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Biogeosciences Discussions

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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Authors Response to the Comments by Reviewer #2

Reviewer's General comments:

Attachment of the pyrite oxidizing bacterium Acidithiobacillus ferrooxidans to the pyrite surface was studied at neutral pH in laboratory experiments under the influence of hydrogen peroxide in different concentrations. Effects were evaluated by measuring pH, iron concentrations, numbers of planktonic cells, as well as SEM and XPS measurements of the pyrite surface.

In my opinion the manuscript does not provide any scientific advancement in understanding attachment of A. ferrooxidans to pyrite and its role in the pyrite oxidation process. The manuscript does not fulfill the standard of a scientific publication because of the following reasons:

Authors' Reply to Reviewer's General comments:

This work represents the first attempt to examine pyrite oxidation in a complex system involving iron/sulfide-oxidizing bacteria, molecular oxygen and intermittent fluxes of hydrogen peroxide at micromolar levels. As also pointed out by Reviewer #4, investigation of such a complex system is quite difficult to perform, we did not intend to set an unrealistic high bar for this study. As stated in the manuscript, the objective was to determine whether H2O2 at micromolar levels has a role to play in microbially mediated pyrite weathering under initially neutral pH conditions. We believe that this goal has been achieved to a certain degree of satisfaction although additional data may be needed to make the arguments more convincing.

This work is part of a larger research project looking at the roles of H2O2 at micromolar levels in both abiotic and biotic acid sulfate-producing weathering at both circumneutral and acidic pH scenarios. There are additional data that can be used to further support the arguments made in this manuscript.

While we partly accept the criticisms by the reviewer, we feel that many of his/her comments are not made based on the facts or correct understanding of the experimental data. These are detailed point-by-point below.

Reviewer: 1. The data do not allow drawing conclusions about the effect of hydrogen peroxide on cell attachment and pyrite oxidation. The data shown do not provide significant differences for the three experimental treatments with different hydrogen peroxide concentrations and the control, besides that the pH in T3 is lower than for the other treatments presumably due to chemical pyrite oxidation by hydrogen peroxide at the highest concentration applied.

Authors: These statements are not true. Apart from the significant difference in pH between the control and T3, marked difference among the control and the treatments was also observable:

(a) The population density of attached cells on the pyrite cube surface was markedly different. No attached cells were observed in T2 and T3; the population density of the attached cells was lower in the control than in T1. This was observed from the entire pyrite cube surface area. Perhaps the resolution of SEM images in Figure 2 is too low to allow a clear comparison. We now provide highresolution SEM images in the supplementary document (Supplementary Figure S1) to assist in demonstrating the difference.

(b) As shown in Figure 2e and 2f, there was marked difference in the shape, size and orientation pattern of the corrosion pits on the pyrite surface.

(c) There was a clear trend showing that the major XPS peaks (Fe2p3/2 and S 2p) for the reacted pyrite surfaces shifted to the lower binding energy side with increasing dosage level of H2O2 (Table 1 and Figure 4).

Reviewer: Fig. 1b shows a similar decrease of planktonic cell numbers over time for all treatments.

Authors: This was consistent with our separate dose-response experiment, which showed that A. ferrooxidans were tolerant to H2O2 up to a level >2000 _M when Fenton-type reaction does not operate (unpublished data). The Fe2+ in the bulk solutions was non-detectable. This means that Fenton reaction-derived free radicals in the bulk solution, if any, must have been very low, which was unable to cause significant oxidative damage/stress in the planktonic cells.

It is quite normal that when examining the effects of an environmental variable on a range of selected environmental parameters, some will respond significantly and others will not.

Reviewer: Fig. 2 only provide qualitative data

Authors: Figure 2 presents SEM images of the control and the treatments. We wonder how the reviewer presents SEM images in a quantitative way. We believe that the difference in the population density among the control, T1, T2 and T3, and the orientation pattern of corrosion pits between the control and T1 can be clearly seen from these SEM images and therefore the objective was satisfactorily achieved.

Reviewer: the bars in Fig. 3 are similar for all treatments, error bars are missing,

Authors: These are composite bar charts. We wonder how the reviewer insert error bars into a composite bar chart.

Again, it is quite normal that when examining the effects of an environmental variable on a range of selected environmental parameters, some will respond significantly and others will not or just respond gently. There was observable difference in Fe/S ratio among the control and the treatments for the reacted surfaces. It must be realized that this study used micromolar level H2O2 that is likely to be encountered in naturally occurring environments. Therefore, significant footprints on pyrite surfaces are not expected to be observed, as opposed to scenarios when higher concentration of H2O2 is used.

Furthermore, the main purpose of this figure was to demonstrate the difference in the chemical composition between the reacted surface and the corroded surface.

Reviewer: and also the XPS spectra in the supplementary material do not reveal significant differences for the different treatments.

Authors: This is not true. As shown in Table 1 (already mentioned above), the difference in the major XPS peak positions was sufficiently clear among the control and the treatments. The XPS spectra in the supplementary material were presented as separate chart for each treatment (as part of the original data set). The direct comparison of XPS spectra is provided in Figure 4, which does show certain degree of visible

difference.

Reviewer: The numbers of planktonic cells shown in Fig. 2b are quantitative but do not tell anything about the colonization of the pyrite surface.

Authors: This is obvious. We cannot understand why the reviewer raised this issue. Figure 2b was to show the presence/evolution of cells in the solution. We had no intention to use this data to "tell anything about the colonization of the pyrite surface" although certain relationship between the planktonic cells and the attached cells is expected.

Reviewer: The decrease of cell numbers over time in all treatments is most likely caused by cell death. A. ferrooxidans is an obligate acidophilic organism (pH maximum at pH 4.5), but cells were exposed to pH > 5 for more than 100 days! No data about the physiological status of the cells is given (e.g. FISH, cultivation), thus it is even unclear if the detected planktonic cells are still alive and active in iron- and sulfur oxidation. As a consequence any statement about the physiological status of the cells are not supported by data, e.g. in the conclusions "The planktonic Acidithiobacillus ferrooxidans were able to survive under the highest H2O2 dosage: : :".

Authors: Only viable planktonic cells were counted in this study. The direct cell counting was performed using a Neubauer hemocytometer. The cells that showed no sign of motion were not counted as viable cells.

Reviewer: Quantitative data for the colonization of the cells on the pyrite surface obtained by fluorescence microscopy after DNA staining or AFM (e.g. Noel et al. 2010 Hydrometallurgy) would have been useful for this study.

Authors: The cell-shaped objects on the pyrite cube surfaces in this study were "fossil cells" that were covered by oxidation products of pyrite (see Supplementary Figure S2). DAPI staining does not work for such materials.

We wonder why the reviewer was so sure that "Quantitative data for the colonization of

the cells on the pyrite surface can be obtained by fluorescence microscopy after DNA staining or AFM". This was not shown in the reference mentioned by the reviewer. Instead, the authors stated that "Statistic counting of cells remains to be difficult because of aggregate formation. At present just an appreciation of the different cell amounts is possible and the method has to be optimized" (Page 374).

We believe that the qualitative data provided by SEM images are sufficient to achieve the research goal set in this study. In the ongoing study aiming at obtain further insights into the cell attachment using polished pyrite plates and shorter experiment duration, AFM and EFM methods have been considered.

Reviewer: The iron data given in Table 1 are all lower than 1 mg per L and not worth to be shown. Iron is almost insoluble at oxic conditions above pH 4 and precipitates as iron(hydr)oxide.

Authors: We don't agree with the reviewer's point. Although the total Fe concentration was below 1 mg/L, these values were above the detection limit of the analytical method used. This information is important because it showed the maintenance of trace amount of Fe in the solution, which indicated that Fe kept releasing from the reacted pyrite surfaces to the solution. The solution-borne Fe was in a state of dynamic equilibrium controlled by the following sequential chemical reaction:

Pyrite-Fe(s)+oxidant –> Fe2+(aq)

Fe2+(aq) + oxidant –> Fe3+(aq)

Fe3+(aq) + H2O –> Fe(OH)3 + 3H+

The total Fe represents the sum of the three different Fe forms in the above equations. The method used for determination of Fe2+ had a detection limit greater than 1 mg/L and consequently failed to detect the presence of Fe2+ in the reaction system. Therefore, presentation of the total Fe data is necessary for demonstrating the liberation of pyrite-bound Fe.

Reviewer: Soluble S species have not been analyzed (e.g. Schippers and Jorgensen 2002 Geochim. Cosmochim Acta) to backup any conclusions about their role in supporting bacterial growth (e.g. Conclusions).

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

Reviewer: 2. The scientific statements are not supported by the data. The first sentence of the abstract is speculative. It has not even shown that the cells oxidize the pyrite, and not at all that "microbial oxidation" is influenced by hydrogen peroxide. Also the second sentence: Colonization of Acidithiobacillus ferrooxidans onto the mineral surface has not been demonstrated (only planktonic cells were counted see 1.). All further sentences in the abstract are speculative as well.

Authors: The reviewer's comments are not valid because they are not based on the facts. The clear cell-shaped corrosion pits (Figure 2e and f) observed for the control and T1 were strong evidence for microbially mediated oxidation of pyrite. Such corrosion pits were absent on the pyrite cube surfaces in T2 and T3 (No cell-shaped objects were observed for these high-dose treatments). Cell attachment were only observed for the control (no added H2O2) and the low-dose treatment (T1) and the population density of the attached cells tended to be higher in T1 than in the control.

Reviewer: 3. The discussion is weak and does not reference to papers on relevant topics such as attachment of A. ferrooxidans to pyrite (e.g. Sand et al. 2001 Hydrometallurgy), the sulfur chemistry and mechanisms of pyrite oxidation (e.g. Sand et al. 2001

Hydrometallurgy; Druschel and Borda 2006 Comment in Geochim. Cosmochim Acta), the role of hydrogen peroxide in pyrite oxidation (e.g. Borda et al. 2003). Instead the discussion proposes a reaction mechanism disconnected from the state-of-the-art and not based on the scientific literature. Beside in the first few lines the entire discussion section does not contain any references.

Authors: We partially accept the criticisms by the reviewer. As stated in the response to comments by Reviewer #1, we intend to add additional data and extend the discussion regarding the effects of biofilm on cell attachment and the related corrosion of pyrite surfaces. The Sand group's findings will certainly be very useful for improving the quality of arguments.

We are aware of the works on pyrite oxidation by hydrogen peroxide that were published in major international journals. These previous works were conducted in abiotic systems and either with a high concentration (millimolar level up) or through spontaneous generation, as well as with different specific focuses. In many ways the experimental conditions in these previous works were quite different from ours. While we did mention some of these references in the Introduction section and efforts were also made to establish connection with these published works, we were not able to locate appropriate references that were highly relevant to what we discussed in this section (Perhaps no authors will refuse to cite references that will enhance their arguments. We were just reluctant to cite references solely for the purpose of showing that some references had been cited). The reviewer ignored the fact that quite a few references were cited in the Experimental Result Analysis section, which provides a theoretical base for the discussions made in the Discussion section.

It will be more useful if the reviewer could point out any errors/mistakes in the proposed reaction mechanisms rather than made judgements based on how many references were cited.

We have reservation about the term "the state-of-the-art" the reviewer used here. There

is no doubt that a few pieces of pioneer works have made important contributions to the understanding of the overriding biogeochemical processes governing pyrite oxidation. Our knowledge obtained so far is far from sufficient for unveiling the complex mechanisms responsible for pyrite oxidation in natural environments. Placing a cap on scientific exploration will not help promote the advancement of knowledge in this field.

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

Supplementary Figure S1

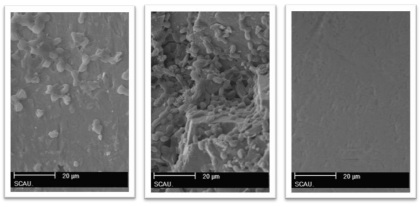

Figure S1 SEM images of the pyrite cube surfaces under different treatments. (a) exposed to *Acidithiobacillus ferrooxidans* only; (b) exposed to *Acidithiobacillus ferrooxidans* and 50 µM H_2O_2; and (c) exposed to *Acidithiobacillus ferrooxidans* and 100 µM H_2O_2. Arrows point to the cells attached to the surfaces of pyrite crystals.

Fig. 1. Supplementary Figure S1

Supplementary Figure S2

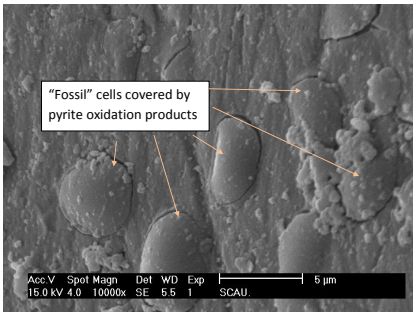

Figure S2 High-resolution SEM image showing the cell-shaped objects observed on the mineral surface in Treatment 1 (T1: H_2O_2 at 50 µM) of Experiment 1. These were the "fossil" cells that were covered by iron oxides/hydroxide/hydroxysulfate formed during attached cell-induced surface corrosion. Note that the size of the cell-shaped object was much greater than the actual size of a cell.

Fig. 2. Supplementary Figure S2