

## Point-by-point response to the comments of the reviewer #1

1. **Comments from Referees:** The section of 2.1 should include more details about the soil sampling.

**Response:** Thanks for this comment. I have rewritten the soil sampling details in the section of 2.1.

**Changes in manuscript:**

(Lines 69-71) "Samples of oxidized mine tailings on the surface (0-20 cm) were collected using a steel corer in October 2015. Mine tailing samples stored in sterilized plastic bags were transported to the laboratory in an ice cooler and stored at -20°C before analysis."

2. **Comments from Referees:** The experiment treatments was also not clear. From section 2.2, we can't judge how many treatments were set. Please specify. Moreover, what's samples were conducted qPCR, sequencing and cloning were also confused.

**Response:** Thanks for this comment. I have rewritten the experiment treatments in sections. And samples that conducted qPCR, sequencing and cloning were also written. And the detail data for qPCR has been showed in the supplementary materials.

**Changes in manuscript:**

(1) For experiment treatment: (Lines 78-85) "There were a total of four treatments in the microcosms of the two mine tailings. For each mine tailing, fresh mine tailings (equivalent to 10.0 g d.w.s.) were mixed with a total of 2 g of sterile pulverized FeS<sub>2</sub> at approximately 60% maximum water-holding capacity as the FeS<sub>2</sub> treatment, followed by incubation at 25°C in the dark for 14 days. Yangshanchong mine tailing samples (YM) cultured with FeS<sub>2</sub> are abbreviated as YM\_FeS<sub>2</sub>, and Shuimuchong mine tailing samples (SM) cultured with FeS<sub>2</sub> are abbreviated as SM\_FeS<sub>2</sub>. In addition, fresh mine tailings at approximately 60% maximum water-holding capacity without any additive were used as the control groups and abbreviated as YM\_ck and SM\_ck. For each treatment, the microcosms were incubated with 10% <sup>13</sup>C-CO<sub>2</sub> or <sup>12</sup>C-CO<sub>2</sub>, and both treatments were constructed in triplicate for DNA-SIP analysis."

(2) For samples that conducted qPCR, sequencing and cloning: (Lines 110-112) "Real-time quantitative PCR analysis was performed on a CFX96 optical real-time detection system (Bio-Rad, Laboratories Inc., Hercules, CA, USA) to determine the copy numbers of the *cbbL*, *cbbM* and 16S rRNA genes in DNA gradient fractions from the YM\_FeS<sub>2</sub> and SM\_FeS<sub>2</sub> DNA-SIP microcosms." (Lines 126-129) "The composition of the bacterial communities in different samples was assessed by pyrosequencing of the 16S rRNA genes. The 16S rRNA gene from the <sup>13</sup>C-labeled DNA fraction, which had CsCl buoyant densities of 1.738 g·mL<sup>-1</sup> in the heavy fraction in YM\_FeS<sub>2</sub> and 1.734 g·mL<sup>-1</sup> in the heavy fraction in SM\_FeS<sub>2</sub>, was also amplified for pyrosequencing." (Lines 144-146) "Clone libraries of the *cbbL* and *cbbM* genes were also constructed from the <sup>13</sup>C-labeled DNA fractions with CsCl buoyant densities of 1.738 g·mL<sup>-1</sup> in the heavy fraction in YM\_FeS<sub>2</sub> and 1.734 g·mL<sup>-1</sup> in the heavy fraction in SM\_FeS<sub>2</sub>."

3. **Comments from Referees:** For section 4.1, authors only described how Carbon sequestration in mine tailings but without any discussion Combining with your own data.

**Response:** Thanks for this comment. I have rewritten the discussion based on this comments.

### Changes in manuscript:

(Lines 263-290) "4.1 The effect of FeS<sub>2</sub> on the whole bacterial community in mine tailings

Acidic polymetallic mine tailings have strong potential for pyrite oxidation. In this study, a large amount of sulfuric acid was generated (increases of approximately 19.95 mg/g and 14.64 mg/g in YM and SM, respectively), and a persistent decline in pH was observed (pH decreased by 0.44 and 0.35 in YM and SM, respectively) in only 14 days. These changes clearly indicated oxidization of pyrite (i.e., acidification) in mine tailings. Previous studies have found that some bacterial phyla, such as Firmicutes and Nitrospirae, significantly increase (Chen et al., 2013; Liu et al., 2014) with the acidification process of mine tailings. In the present study, the bacterial composition in the different mine tailings varied greatly, and only the Firmicutes phylum increased in both tested mine tailings under pyrite addition. This group might participate in the oxidization of sulfide minerals (Chen et al., 2013), such as *Sulfobacillus*, which accounted for the majority of Firmicutes. Many other microorganisms might be inhibited under pyrite addition. Korehi et al. (2014) and Liu et al. (2014) also indicated that the ongoing oxidation process in mine tailings was accompanied by an increase in Firmicutes and a decrease in Actinobacteria and all classes of Proteobacteria except Gammaproteobacteria. In addition, Chen et al. (2013) and Liu et al. (2014) found that the relative abundances of Euryarchaeota belonging to archaea significantly increased with decreasing pH, which indicates that this taxon is an indicator of metal contamination (Hur et al., 2011). Euryarchaeota compete with  $\beta$ -Proteobacteria for ecological niches under such acidic conditions (Liu et al., 2014). However, in this study, only a few archaea were detected, which might be related to differences in primer affinities and samples.

The growth of microorganisms in bare mine tailings is usually limited by the availability of organic carbon (Schimel and Weintraub, 2003). Pyrite oxidization in mine tailings further enhanced the acidity of the mine tailings (pH decreased to 2.77 and 2.57 in YM\_FeS<sub>2</sub> and SM\_FeS<sub>2</sub>, respectively). As a result, only microorganisms that were resistant to infertility and/or acidophilic conditions could maintain high activities. In the present study, *Conexibacter*, *Alicyclobacillus*, *Bacillus*, *Sulfobacillus*, *Leptospirillum*, *Rhodoplanes*, *Methylobacterium*, *Acidiphilium*, *Novosphingobium* and *Methyloversatilis* were the top genera with relative abundances above 0.2% in the mine tailings. Some specific taxa, including the genera *Alicyclobacillus*, *Sulfobacillus*, *Leptospirillum* and *Acidiphilium*, increased in both of the tested mine tailings under pyrite addition, indicating high consistency of dominant bacterial genera in different mine tailings. It is possible that in the case of pyrite oxidization and the availability of organic carbon, acidophilic and/or autotrophic bacteria could be stimulated (Deng et al., 2016; Antonelli et al., 2018), and the main carbon fixers found in these two mine tailings may be derived from the same groups."

## **Point-by-point response to the comments of the reviewer #2**

1. **Comments from Referees:** The manuscript explored the autotrophic microbes and the FeS<sub>2</sub> facilitation role in acidic mine tailings using stable isotope and molecular methods. The results showed that FeS<sub>2</sub> facilitated CO<sub>2</sub>-fixing by microbes and increased the abundances of relevant autotrophs. The study is very interesting, which could provide new insights into the autotrophic roles in extreme environments. However, the article writing is awful in logic, result description and interpretation.

**Response:** We thank the reviewer for pointing out the importance of this study and giving me a number of useful advices. And I make major revisions according to these comments.

2. **Comments from Referees:** The introduction did not show some key points relevant to the research, such as possible CO<sub>2</sub>-fixing pathways and autotrophs in acidic sulfur-enriched environments. The introduction was not well structured and really needs rewrite.

**Response:** Thanks for this comment. I have rewritten the introduction details according to this comment.

### **Changes in manuscript:**

(Lines 29-64) "Soil ecosystems have great potential as carbon sinks to stabilize CO<sub>2</sub> and regulate climate change (White et al., 2000), and atmospheric CO<sub>2</sub> can be fixed into plants by photosynthesis and assimilated into soils as soil organic carbon (SOC) by decomposition and microbial activity (Deng et al., 2016;Antonelli et al., 2018). Currently, chemolithoautotrophic organisms fix atmospheric CO<sub>2</sub> by six pathways, including the widely distributed Calvin-Benson-Bassham (CBB) cycle, the reductive tricarboxylic acid (rTCA) cycle, the reductive acetyl-CoA pathway and the recently discovered 3-hydroxypropionate/4-hydroxybutyrate (HP/HB) cycles (Berg, 2011;Alfreider et al., 2017).

The ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) enzyme is the most prominent enzyme on earth (Raven, 2013) and is important in the CBB cycle. The CBB cycle is the most prevalent means of CO<sub>2</sub> fixation by autotrophs (Tabita, 1999;Berg, 2011). In addition, the genes encoding the large subunit of RuBisCO serve as autotroph markers (Alfreider et al., 2017;Berg, 2011). The *cbbL* and *cbbM* genes encode RuBisCO form I and form II, respectively, with 25 to 30% amino acid sequence identity (Tabita et al., 2008).

Compared with soil ecosystems, polymetallic mine tailings exhibit specific features, including a lack of organic matter, nutrients and nutrient-holding capacity (Lottermoser, 2010;Young et al., 2015), that restrict plant growth, and plant productivity is generally difficult to restore in mining wastelands (Hu et al., 2018;Li et al., 2017). The limited amount of organic matter in mine tailings also inhibits the activities of heterotrophic microorganisms, and therefore the microorganisms in these environments are lithotroph-dominant (Li et al., 2015). Therefore, these autotrophic microbes may play a role that cannot be ignored for organic carbon sequestration in mine tailings. In addition, polymetallic mine tailings have also considerable potential to stabilize the levels of atmospheric CO<sub>2</sub> (Harrison et al., 2013) through the carbonation of noncarbonate minerals, including the dissolution of silicates, hydroxides and oxides and the precipitation of carbonate minerals (McCutcheon et al., 2016;Meyer et al., 2014;McCutcheon et al., 2014). However, previous studies focused on carbonate mineral precipitation, while the role of autotrophs in carbon sequestration by mine tailings has been neglected.

Polymetallic mine tailings contain sulfide minerals (e.g., pyrite), and the oxidation of these sulfide minerals leads to a decrease in pH, also known as mine tailing acidification. Previous studies have noted that polymetallic mine tailings have lithotroph-dominant microbial compositions (Li et al., 2015) due to the limited amount of organic matter. Consequently, acidophilic, chemoautotrophic bacteria, including *Acidithiobacillus*, *Leptospirillum* and *Sulfobacillus* (Chen et al., 2013; Liu et al., 2014), can promptly participate in ferrous and sulfur oxidation in mine tailings, and these autotrophic taxa play leading roles in carbon cycling and energy flow during the mine tailing acidification process. However, the relationship between the oxidation of sulfide minerals and carbon sequestration by these acidophilic, chemoautotrophic bacteria is still unknown. In the present study, we conducted a microcosm experiment using mine tailings collected from two mines to determine the effects of sulfide mineral (pyrite) oxidation on carbon sequestration in mine tailings through the addition of pyrite. In addition, the main carbon fixers were analyzed through DNA-based stable isotope probing (DNA-SIP) and *cbbL* and *cbbM* gene analysis. Our objectives were to investigate whether sulfide mineral oxidation can stimulate carbon sequestration in mine tailings and to identify the key carbon sequestration groups in mine tailings during the acidification process.”

**3. Comments from Referees:** The method section failed to describe key details:

1) weather the samples were washed by acid prior to measuring isotope compositions? 2) no descriptions on chemical analysis in samples, i.e. solutes for Fe<sup>2+</sup>, Fe<sup>3+</sup>, SO<sub>4</sub><sup>2-</sup>; 3) no citations for the primer sets, which were apparently designed in the study; 4) no informations on PCR reactions 5) how did the authors determine the PCR efficiency? 6) how did the authors qualify gene abundance? standard curves? 7) no statistic software informations; 8) how many replicates were for each treatment?

**Response:** Thanks for this comment. I have rewritten and added more information according to this comment.

**Changes in manuscript:**

(1) (Lines 88-89) “Carbon isotope composition was analyzed by a Delta V Advantage Mass Spectrometer (Thermo Fisher Scientific, Inc., USA) coupled with an elemental analyzer (Flash2000; HT Instruments, Inc., USA) in continuous flow mode.” (Lines 92-94) “The carbon isotope composition and TOC content were analyzed after performing a soil acidification pretreatment method to remove inorganic carbon as described previously (Wang et al., 2015)”

(2) (Lines 96-99) “The Fe<sup>2+</sup> and Fe<sup>3+</sup> in the soils were extracted by HCl. The Fe<sup>2+</sup> in the extract was measured by a spectrophotometric method after mixing with phenanthroline and trisodium citrate, and the Fe<sup>3+</sup> in the extract was reduced to Fe<sup>2+</sup> by hydroxylammonium chloride and measured by a spectrophotometric method (Heron et al., 1994). The total sulfate ion content was determined via ion chromatography after extraction by sodium hydroxide, as described previously (Yin and Catalan, 2003).”

(3) Citations for the primer sets have been added. (Lines 112-117) “The K2f/V2r primer pair (K2f: 5'-ACC AYC AAG CCS AAG CTS GG-3' and V2r: 5'-GCC TTC SAG CTT GCC SAC CRC-3') (Nanba et al., 2004), the *cbbMF/cbbMR* primer pair (*cbbMF*: 5'-TTC TGG CTG GGB GGH GAY TTY ATY AAR AAY GAC GA-3' and *cbbM-R*: 5'-CCG TGR CCR GCV CGR TGG TAR TG-3') (Campbell and Cary, 2004) and the 515F/907R primer pair (515F: 5'-GTG CCA GCM GCC GCG G-3' and 907R: 5'-CCG TCA ATT CMT TTR AGT TT-3') (Zhou et al., 2011) were used to amplify the *cbbL*, *cbbM* and 16S rRNA genes,

respectively.”

(4) Informations on PCR reactions have been added. (Lines 119-120) “qPCR analysis of the *cbbL*, *cbbM* and 16S rRNA genes was performed under the following conditions: 40 cycles of 30 s at 95°C, 30 s at 55°C (*cbbL* and 16S rRNA genes) or 57°C (*cbbM* gene), and 45 s at 72°C.”

(5) The PCR efficiency was determined according to the standard curves. (Lines 120-122) “Standard curves were obtained using 10-fold serial dilutions of linearized recombinant plasmids containing the *cbbL*, *cbbM* and 16S rRNA genes with known copy numbers. The amplification efficiencies were 90-100%, which were obtained with  $R^2$  values greater than 0.99.”

(6) The gene abundance was calculated based on the standard curves. (Lines 120-122) “Standard curves were obtained using 10-fold serial dilutions of linearized recombinant plasmids containing the *cbbL*, *cbbM* and 16S rRNA genes with known copy numbers.”

(7) Statistic software informations has been added. (Lines 154-159) “Bray-Curtis distance matrices for the overall bacterial community composition among the given samples were calculated in R v.3.3.2 using the ‘vegdist’ function of the vegan package and visualized by nonmetric multidimensional scaling (NMDS) in Origin 8. A heatmap of dominant genera with relative abundances above 0.2% was applied for plotting in the R environment with the pheatmap package. The translated *cbbL* and *cbbM* sequences from the heavy fractions were used to construct a phylogenetic tree by the neighbor-joining method using the MEGA package, version 7.0.”

(8) I have rewritten the experiment treatments in sections. (Lines 175-182) “There were a total of four treatments in the microcosms of the two mine tailings. For each mine tailing, fresh mine tailings (equivalent to 10.0 g d.w.s.) were mixed with a total of 2 g of sterile pulverized FeS<sub>2</sub> at approximately 60% maximum water-holding capacity as the FeS<sub>2</sub> treatment, followed by incubation at 25°C in the dark for 14 days. Yangshanchong mine tailing samples (YM) cultured with FeS<sub>2</sub> are abbreviated as YM\_FeS<sub>2</sub>, and Shuimuchong mine tailing samples (SM) cultured with FeS<sub>2</sub> are abbreviated as SM\_FeS<sub>2</sub>. In addition, fresh mine tailings at approximately 60% maximum water-holding capacity without any additive were used as the control groups and abbreviated as YM\_ck and SM\_ck. For each treatment, the microcosms were incubated with 10% <sup>13</sup>C-CO<sub>2</sub> or <sup>12</sup>C-CO<sub>2</sub>, and both treatments were constructed in triplicate for DNA-SIP analysis.”

**4. Comments from Referees:** Fig1 symbols are very confusing, and no descriptions on the above and bottom columns.

**Response:** Thanks for this comment. I have redrawn the figure and have rewritten the figure legend.

**Changes in manuscript:**

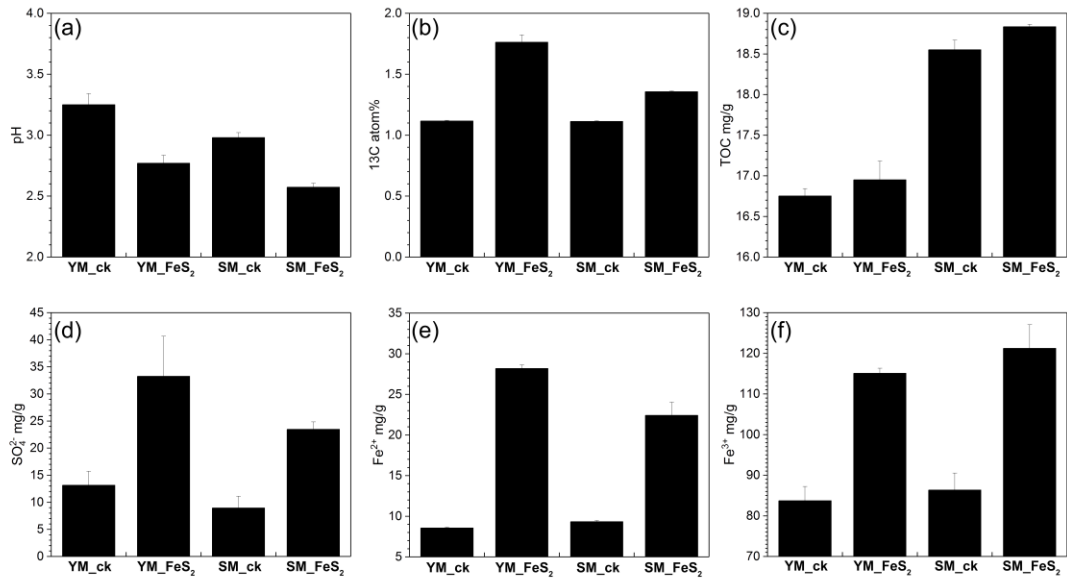


Fig. 1 pH values (a), <sup>13</sup>C atom % (b), TOC (c) contents, SO<sub>4</sub><sup>2-</sup> (d), Fe<sup>2+</sup> (e) and Fe<sup>3+</sup> (f) in mine tailings. The error bars indicate the standard errors of three subsamples for each tailing sample. To determine <sup>13</sup>C atom % (b), all analyzed samples were treated with <sup>13</sup>C-CO<sub>2</sub> in microcosms. YM\_ck, control group of Yangshanchong mine tailings; SM\_ck, control group of Shuimuchong mine tailings; YM\_FeS<sub>2</sub>, Yangshanchong mine tailings treated with FeS<sub>2</sub>; SM\_FeS<sub>2</sub>, Shuimuchong mine tailings treated with FeS<sub>2</sub>.

**5. Comments from Referees:** No specific legends or descriptions on the two inserts in Fig. 3, and the color differences are not clear

**Response:** Thanks for this comment. I have redrawn the figure and have rewritten the figure legend.

**Changes in manuscript:**

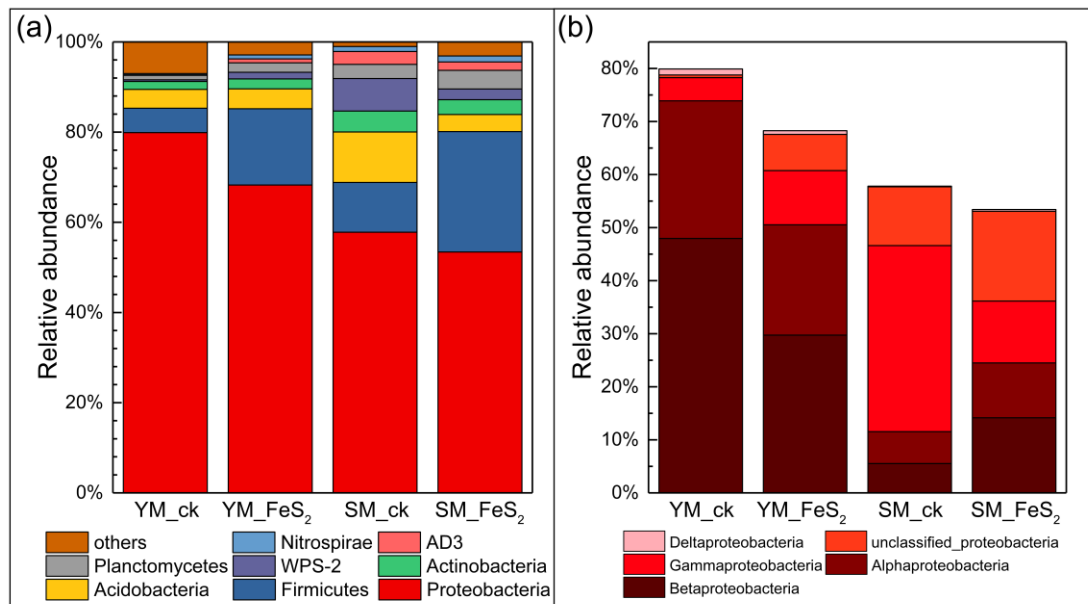


Fig. 3 Relative abundances (percentages) of the main identified bacterial taxonomic groups, i.e.,

the phyla Proteobacteria, Firmicutes, Acidobacteria, Actinobacteria, WPS-2, Planctomycetes, AD3 and Nitrospirae (a); and the classes Alphaproteobacteria, Betaproteobacteria, Deltaproteobacteria and Gammaproteobacteria (within the phylum Proteobacteria) (b). For each tailing sample, the relative abundances of the sequences assigned to a given taxonomic unit were calculated for each of three subsamples, and the average value was then used to represent the relative abundance of each tailing sample.

**6. Comments from Referees:** L252-261, 284-291, there are many super long sentences. A sentence usually contains maximum 22 words.

**Response:** Thanks for this comment. I have rewritten these sentences.

**Changes in manuscript:**

(1) (Lines 193-200) "In the Yangshanchong mine tailings, pyrite addition significantly increased the relative abundances of AD3, Nitrospirae and unclassified Proteobacteria by 0.75% (P=0.008), 0.59% (P=0.019) and 6.33% (P<0.001), respectively. In parallel, in Shuimuchong mine tailings, FeS<sub>2</sub> addition significantly increased the relative abundances of Firmicutes, Planctomycetes, unclassified Proteobacteria, Alphaproteobacteria, Betaproteobacteria, and Deltaproteobacteria by 15.69% (P<0.001), 0.97% (P<0.001), 5.88% (P=0.002), 4.35% (P=0.001), 8.61% (P<0.001) and 0.21% (P=0.003), respectively. However, in SM, the percentages of AD3, Acidobacteria, Actinobacteria and Gammaproteobacteria in SM by 0.97% (P=0.002), 7.43% (P=0.002), 1.35% (P=0.016) and 4.85% (P=0.002) were decreased under pyrite addition."

(2) (Lines 217-223) "In the Yangshanchong mine tailings, pyrite addition significantly increased the relative abundances of the genera Alicyclobacillus, Leptospirillum, Sulfobacillus and Acidiphilium by 0.67% (P=0.027), 0.74% (P=0.002), 8.86% (P=0.043) and 1.57% (P<0.001), respectively. In the Shuimuchong mine tailings, FeS<sub>2</sub> addition significantly increased the relative abundances of Alicyclobacillus, Bacillus, Sulfobacillus, Methylobacterium, Novosphingobium and Methyloversatilis by 0.57% (P<0.001), 9.94% (P<0.001), 5.99% (P<0.001), 0.15% (P<0.001), 2.06% (P=0.004) and 0.56% (P<0.001), respectively."

**7. Comments from Referees:** The CO<sub>2</sub>-fixing capacity by autotrophs should be calculated.

**Response:** Thanks for this comment. I have calculated the CO<sub>2</sub>-fixing capacity by autotrophs in the result.

**Changes in manuscript:**

(Lines 173-174) "The CO<sub>2</sub>-fixing capacities of autotrophs under FeS<sub>2</sub> addition were 9.50±0.91 mg/kg-d in YM and 3.69±0.11 mg/kg-d in SM."

**8. Comments from Referees:** L306-307, the statement is problematic: <sup>12</sup>CO<sub>2</sub> is a control relative to <sup>13</sup>CO<sub>2</sub>, so the shift to heavy fractions should not be observed in <sup>12</sup>CO<sub>2</sub>. L307-311, the statements are not correct: for the peak in <sup>13</sup>CO<sub>2</sub> occurred in the density of 1.72 rather than 1.73 in both <sup>12</sup>CO<sub>2</sub> and <sup>13</sup>CO<sub>2</sub>.

**Response:** Thanks for this comment. I have written the sentences.

**Changes in manuscript:**

(Lines 235-238) "*cbbL* and *cbbM* gene levels under <sup>13</sup>C-CO<sub>2</sub> treatment peaked at a density of 1.72 in both the <sup>12</sup>C-CO<sub>2</sub> and <sup>13</sup>C-CO<sub>2</sub> treatments. In addition, a shift toward heavy fractions was observed for the *cbbL* and *cbbM* gene abundances in the <sup>13</sup>C-CO<sub>2</sub> treatment (Fig. 5), with buoyant

densities of 1.738 g·mL<sup>-1</sup> in YM\_FeS<sub>2</sub> and 1.734 g·mL<sup>-1</sup> in SM\_FeS<sub>2</sub>.”

**9. Comments from Referees:** L311-314, the statements should go to discussion section

**Response:** Thanks for this comment. I have move the works into the discussion section.

**Changes in manuscript:**

(Lines 305-309) “In addition, only a few archaea were detected based on 16S rRNA gene sequencing, and the clone libraries of the *cbbL* and *cbbM* genes in the <sup>13</sup>C-labeled heavy fraction did not show archaeal sequences for Calvin cycle genes. These results indicated that bacterial carbon sequestration is mainly attributable to chemoautotrophic bacteria in pyrite oxidation of mine tailings. However, archaea may have higher activities in RuBisCO-mediated carbon metabolic pathways (Kono et al., 2017), which will require further study.”

**10. Comments from Referees:** Fig. 6, Cultured genus most related to OTU1, 2, 3 and 4 should be given for identifying purpose

**Response:** Thanks for this comment. I have added more information about the cultured genus most related to OTU1, 2, 3 and 4 in the supply materials

**Changes in manuscript:**

**Fig. 6** Phylogenetic tree of the translated *cbbL* and *cbbM* sequences in the heavy fractions from YM and SM treated with FeS<sub>2</sub>. Relative frequencies (%) are marked in the bar graph. Bootstrap values of >50% are indicated at branch points. The *cbbL* and *cbbM* gene copy numbers in the heavy fractions from FeS<sub>2</sub>-treated YM and SM are shown in the middle of the figure. The cultured genera most related to OTUs from the *cbbL* and *cbbM* clone libraries are shown in Table S1.

Table S1 Cultured genus most related to OTUs from *cbbL* and *cbbM* clone libraries

| cbbL OTU  | Closest affiliation                              | Max identity | cbbM OTU  | Closest affiliation                                | Max identity |
|-----------|--|--------------|-----------|--|--------------|
| cbbL-OTU1 | <i>Sulfobacillus acidophilus</i> (DQ139404)      | 99           | cbbM-OTU1 | Gamma proteobacterium (HQ675075)                   | 98           |
| cbbL-OTU2 | <i>Thiohalospira halophila</i> (GQ888600)        | 83           | cbbM-OTU2 | <i>Magnetospirillum moscoviense</i> (KF712469)     | 88           |
| cbbL-OTU3 | <i>Chromatium vinosum</i> (M26396)               | 78           | cbbM-OTU3 | Gamma proteobacterium (HQ675084)                   | 98           |
| cbbL-OTU4 | <i>Acidithiobacillus caldus</i> (GQ409763)       | 83           | cbbM-OTU4 | <i>Magnetospirillum magnetotacticum</i> (AF442517) | 90           |
| cbbL-OTU5 | <i>Thiobacillus denitrificans</i> (L42940)       | 86           | cbbM-OTU5 | <i>Acidithiobacillus ferrooxidans</i> (KT884974)   | 98           |
| cbbL-OTU6 | <i>Acidithiobacillus ferrooxidans</i> (AB362170) | 100          | cbbM-OTU6 | Thiobacillus sp. ( AB032829)                       | 99           |
| cbbL-OTU7 | <i>Chromatium vinosum</i> (M26396)               | 89           |           |  |              |
| cbbL-OTU8 | <i>Thioalkalivibrio versutus</i> (KY452013)      | 82           |           |  |              |
| cbbL-OTU9 | <i>Azospirillum lipoferum</i> (DQ787337)         | 100          |           |  |              |

**11. Comments from Referees:** cbb is not a correct gene name, it should be cbbL or cbbM.

**Response:** Thanks for this comment. I have rewritten the gene name across the manuscript.

**Changes in manuscript:**



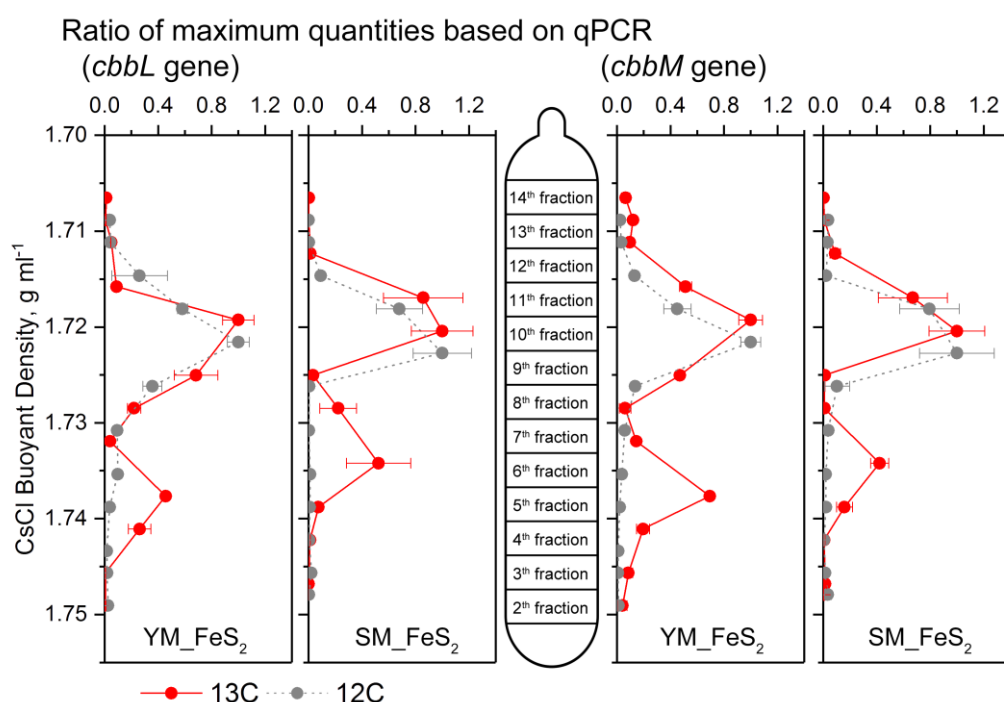
(Lines 240-241) “The *cbbL* and *cbbM* gene sequences from the clone libraries in the  $^{13}\text{C}$ -DNA heavy fraction treated with  $^{13}\text{C}$ - $\text{CO}_2$  were used for phylogenetic analysis (Fig. 6).”

(Lines 233-235) “For the quantitative analysis of *cbbL* and *cbbM* gene abundances, the buoyant densities of the DNA in isopycnic centrifugation gradients were used to assess the labeling efficiencies of *cbbL* or *cbbM* gene-carrying carbon fixers in the DNA-SIP microcosms (Fig. 5).”

**12. Comments from Referees:** Is Fig. 5 for FeS<sub>2</sub> treatments or raw mine tailings?

**Response:** Thanks for this comment. The figure was for FeS<sub>2</sub> treatment and I have redrawn the figure. And *cbbL* and *cbbM* gene abundance qPCR data are shown in the supplementary materials.

**Changes in manuscript:**



**Fig. 5** Quantitative distribution of *cbbL* and *cbbM* gene fragments across the entire buoyant density gradients of the DNA fractions from microcosms treated with FeS<sub>2</sub> and incubated with  $^{12}\text{C}$ - $\text{CO}_2$  or  $^{13}\text{C}$ - $\text{CO}_2$ . The normalized data consist of the ratio of the gene copy number for each DNA gradient to the maximum quantity for each treatment. The error bars represent the standard errors of triplicate microcosms, and each contains three technical replicates. *cbbL* and *cbbM* gene abundance qPCR data are shown in the supplementary materials.

**13. Comments from Referees:** L351-371, the paragraph should go to introduction section.

The discussion is far from the results, i.e. discuss why and how FeS<sub>2</sub> facilitates microbial CO<sub>2</sub>-fixing and changes the whole bacterial community.

**Response:** Thanks for this comment. I have rewritten the Introduction and Discussion according to this comment.

**Changes in manuscript:**

(1) Introduction: “Soil ecosystems have great potential as carbon sinks to stabilize CO<sub>2</sub> and regulate

climate change (White et al., 2000), and atmospheric CO<sub>2</sub> can be fixed into plants by photosynthesis and assimilated into soils as soil organic carbon (SOC) by decomposition and microbial activity (Deng et al., 2016;Antonelli et al., 2018). Currently, chemolithoautotrophic organisms fix atmospheric CO<sub>2</sub> by six pathways, including the widely distributed Calvin-Benson-Bassham (CBB) cycle, the reductive tricarboxylic acid (rTCA) cycle, the reductive acetyl-CoA pathway and the recently discovered 3-hydroxypropionate/4-hydroxybutyrate (HP/HB) cycles (Berg, 2011;Alfreider et al., 2017).

The ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) enzyme is the most prominent enzyme on earth (Raven, 2013) and is important in the CBB cycle. The CBB cycle is the most prevalent means of CO<sub>2</sub> fixation by autotrophs (Tabita, 1999;Berg, 2011). In addition, the genes encoding the large subunit of RuBisCO serve as autotroph markers (Alfreider et al., 2017;Berg, 2011). The *cbbL* and *cbbM* genes encode RuBisCO form I and form II, respectively, with 25 to 30% amino acid sequence identity (Tabita et al., 2008).

Compared with soil ecosystems, polymetallic mine tailings exhibit specific features, including a lack of organic matter, nutrients and nutrient-holding capacity (Lottermoser, 2010;Young et al., 2015), that restrict plant growth, and plant productivity is generally difficult to restore in mining wastelands (Hu et al., 2018;Li et al., 2017). The limited amount of organic matter in mine tailings also inhibits the activities of heterotrophic microorganisms, and therefore the microorganisms in these environments are lithotroph-dominant (Li et al., 2015). Therefore, these autotrophic microbes may play a role that cannot be ignored for organic carbon sequestration in mine tailings. In addition, polymetallic mine tailings have also considerable potential to stabilize the levels of atmospheric CO<sub>2</sub> (Harrison et al., 2013) through the carbonation of noncarbonate minerals, including the dissolution of silicates, hydroxides and oxides and the precipitation of carbonate minerals (McCutcheon et al., 2016;Meyer et al., 2014;McCutcheon et al., 2014). However, previous studies focused on carbonate mineral precipitation, while the role of autotrophs in carbon sequestration by mine tailings has been neglected.

Polymetallic mine tailings contain sulfide minerals (e.g., pyrite), and the oxidation of these sulfide minerals leads to a decrease in pH, also known as mine tailing acidification. Previous studies have noted that polymetallic mine tailings have lithotroph-dominant microbial compositions (Li et al., 2015) due to the limited amount of organic matter. Consequently, acidophilic, chemoautotrophic bacteria, including *Acidithiobacillus*, *Leptospirillum* and *Sulfobacillus* (Chen et al., 2013;Liu et al., 2014), can promptly participate in ferrous and sulfur oxidation in mine tailings, and these autotrophic taxa play leading roles in carbon cycling and energy flow during the mine tailing acidification process. However, the relationship between the oxidation of sulfide minerals and carbon sequestration by these acidophilic, chemoautotrophic bacteria is still unknown. In the present study, we conducted a microcosm experiment using mine tailings collected from two mines to determine the effects of sulfide mineral (pyrite) oxidation on carbon sequestration in mine tailings through the addition of pyrite. In addition, the main carbon fixers were analyzed through DNA-based stable isotope probing (DNA-SIP) and *cbbL* and *cbbM* gene analysis. Our objectives were to investigate whether sulfide mineral oxidation can stimulate carbon sequestration in mine tailings and to identify the key carbon sequestration groups in mine tailings during the acidification process.”

(2) Discussion: “4.1 The effect of FeS<sub>2</sub> on the whole bacterial community in mine tailings

Acidic polymetallic mine tailings have strong potential for pyrite oxidation. In this study, a large amount of sulfuric acid was generated (increases of approximately 19.95 mg/g and 14.64 mg/g in YM and SM, respectively), and a persistent decline in pH was observed (pH decreased by 0.44 and 0.35 in YM and SM, respectively) in only 14 days. These changes clearly indicated oxidization of pyrite (i.e., acidification) in mine tailings. Previous studies have found that some bacterial phyla, such as Firmicutes and Nitrospirae, significantly increase (Chen et al., 2013; Liu et al., 2014) with the acidification process of mine tailings. In the present study, the bacterial composition in the different mine tailings varied greatly, and only the Firmicutes phylum increased in both tested mine tailings under pyrite addition. This group might participate in the oxidization of sulfide minerals (Chen et al., 2013), such as *Sulfobacillus*, which accounted for the majority of Firmicutes. Many other microorganisms might be inhibited under pyrite addition. Korehi et al. (2014) and Liu et al. (2014) also indicated that the ongoing oxidation process in mine tailings was accompanied by an increase in Firmicutes and a decrease in Actinobacteria and all classes of Proteobacteria except Gammaproteobacteria. In addition, Chen et al. (2013) and Liu et al. (2014) found that the relative abundances of Euryarchaeota belonging to archaea significantly increased with decreasing pH, which indicates that this taxon is an indicator of metal contamination (Hur et al., 2011). Euryarchaeota compete with  $\beta$ -Proteobacteria for ecological niches under such acidic conditions (Liu et al., 2014). However, in this study, only a few archaea were detected, which might be related to differences in primer affinities and samples.

The growth of microorganisms in bare mine tailings is usually limited by the availability of organic carbon (Schimel and Weintraub, 2003). Pyrite oxidization in mine tailings further enhanced the acidity of the mine tailings (pH decreased to 2.77 and 2.57 in YM<sub>FeS<sub>2</sub></sub> and SM<sub>FeS<sub>2</sub></sub>, respectively). As a result, only microorganisms that were resistant to infertility and/or acidophilic conditions could maintain high activities. In the present study, *Conexibacter*, *Alicyclobacillus*, *Bacillus*, *Sulfobacillus*, *Leptospirillum*, *Rhodoplanes*, *Methylobacterium*, *Acidiphilium*, *Novosphingobium* and *Methyloversatilis* were the top genera with relative abundances above 0.2% in the mine tailings. Some specific taxa, including the genera *Alicyclobacillus*, *Sulfobacillus*, *Leptospirillum* and *Acidiphilium*, increased in both of the tested mine tailings under pyrite addition, indicating high consistency of dominant bacterial genera in different mine tailings. It is possible that in the case of pyrite oxidization and the availability of organic carbon, acidophilic and/or autotrophic bacteria could be stimulated (Deng et al., 2016; Antonelli et al., 2018), and the main carbon fixers found in these two mine tailings may be derived from the same groups.

#### 4.2 The effect of FeS<sub>2</sub> on bacterial carbon sequestration in mine tailings

Previous studies have shown that mine tailings provide an excellent substrate for carbon sequestration through the formation of carbonate due to the large surface area of the material grains (McCutcheon et al., 2016). Compared to soils and natural bedrock, mine tailings may possess higher carbonate precipitation rates (Wilson et al., 2009). However, in this study, the <sup>13</sup>C content and TOC content in mine tailings increased slightly. DNA-SIP analysis demonstrated that a considerable amount of <sup>13</sup>C-CO<sub>2</sub> was assimilated by carbon fixers in the <sup>13</sup>C-CO<sub>2</sub>-labeled mine tailing samples, leading to a significant shift of *cbbL* or *cbbM* gene-carrying genomic DNA into the heavy fraction. Both of these results indicated the contribution of microbial activities to carbon sequestration in mine tailings. To the best of our present knowledge, this report is the first to elucidate carbon sequestration by autotrophic groups in mine tailings based on isotope tracers and DNA-SIP. Previous studies have found that microbial photosynthesis accelerates carbonate mineral

precipitation and further induces mineralization (McCutcheon et al., 2014; McCutcheon et al., 2016). However, in the present study, the microcosms were not cultured in the presence of illumination, and as a result, chemoautotrophic microorganisms, particularly iron and/or sulfide oxidizers (such as *Sulfobacillus*), were the dominant carbon fixers. In addition, only a few archaea were detected based on 16S rRNA gene sequencing, and the clone libraries of the *cbbL* and *cbbM* genes in the <sup>13</sup>C-labeled heavy fraction did not show archaeal sequences for Calvin cycle genes. These results indicated that bacterial carbon sequestration is mainly attributable to chemoautotrophic bacteria in pyrite oxidization of mine tailings. However, archaea may have higher activities in RuBisCO-mediated carbon metabolic pathways (Kono et al., 2017), which will require further study.

During pyrite oxidization in mine tailings, some acidophilic autotrophic microorganisms have very high activity levels. For example, the *Sulfobacillus* and *Leptospirillum* genera, both of which are vital ferrous and sulfur oxidizers, increased significantly during pyrite oxidization. Zhang et al. (2016) found genes for the CBB pathway and rTCA, but no other CO<sub>2</sub> fixation pathways, in a copper bioleaching microbial community. For the CBB pathway in this study, the *Sulfobacillus*-like *cbbL* gene was the primary carbon fixing-associated gene. Nancucheo and Johnson (2012) found that among acidophilic prokaryotes isolated from mine-impacted environments, the ability to metabolize glycolic acid appeared to be restricted to Firmicutes (e.g., *Sulfobacillus*), and the glycolic acid in all of these acidophiles might be due to the activity of RuBisCO (Nancucheo and Johnson, 2012). Previous studies have confirmed the presence of *Sulfobacillus* in mine tailings (Coral et al., 2018; Yu et al., 2018); *Sulfobacillus* has the ability to oxidize or reduce Fe(III) and oxidize sulfur (Dold et al., 2005). This ability is important, as this genus likely leads to a high mineral dissolution rate by adhering to mineral surfaces and further enhancing sulfide mineral oxidation (Li et al., 2016; Becker et al., 2011). None of the *cbbL* or *cbbM* genes identified were highly homologous to genes in *Leptospirillum*, but this may be due to primer specificity. Nevertheless, Marín et al. (2017) found that the rTCA carbon fixation pathway genes were mainly found in by *Leptospirillum* spp. RuBisCO is the most prominent enzyme, and the gene coding for the large subunit of RuBisCO serves as a marker for the analysis of autotrophic organisms, including bacteria, using the CBB cycle (Berg, 2011). In addition, the *Sulfobacillus*-like *cbbL* gene dominated the <sup>13</sup>C-labeled DNA of the carbon-fixing taxa. Furthermore, the higher relative abundance of *Sulfobacillus* than *Leptospirillum*, according to 16S rRNA analysis, demonstrates the contribution of the *Sulfobacillus*-like *cbbL* gene to carbon sequestration. Even so, while the number (or relative abundance) of autotrophs demonstrated their ability to sequester carbon, it did not reflect their ability to perform or their importance in ferrous and sulfur oxidation. For example, the reduced percentage of the genus *Acidithiobacillus* in the two mine tailings did not reflect the contribution of this genus to the oxidation of iron and sulfur. Falagán et al. (2017) highlighted the importance of thermotolerant acidophiles, such as *Acidithiobacillus* and the genus *Sulfobacillus*, in extracting and recovering metals from mine tailings. Furthermore, it has been known for many years that *Acidithiobacillus* can obtain energy by catalyzing the oxidation reaction of Fe<sup>2+</sup> to Fe<sup>3+</sup> from sulfites (Dold, 2014), and this may significantly speed up the rate of ferrous oxidization. The decreased level of total carbon, including low-molecular-weight carboxylic acids LMWCA, may also limit the activity of this bacterium (Dold et al., 2005).

In conclusion, this study was the first to elucidate carbon sequestration by autotrophic groups in the acidification process of mine tailings based on isotope tracers and DNA-SIP. Our results

demonstrated higher  $^{13}\text{C}$  atom % values with the addition of pyrite than in control groups after a 14-day incubation, as well as a significant increase in the total organic carbon content. The *Sulfobacillus* genus was dominant in the pyrite-treated bacterial communities and was also the primary carbon fixer with the RuBisCO form I-encoding gene *cbbL*. Finally, the *cbbL* gene may play a vital role in carbon sequestration in the sulfide mineral oxidation of mine tailings.”