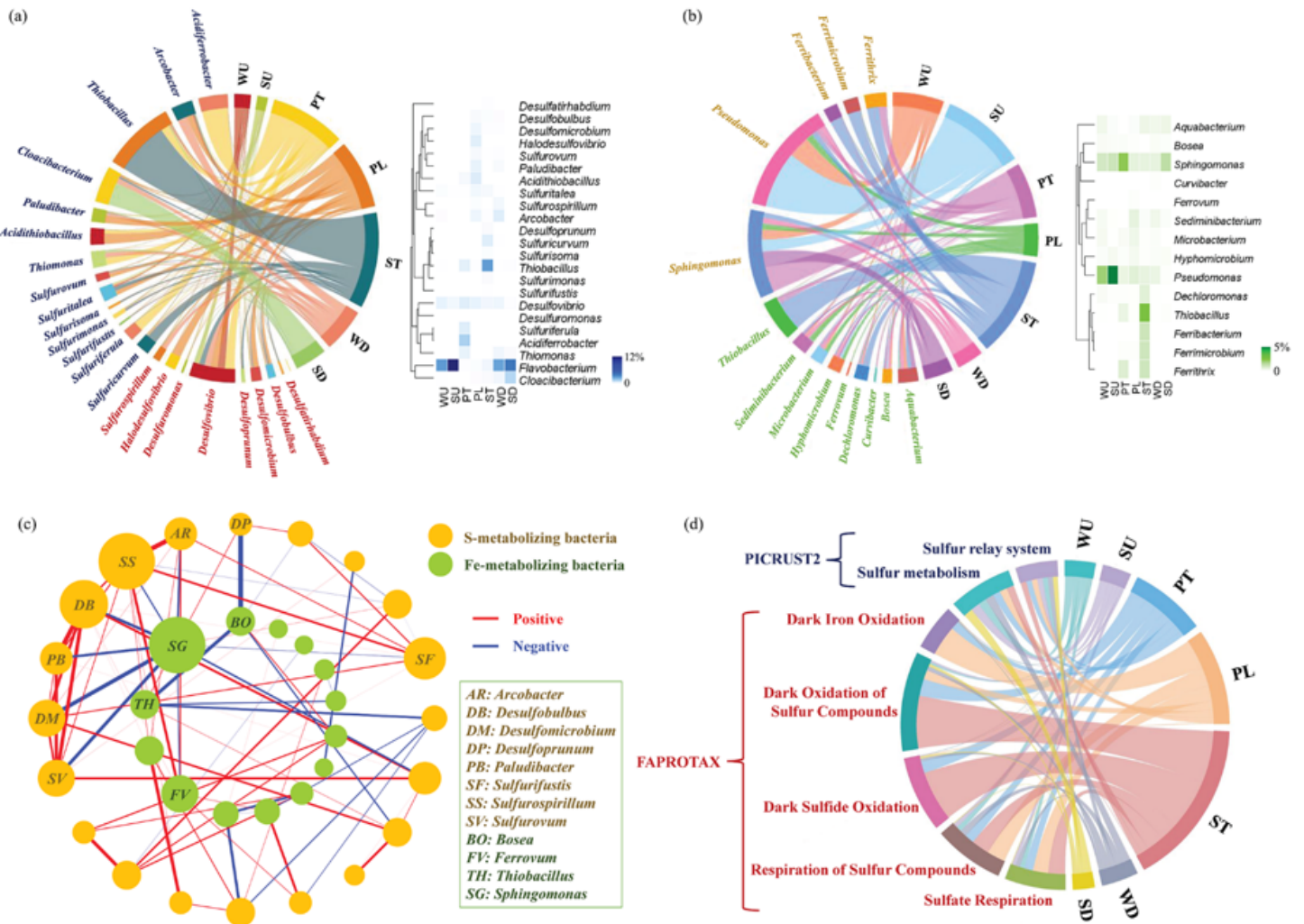


Figure 4

The distribution of sulfur-metabolizing bacteria (a) and iron-metabolizing bacteria (b). The network of sulfur-metabolizing bacteria and iron-metabolizing bacteria (c) and predicted function of sulfur and iron metabolism (d). The diameter of nodes in (c) means node degree.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [AppendixA.Supplementary.docx](#)