

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

1. **Comments from Referees:** I know it is a revised manuscript, which was substantially improved. However, I still have some concerns. The results between bacterial community structure changes and carbon fixing bacteria were disconnected. Furthermore, the manuscript is written with plenty of grammatical mistakes, therefore I suggest authors to seek assistance from a native speaker for proofreading.

Response: We thank the reviewer for giving me a number of useful advices. And I make major revisions according to these comments. Firstly, the dominant genera analysis from results section 3.2 was rewritten, which pointed out the dominant genera in the pyrite oxidation of mine tailings and also highlighted the importance of the autotrophic genera in samples. Similarly, the fig.4 has been redrawn to reflect the dominant genera including autotrophic taxon in mine tailings. Secondly, the English Language is revised by the highly qualified native English speaking editors at American Journal Experts after we rewrote the manuscript.



Changes in the result 3.2 from manuscript:

(Lines 207-221) "The total number of genera assigned to known taxa accounted for 29.89% of the total bacterial communities. In addition, we constructed a heatmap diagram (Fig. 4) that shows the top 51 dominant genera with relative abundances above 0.02% in the mine tailings, accounting for 29.16% of the total bacterial communities. Specifically, *Sulfobacillus* (8.04%) and *Novosphingobium* (8.60%) accounted for 16.64% of the total bacterial communities and were the dominant taxa in the mine tailings. In contrast, autotrophic bacteria including *Rhodanobacter* (0.04%), *Pseudomonas* (0.02%), *Acidithiobacillus* (0.02%), *Thiobacillus* (0.04%), *Ralstonia* (0.02%), *Thiomonas* (0.04%), *Burkholderia* (0.09%), *Acidiphilium* (1.49%), *Rhodobacter* (0.04%), *Rhodoplanes* (0.59%), *Nitrospira* (0.02%), *Leptospirillum* (0.80%), *Sulfobacillus* (8.04%), *Clostridium* (0.04%) and *Corynebacterium* (0.04%) accounted for 11.33% of the total bacterial communities. Whereby, *Thiobacillus*, *Acidiphilium*, *Leptospirillum*, *Acidithiobacillus* and *Sulfobacillus* are ferrous and sulfur-oxidizing bacteria. For the Yangshanchong mine tailings, pyrite addition significantly increased the relative abundances of the autotrophic genera *Acidithiobacillus*, *Leptospirillum*, *Sulfobacillus* and *Acidiphilium* by 0.02% ($P=0.001$), 0.74%

($P=0.002$), 8.86% ($P=0.043$) and 1.57% ($P<0.001$), respectively. FeS₂ addition also significantly increased the relative abundances of autotrophic genera in the Shuimuchong mine tailings: *Rhodanobacter*, *Acidithiobacillus*, *Thiobacillus* and *Sulfobacillus* by 0.07% ($P=0.016$), 0.03% ($P=0.034$), 0.02% ($P=0.030$) and 5.99% ($P<0.001$), respectively.”

2. **Comments from Referees:** line 30 'chemolithoautotrophic organisms fix atmospheric CO₂ by six pathways' this sentence is not connected to the previous sentence, the last sentence introduced plant photosynthesis, then it jumped into chemolithoautotrophs. Furthermore, there was no mention of autotrophic bacteria which also use cbb cycle.

Response: Thanks for this comment. I rewrote the first paragraph of the introduction to improve the logicity of the article, and I also pointed out that “the CBB cycle is the most prevalent means of CO₂ fixation by autotrophs including autotrophic bacteria” in Lines 34-35.

Changes in manuscript:

(Lines 28-38) “Soil ecosystems have great potential as carbon sinks to stabilize CO₂ and regulate climate change (White et al., 2000). Atmospheric CO₂ can be fixed in plants via photosynthesis and assimilated into soils via decomposition and microbial activity (Deng et al., 2016;Antonelli et al., 2018), and autotrophic bacteria play a significant role in carbon sequestration in soil ecosystems (Berg, 2011;Alfreider et al., 2017). Six autotrophic carbon sequestration mechanisms are widespread, including the 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). Among them, the CBB cycle is the most prevalent means of CO₂ fixation by autotrophs including autotrophic bacteria (Tabita, 1999;Berg, 2011). The enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) is important in the CBB cycle and is in fact the most prominent enzyme on Earth (Raven, 2013). The *cbbL* and *cbbM* genes encoding the large subunit of RuBisCO, with 25 to 30% amino acid sequence identity (Tabita et al., 2008), serve as autotroph markers (Berg, 2011;Alfreider et al., 2017).”

3. **Comments from Referees:** line 35-40 The *cbbL* and *cbbM* genes encode RuBisCO form I and form II, this is incorrect, they encode the large subunit of RuBisCO

Response: Thanks for this comment and point out mistakes in my limited knowledge. And I have written the sentences.

Changes in manuscript:

(Lines 37-38) “The *cbbL* and *cbbM* genes encoding the large subunit of RuBisCO, with 25 to 30% amino acid sequence identity (Tabita et al., 2008), serve as autotroph markers (Berg, 2011;Alfreider et al., 2017).”

4. **Comments from Referees:** line 80 For ¹³C-CO₂ labelling experiment, how the ¹³C-CO₂ were delivered?

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

Changes in manuscript:

(Lines 78-80) “The microcosms were incubated with 10% ¹³C-CO₂ or ¹²C-CO₂, and both

treatments were constructed in triplicate for DNA-SIP analysis.”

5. **Comments from Referees:** line 170-175 'Furthermore, the ^{13}C atom % values in YM_FeS₂ and SM_FeS₂ were higher than those in the control groups YM_ck and SM_ck, which exhibited ^{13}C atom % values of 1.76 ± 0.06 and 1.36 ± 0.01 .' there were four sample groups (YM_FeS₂, SM_FeS₂, YM, and SM, both with ^{13}C labelling and without) but only two ^{13}C % were provided.

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

Changes in manuscript:

(Lines 165-167) “and the ^{13}C atom % values in YM_FeS₂ (1.76 ± 0.06 ^{13}C atom %) and SM_FeS₂ (1.76 ± 0.06 ^{13}C atom %) were higher than those in the controls YM_ck (1.12 ± 0.01 ^{13}C atom %) and SM_ck (1.11 ± 0.01 ^{13}C atom %).”

6. **Comments from Referees:** line 190-195 'a total of 8 bacterial phyla and 4 proteobacterial classes were frequently identified in the two mine tailings' please define frequently

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

Changes in manuscript:

(Lines 185-186) “In this study, 8 dominant bacterial phyla/candidate divisions (relative abundance >1%) and 4 proteobacterial classes were identified in the two mine tailings,”

7. **Comments from Referees:** line 210-215 '...that shows the top 39 dominant genera with relative abundances above 0.20% from the mine tailings.' some of these taxa are not at genus level (i.e., all those unclassified taxa), they are sequences unclassified at various taxonomic levels.

line 210-215 'only a small number of genera were assigned to known taxa' If they can't be classified at genus level, how can author be certain that they are indeed bacterial genus?

Fig.4 there are some taxonomic units that presented at very high relative abundances (such as unclassified Commamonadaceae, Unclassified Xanthomonadaceae, Novosphingobium, Unclassified proteobacteria), which were much higher than the dominant carbon fixers such as Sulfobacillum, Leptospirillum. Is there any explanation for this?

Response: Thanks for these comments. And I have written the results section 3.2 which pointed out the dominant genera in the pyrite oxidation of mine tailings and also highlighted the importance of the autotrophic genera in samples. Those unclassified taxa have been removed, and the Fig.4 has been redrawn.

Changes in manuscript:

(Lines 207-221) “The total number of genera assigned to known taxa accounted for 29.89% of the total bacterial communities. In addition, we constructed a heatmap diagram (Fig. 4) that shows the top 51 dominant genera with relative abundances above 0.02% in the mine tailings, accounting for 29.16% of the total bacterial communities. Specifically, *Sulfobacillus* (8.04%) and *Novosphingobium* (8.60%) accounted for 16.64% of the total bacterial communities and were the dominant taxa in the mine tailings. In contrast, autotrophic bacteria including *Rhodanobacter* (0.04%), *Pseudomonas* (0.02%),

Acidithiobacillus (0.02%), *Thiobacillus* (0.04%), *Ralstonia* (0.02%), *Thiomonas* (0.04%), *Burkholderia* (0.09%), *Acidiphilium* (1.49%), *Rhodobacter* (0.04%), *Rhodoplanes* (0.59%), *Nitrospira* (0.02%), *Leptospirillum* (0.80%), *Sulfobacillus* (8.04%), *Clostridium* (0.04%) and *Corynebacterium* (0.04%) accounted for 11.33% of the total bacterial communities. Whereby, *Thiobacillus*, *Acidiphilium*, *Leptospirillum*, *Acidithiobacillus* and *Sulfobacillus* are ferrous and sulfur-oxidizing bacteria. For the Yangshanchong mine tailings, pyrite addition significantly increased the relative abundances of the autotrophic genera *Acidithiobacillus*, *Leptospirillum*, *Sulfobacillus* and *Acidiphilium* by 0.02% ($P=0.001$), 0.74% ($P=0.002$), 8.86% ($P=0.043$) and 1.57% ($P<0.001$), respectively. FeS_2 addition also significantly increased the relative abundances of autotrophic genera in the Shuimuchong mine tailings: *Rhodanobacter*, *Acidithiobacillus*, *Thiobacillus* and *Sulfobacillus* by 0.07% ($P=0.016$), 0.03% ($P=0.034$), 0.02% ($P=0.030$) and 5.99% ($P<0.001$), respectively.”

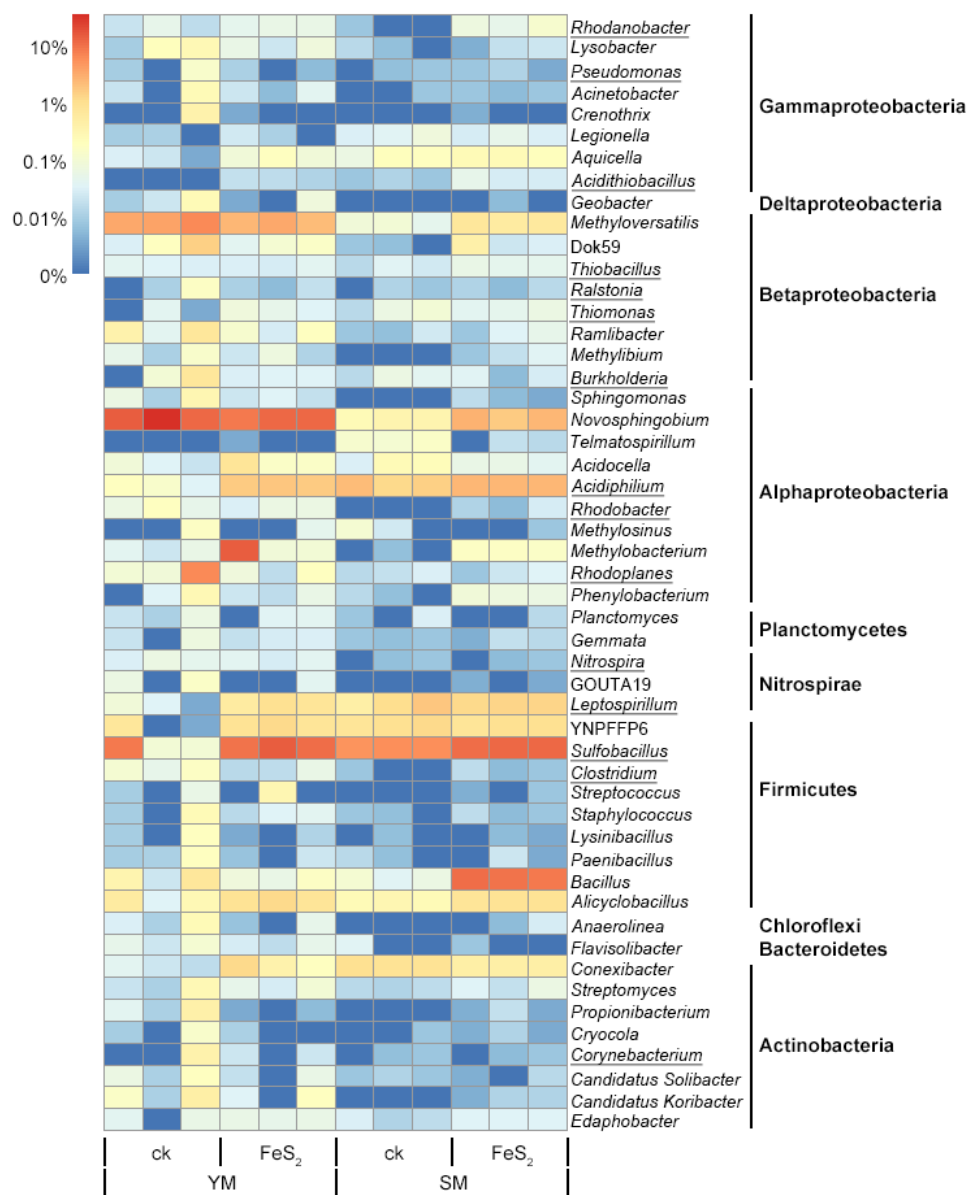


Fig. 4 Heatmap of the top genera with relative abundances above 0.02% in mine tailings. Autotrophic bacteria were marked with underlining.

8. **Comments from Referees:** line 265-270 '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.' Inconsistent with results, results paragraph 1 stated no significant changes in pH

Response: Thanks for this comment. Due to the unclear statement in previous paper version, reviewers and readers do not clear what I mean. In the result, I mean that no significant changes in chemical properties were found for the control groups compared to the original Yangshanchong and Shuimuchong acidic samples, it was pointed out the chemical properties in the YM_ck and SM_ck samples compared to the original samples showed in Lines 69-73 ("The properties of the mine tailings were as follows: Yangshanchong acidic samples, pH 3.21, total nitrogen (TN) 0.11 g·kg⁻¹, total organic carbon (TOC) 16 g·kg⁻¹, SO₄²⁻ 13.32 g·kg⁻¹, As_T 63.29 mg·kg⁻¹, Fe_T 133.46 g·kg⁻¹, Cu_T 1.95 g·kg⁻¹, Pb_T 27.58 mg·kg⁻¹, and Zn_T 205.44 mg·kg⁻¹; Shuimuchong acidic samples, pH 2.92, TN 0.11 g·kg⁻¹, TOC 18 g·kg⁻¹, SO₄²⁻ 8.84 g·kg⁻¹, As_T 51.77 mg·kg⁻¹, Fe_T 117.59 g·kg⁻¹, Cu_T 2.53 g·kg⁻¹, Pb_T 30.43 mg·kg⁻¹, and Zn_T 176.59 mg·kg⁻¹."). And pyrite addition decreased pH values in the YM and SM samples by 0.48±0.16 and 0.41±0.07, respectively. Pyrite addition also increased the SO₄²⁻ content by 252.96% and 262.35%, Fe²⁺ content by 329.47% and 240.38%, and Fe³⁺ content by 137.47% and 140.37% in the YM and SM samples, respectively.

Changes in manuscript:

(Lines 157-164) "No significant changes in chemical properties, pH values (3.25±0.09 in YM_ck and 2.98±0.04 in SM_ck), sulfate (SO₄²⁻) contents (13.15±2.58 mg/g in YM_ck and 8.95±2.19 mg/g in SM_ck), and TOC contents (16.75±0.09 mg/g in YM_ck and 18.55±0.12 mg/g in SM_ck), were found for the control groups compared to the original Yangshanchong and Shuimuchong acidic samples after 14 days of incubation (Fig. 1). However, the addition of pyrite decreased pH values in the YM and SM samples by 0.48±0.16 and 0.41±0.07, respectively. Pyrite addition also increased the SO₄²⁻ content by 252.96% and 262.35%, Fe²⁺ content by 329.47% and 240.38%, and Fe³⁺ content by 137.47% and 140.37% in the YM and SM samples, respectively. Together, these data indicate the occurrence of pyrite oxidization and acidification in mine tailings after pyrite addition."

9. **Comments from Referees:** line 285-290 'Some specific taxa, including the genera Alicyclobacillus, Sulfobacillus, Leptospirillum and Acidiphilium, increased in both of the tested mine tailings under pyrite addition' which figure does this statement refer to? Fig 4? please specify

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

Changes in manuscript:

(Lines 280-283) "The level of some specific taxa, including the autotrophic genera *Acidithiobacillus* and *Sulfobacillus* increased in both of the tested mine tailings under pyrite addition (Fig.4), indicating high consistency of dominant autotrophic bacterial genera in different mine tailings."

10. **Comments from Referees:** line 295-300 the 13C content and TOC content in mine tailings

increased slightly which figure/table does this result refer to fig 1? please specify

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

Changes in manuscript:

(Lines 293-294) “However, in this study, the ^{13}C and TOC contents in mine tailings increased slightly (Fig.1).”

11. **Comments from Referees:** line 295 to 300 'DNA-SIP analysis demonstrated that a considerable amount of ^{13}C -CO₂ was assimilated by carbon fixers in the ^{13}C -CO₂-labeled mine tailing samples, leading to a significant shift of cbbL or cbbM gene-carrying genomic DNA into the heavy fraction.' There was a new peak generated at Buoyant density of ~1.72, with density lower than the peak in control experiment, any explanation for the appearance of this peak? To me it is like a large proportion of autotrophic microorganisms detected in ^{13}C sample did not fix any carbon, this might explain why the intensity of ^{13}C peak at higher density (1.735-1.74) was much lower compared with ^{12}C peak.

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

Changes in manuscript:

(Lines 296-300) “In addition, a peak at a buoyant density of 1.72 g·mL⁻¹ in ^{13}C -CO₂-labeled mine tailing samples was observed, with a density lower than the peak in the ^{12}C -CO₂-labeled control experiment (see supply material.xlsx); the intensity of the ^{13}C peak at a higher density of 1.738 g·mL⁻¹ was also much lower than the ^{12}C peak at a higher density of 1.72 g·mL⁻¹. This suggests that a large proportion of the autotrophic microorganisms detected in the mine tailings samples did not fix carbon.”

12. **Comments from Referees:** line 305 '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 ^{13}C -labeled heavy fraction did not show archaeal sequences for Calvin cycle genes. i does the primer used in this study target Archaea sequence?

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

Changes in manuscript:

(Lines 308-309) “Nonetheless, archaea may have higher activities in RuBisCO-mediated carbon metabolic pathways (Kono et al., 2017), which will require further study.”

13. **Comments from Referees:** line 315-320 '...and the glycolic acid in all of these acidophiles might be due to the activity of RuBisCO' RuBisCO produce glycerate-3-phosphate, not glycolic acid

Response: Thanks for this comment. And I have written the sentences to correct my understanding.

Changes in manuscript:

(Lines 314-317) “among acidophilic prokaryotes isolated from mine-impacted environments, the ability to metabolize glycerate-3-phosphate appeared to be restricted to Firmicutes (e.g., *Sulfobacillus*) and that the glycerate-3-phosphate present in all of these acidophiles might be due to the activity of RuBisCO.”

14. **Comments from Referees:** line 320-325 'None of the cbbL or cbbM genes identified were

highly homologous to genes in *Leptospirillum*, but this may be due to primer specificity' later author mentioned that *Leptospirillum* only had rTCA, then there is no surprise that no *cbbL* gene classified as *Leptospirillum* been identified, and this has nothing to do with primer specificity.

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

Changes in manuscript:

(Lines 321-327) "Although none of the *cbbL* or *cbbM* genes identified in our study were highly homologous to genes in *Leptospirillum*, Marín et al. (2017) reported that rTCA carbon fixation pathway genes were mainly found in *Leptospirillum* spp. RuBisCO is the most prominent enzyme, and the genes encoding the large subunit of RuBisCO serve as a marker for the analysis of autotrophic organisms, including bacteria, using the CBB cycle (Berg, 2011). The *Sulfobacillus*-like *cbbL* gene dominated the ¹³C-labeled DNA among carbon-fixing taxa, and 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."

15. **Comments from Referees:** line 335-340 'Our results demonstrated higher ¹³C 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.' In the results part author said "total organic carbon contents (16.75±0.09 mg/g in YM_ck and 18.55±0.12 mg/g in SM_ck) exhibited no significant changes after 14 days", so was there significant changes in TOC or not?

Response: Thanks for this comment. Due to the unclear statement in previous paper version, reviewers and readers do not clear what I mean. In the result, I mean that no significant changes in chemical properties were found for the control groups compared to the original Yangshanchong and Shuimuchong acidic samples, it was pointed out the chemical properties in the YM_ck and SM_ck samples compared to the original samples showed in Lines 69-73 ("The properties of the mine tailings were as follows: Yangshanchong acidic samples, pH 3.21, total nitrogen (TN) 0.11 g·kg⁻¹, total organic carbon (TOC) 16 g·kg⁻¹, SO₄²⁻ 13.32 g·kg⁻¹, As_T 63.29 mg·kg⁻¹, Fe_T 133.46 g·kg⁻¹, Cu_T 1.95 g·kg⁻¹, Pb_T 27.58 mg·kg⁻¹, and Zn_T 205.44 mg·kg⁻¹; Shuimuchong acidic samples, pH 2.92, TN 0.11 g·kg⁻¹, TOC 18 g·kg⁻¹, SO₄²⁻ 8.84 g·kg⁻¹, As_T 51.77 mg·kg⁻¹, Fe_T 117.59 g·kg⁻¹, Cu_T 2.53 g·kg⁻¹, Pb_T 30.43 mg·kg⁻¹, and Zn_T 176.59 mg·kg⁻¹."). "Additionally, the TOC content increased by 0.20±0.11 mg/g in YM_FeS₂ and 0.28±0.14 mg/g in SM_FeS₂, and the ¹³C atom % values in YM_FeS₂ (1.76±0.06 ¹³C atom %) and SM_FeS₂ (1.76±0.06 ¹³C atom %) were higher than those in the controls YM_ck (1.12±0.01 ¹³C atom %) and SM_ck (1.11±0.01 ¹³C atom %). This result shows that fixation of ¹³C-CO₂ occurred in these mine tailings with the addition of pyrite; the CO₂-fixing capacities of autotrophs under FeS₂ addition were 9.50±0.91 mg/kg·d in YM and 3.69±0.11 mg/kg·d in SM."

Changes in manuscript:

(Lines 157-160) "No significant changes in chemical properties, pH values (3.25±0.09 in YM_ck and 2.98±0.04 in SM_ck), sulfate (SO₄²⁻) contents (13.15±2.58 mg/g in YM_ck and 8.95±2.19 mg/g in SM_ck), and TOC contents (16.75±0.09 mg/g in YM_ck and 18.55±0.12 mg/g in SM_ck), were found for the control groups compared to the original

Yangshanchong and Shuimuchong acidic samples after 14 days of incubation (Fig. 1).”
(Lines 164-169) “Additionally, the TOC content increased by 0.20 ± 0.11 mg/g in YM_FeS₂ and 0.28 ± 0.14 mg/g in SM_FeS₂, and the ¹³C atom % values in YM_FeS₂ (1.76 ± 0.06 ¹³C atom %) and SM_FeS₂ (1.76 ± 0.06 ¹³C atom %) were higher than those in the controls YM_ck (1.12 ± 0.01 ¹³C atom %) and SM_ck (1.11 ± 0.01 ¹³C atom %). This result shows that fixation of ¹³C-CO₂ occurred in these mine tailings with the addition of pyrite; the CO₂-fixing capacities of autotrophs under FeS₂ addition were 9.50 ± 0.91 mg/kg·d in YM and 3.69 ± 0.11 mg/kg·d in SM.”