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## ***Interactive comment on “Pyrite Oxidation under initially neutral pH conditions and in the presence of *Acidithiobacillus ferrooxidans* and micromolar hydrogen peroxide” by Y. Ma and C. Lin***

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Reviewer’s General comments:

Studies on circumneutral biooxidation of sulfur minerals as pyrite, are rare. Thus, this manuscript represents an opportune research.

However, it is no clear why the authors used the acidophilic *Acidithiobacillus ferrooxidans* instead other chemolithotrophic but circumneutral microorganisms, as *Starkeya novella* (formerly *Thiobacillus novellas*, optimal pH 6.5-8), *Halothiobacillus neapolitanus* (formerly *Thiobacillus neapolitanus*, pH 6.6-7), *Thiobacillus thioparus* and *Thiomonas intermedia* (5-8).

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In this research an interfacial characterization of the pyrite was done. Yet, the biooxidation activity is well explained if the interfacial process is carefully analyzed. Thus, I recommended to request to the authors, to include the ESEM-EDX analysis of the pyrite surface after and before the assays, for each trial, vr. gr. C, T1, T2 and T3, in order to improve the surface characterization and therefore, the discussion of their results and the conclusion of the overall work. Also, I recommended to the authors a more detailed description of the observed microorganism in the pyrite surface and to discuss the presence of such biofilms in terms of the physical and chemical characteristics of the insoluble species presented on the pyrite surface.

Authors' Reply to Reviewer's General comments:

We agree with the reviewer that it is important to investigate pyrite oxidation by neutrophilic iron/sulfide oxidizing bacteria under circumneutral pH conditions, which is indeed part of our ongoing research efforts.

We investigated *Acidithiobacillus ferrooxidans* first because this component was in close connection with the parallel component to investigate the pyrite oxidation in acidic scenarios (pH 2), which required acidophilic bacterial strains to be used. We tried to keep the consistency between the two components in terms of the dosage levels of hydrogen peroxide and the bacterial strain used for the experiment.

The interesting work by Mielke et al. (2003) showed that *Acidithiobacillus ferrooxidans* were able to colonize on pyrite mineral surface at circumneutral pH. This suggests that the pioneer colonizing microbes for grazing on the pyrite surfaces are not exclusively of neutrophilic iron/sulfide oxidizing bacteria. In this work, we examined the colonization of *Acidithiobacillus ferrooxidans* on the surfaces of pyrite cubes when the reaction system is exposed to intermittent fluxes of H<sub>2</sub>O<sub>2</sub> at micromolar levels, which are likely to be encountered in natural environments. This has implications for better understanding the microbially mediated oxidation mechanisms in the real world.

We accept the reviewer's suggestions and the data on ESEM-EDX analysis of the pyrite

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surface will be added.

Biofilm characterization was not performed in this experiment that used natural pyrite cubes (as grown surface). In separate experiments, we examined the colonization of the cells on polished pyrite surfaces under similar environmental conditions. These data can be added to the manuscript to provide further insights in bridging the observed distribution of biofilms and the chemical composition/states of the corroded surfaces.

#### Reviewer's Specific comments

##### Introduction

Reviewer's Comments: Page 559, lines 17-18. This statement comprise the justification of studies like the presented in this manuscript. Please, support it with more references, if is possible.

Authors' Reply: More references will be added to support the statements.

##### Materials and methods

Reviewer's Comments: Section 2.2. It really worried me: the counted cells were live cells? Section 2.3. Please justify the assays duration, (51 days) as well as the sampled times

Authors' Reply: Only viable cells were counted. More information on the method/procedure for cell counting will be provided.

For the pyrite-solution contact experiments involving iron/sulfide-oxidizing bacteria, lengthy experiments were frequently used to create deep footprints to allow adequate characterization of the reacted surfaces of the investigated sulfide minerals. For example: 1. Pisapia et al. 2008, Geomicrobiology Journal, 25(6): 261-273 (experiment length: 22 months) 2. Rojas-Chapana and Tributsch, 2004, FEMS Microbiology Ecology, 47:19-29 (experiment length: 6 months) 3. Rodríguez-Agüez et al., 2003, Hydrometallurgy, 71: 37-46 (experiment length: 40 days) 4. Jones et al., 2003, Geochimica

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et Cosmochimica Acta, 67: 955-965 (experiment length: 3 weeks) 5. Edwards et al., 2001, FEMS Microbiology Ecology, 34: 197–206 (experiment length: 4 weeks) 6. Mustin et al. 1992, Applied and Environmental Microbiology, 58: 1175-1182 (experiment length: 55 days)

### Experiment Results analysis

Reviewer's Comments: Section 3.1. After 51 days, the final cell concentration was practically the same in trials T1 to T3, but there were only attached cells in trial T1 and in the control, nevertheless the pyrite surface presented S<sub>0</sub> and Sn<sup>2-</sup>. Please, explain such results.

Authors' Reply: In this section, only the facts (experimental results) and primary data analysis were presented. Interpretation and discussion of these results were made in the Discussion section. We will further explain these results in the revised version of the manuscript.

### Discussion section

In my opinion this paper should take more care of the basic fundamentals in order to improve the discussion of the obtained results, specifically regarding:

Reviewer's Comments: (1) About the Fe and S species that has been reported by other researchers in the oxidized pyrite surface and thus species registered via XPS in this research. In my opinion, it is also possible that the attachment of cells onto the pyrite surface is deeply influenced by such reduced species, as the registered S<sub>0</sub> and the Sn<sup>2-</sup> (See Page 566, lines 17 and 18). Please, discuss such possibility.

Authors' Reply: We agree with the reviewer's point that the attachment of cells onto the pyrite surface may be closely related to the chemical states of surface S and efforts will be made to discuss this.

Reviewer's Comments: (2) About structure, function and development of biofilms of A. ferrooxidans in both acidic and circumneutral media.

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Authors' Reply: The biofilms were not characterized in this experiment. However, biofilm characterization has been considered in the polished pyrite experiment. We will make discussion on this using the new experimental data, in combination with research findings reported in literature.

Reviewer's Comments: (3) The species of Fe(II) or Fe(III) and reduced sulfur species that should be present at the obtained pH and redox potential (Eh) in each trial (C, T1, T2 and T3) and each tested time. In Page 567, lines 19-20 the authors indicated that planktonic cells only oxidize aqueous Fe(II) and soluble reduced-S species. A reference is needed; and the discussion must be more stressed if the authors inform if such species are present at the pH and Eh registered in the performed assays.

Authors' Reply: At neutral pH, Fe<sup>2+</sup> liberated from the mineral surfaces (either by cell attack or H<sub>2</sub>O<sub>2</sub> attack) could be rapidly converted to Fe<sup>3+</sup>, followed by precipitation as "insoluble" iron compounds and simultaneous release of H<sup>+</sup>. Therefore, pH is a more convenient parameter to indicate the pyrite oxidation. It was considered that the use of a small volume of solution allowed the change in pH being more easily observed (in-situ measurement of pH doesn't require solution sampling).

The total volume of the solution in each reactor was only 40 mL, which limited the frequency of sampling and the amount of solution sample that could be taken each time without markedly disturbing the solution equilibrium system. Therefore, sample collection for determinations of planktonic cell population and aqueous Fe species were only performed at selected times. Sulfur species were not determined due to insufficient solution sample.

In the polished pyrite experiment, we increased the volume of solution, which allow more parameters being more frequently measured.

Reviewer's Comments: (4) The role of the EPS in issues as Fe(II)/Fe(III) retention and Fe(II) biooxidation. S Certainly, since 1995 Sand, Gehrker and collaborators have highlighted the importance of biofilms matrix as a reactive space wherein the electro-

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chemical mechanisms/surface reaction takes place, specifically the initial attachment of EPS-complexed Fe ions to the pyrite surface by electrostatic interactions and their acceleration of the dissolution rate of this mineral.

**Authors' Reply:** We agree with the reviewer that EPS could play a significant role in these processes, which is part of our current research efforts. At the time when this experiment was conducted, this level of study was not considered due to the unavailability of the required research facilities. Our research objective was primarily to examine the role of intermittent fluxes of H<sub>2</sub>O<sub>2</sub> at micromolar levels in microbially mediated pyrite oxidation by comparing a few major chemical and biological parameters in the reaction systems with the same (comparable) background interferences. This goal has been satisfactorily achieved by this experiment. The subsequent polished pyrite experiment will provide further data to support the arguments. It is our intention to enhance the discussion on this aspect by adding the new data in combination with the research findings obtained by the above-mentioned authors.

**Reviewer's Comments:** (5) About the antioxidative response of the microorganism to H<sub>2</sub>O<sub>2</sub>; perhaps about the superoxide-dismutases (SOD) activity. In Page 567, lines 12-13 the authors suggested that *A. ferrooxidans* developed H<sub>2</sub>O<sub>2</sub> tolerance, however such tolerance could be an intrinsic resistance: the SOD is a key enzyme in *A. ferrooxidans* since the typical environment of this microorganism is oxidant. Page 567, lines 23-25: Please, justify why the pyrite cubes of the mentioned dimensions were used in long-term assays. (In my opinion the pyrite weight was quiet enough (ca. 36 g)). Page 568, lines 2: Please, inform if in the bottom of each culture flask was observed precipitated of the compounds that may explain the drop in the Fe concentration.

Section 4.3. In my opinion, this section needs references, to complete the discussion of the results obtained by XPS.

**Authors' Reply:** We did conduct dose-response experiments to observe the growth and biochemical responses of *A. ferrooxidans* to micromolar H<sub>2</sub>O<sub>2</sub> and antioxidant

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enzymes were part of the study. These data were not presented in this manuscript to avoid making a lengthy article. However, we can present these data as part of the supplementary document.

The use of pyrite cubes rather than powdered pyrite was to avoid introducing spontaneous H<sub>2</sub>O<sub>2</sub> into the reaction system since oxidation of powdered pyrite by molecular oxygen can produce H<sub>2</sub>O<sub>2</sub> (Schoonen et al., 2010). Reference Schoonen, M.A.A.; Harrington, A. D.; Laffers, R.; Strongin, D.R. Role of hydrogen peroxide and hydroxyl radical in pyrite oxidation by molecular oxygen. *Geochim. Cosmochim. Acta.* 2010, 74(17), 4971-4987.

The experiment required over 25 pyrite cubes with similar size, which was a challenge. The dimension of the pyrite cubes was not selected on purpose but based on the availability of such pyrite cubes from the available pyrite specimens that met this number requirement.

There were trace amounts of precipitates on the bottom of the conical flasks, especially in T3 (the highest-dose treatment). This visible observation will be added to the manuscript.

More references will be added to Section 4.3 for the discussion on XPS results.

Reviewer's Comments: Table 1. Include the species associated to each peak and the corresponding reference, in two new columns.

Figure 3. I recommended present the information showed in Fig. 3 in a Table, in order to focus on the absolute data and thus, to facilitate the lecture to these interesting results.

Technical corrections Page 566, line 14: Please, change “extract mechanisms” by “exact mechanism” Page 567, lines 19: Please, use “oxidize” instead “feed” Page 567, line 23: use “source of Fe and/or S” instead “foods”

Authors' Reply: Changes will be made accordingly.

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Interactive comment on Biogeosciences Discuss., 9, 557, 2012.

**BGD**

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