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Interactive comment on "Iron minerals inhibit the growth of bacteria via a free-radical mechanism: Implications for soil carbon storage" *by* Hai-Yan Du et al.

Anonymous Referee #2

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I. General comments: This study aimed to study (i) the impact of Al- and Fe-containing minerals (montmorillonite, kaolinite, hematite, goethite and ferrihydrite) on bacterial growth using cultural approach on Pseudomonas brassicacearum J12 and (ii) the involvement of ROS, produced via fenton reactions, on Pseudomonas brassicacearum J12 growth. The subject is clearly interesting and is in accordance with researches published in Biogeosciences journal. Such researches on interactions between biotic and abiotic compartments are essential for our understanding of nutrients fluxes in soils and I encourage the publication of this manuscript in Biogeosciences journal. However, some points need to be clarified before publication.

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II. Major comments:

- Major comment 1: Tilte: “Iron minerals inhibit the growth of bacteria via a free-radical mechanism: Implication for soil carbon storage”: You cannot generalize your results to the domain of bacteria. I recognize that we will never be satisfied enough with the number of species studied, but I think that before expanding your results to the domain of bacteria, you should confirm them on other species from different phylum which show important genetic and phenotypic distances.

- Major comment 2: Integrate your statistical results in the description of the results and in your figures.

III. Specific comments:

Abstract

- “Together, these findings indicate that the reduced surface Fe(II) derived from Fe(III)-containing minerals inhibit bacteria via a free-radical mechanism, which may further contribute to soil carbon storage.” : see Major comment 1. Free-radicals may lead to organic matter degradation-mineralization, you do not develop this idea in the manuscript.

Introduction

- This is a clear introduction which provide a good representation to the overall situation.

- “The bacterial inhibition property of a mineral is associated with the particular chemistry and with the mineral properties, 45 resulting in the various bacterial inhibition mechanisms of minerals” [l. 43-45]: can you please give more precisions on the various inhibition mechanisms?

- Please, name the Al- and the Fe-contaning minerals that you used in this study, it is hard to understand for non-chemists to which category belong kaolinite, montmorillonite, hematite, goethite, ferrihydrite.

Material and Methods

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- I suggest to separate the paragraphe 2.1 into two parts: “2.1 Mineral preparation” [l.106], “2.2 Pseudomanas cultivation experiments” [l.121]

- Suppress “which is a major group of rhizobacteria that aggressively colonize plant roots, has been considered an important group for sustainable agriculture” [l.122-123]: the information is already given [l.90].

- Why didn’t you chose to have a control [NB + Mineral]? The OD of this control can be subtracted from the OD measured in [NB + Mineral + Bacteria] and give you the OD of your bacteria without the disturbance induced by the mineral?

- Did you measure the kinetic of bacterial growth during the 12h? Are you sure that the bacteria is still in exponential phase of growth? Why did you chose 12h for the first incubation and 8h for the second one?

- pH measurement should be explain in “2.6 Chemical analysis”

- I do not understand the choice of an ANOVA, when did you used that test.

- Figure 1, 3, S5, S6, S7, S8 should integrate your statistical analysis.

- Explain/describe the “one-sample Kolmogorov-Smirnov Test”

- Which software did you use to find and represent the model that best fits with your data (Fig.4)?

Results

- In this part you should not interpret your results: [l.230-231], [l.244-247], [l.294-295], [l.268-269], [l.280-281], [l.313], [l.318-319], [l.327-328].

- Here, we are waiting for an exhaustive description of the results obtained during the study: give some values (mean $\pm$ SE) and precise when values are significantly (or not) different between the different conditions tested.

Paragraph [l.224-234]:

- “3.1. Bacterial inhibition by minerals” [l.223]: This title does not correspond in case of montmorillonite. I suggest something like: “Effect of mineral nature and their concentrations on P. brassicacearum J12 development”.

- Suppress “The effects of the nature and content of tested minerals on the OD 600 of Pseudomonas brassicacearum J12 subcultures taken after 12 h growth are shown in Fig. 1.”: it should be in the “Material and Methods” part.

- “Compared to Control (i.e., no minerals), the presence of montmorillonite significantly increased OD 600 .”: give values.

- Suppress “On the other hand,” [l. 227]

- [l.227-230]: “Presence of all other investigated minerals decreased OD 600 in the following order: ferrihydrite > goethite > hematite > kaolinite at 5 and 25 mg mL -1, and ferrihydrite > goethite > kaolinite > hematite at 10 mg mL -1”: Please give some values.

- Suppress “Meanwhile” [l.232]

- “An increase in mineral concentration resulted in a significant decrease in OD 600, except for montmorillonite” [l. 232-233]: Give some values.

- Suppress “as the OD 600 seemed to be independent of its concentration” [l.233-234]: it is an interpretation. You can replace it by something like: “However, in presence of montmorillonite the OD600 is stable at 0.43 $\pm$ SE for all the mineral concentration studied”

- Fig.1: we do not see bottom bar of the SE

- Fig.1 text/description: you should mention the mineral concentrations.

Paragraph [l.235-247]:

- It represents a new idea: you should give it a title (e.g. “chemical structure of minerals”)

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- [l.235-247]: why didn't you describe the EPR profiles of ferrihydrite, goethite and hematite?

Paragraph [l.249-258]:

- "A 12 h cultivation of Pseudomonas brassicacearum J12 in the presence of different minerals revealed that generation of HOâĂć 250 radicals in the cases of montmorillonite, kaolinite and hematite was almost similar to the control (Fig. 3)": "Almost"? You should precise if the difference are significant or not. To precise my comment, you should study the significance of the difference between the control and montmorillonite for the three concentrations, and kaolinite 25 mg.mL-1. Moreover, I think that the difference is significant between (i) montmorillonite 25mg.mL-1 and kaolinite 25mg.mL-1 and (ii) montmorillonite 25mg.mL-1 and hematite 25mg.mL-1.

- [l255]: replace "rapidly" by significantly: there is no notion of time.

- Fig.3 text/description: you should mention the mineral concentrations

Paragraph [l.259-295]:

- Globally, I encourage the authors to reorganize this part of the manuscript. You should describe all your results (Fig. 4) not only those which are consistent with your interpretation. Just for example: I observe a significant increase of soluble Fe in the treatment containing goethite with the increase of goethite concentration but this results is missing from the test.

- Given the importance of the pH in the results description, I think that this result may be integrated in Fig.4.

- [l. 261-263]: "Much more soluble Fe was released from Fe(III)-containing minerals than from montmorillonite, kaolinite, and control (Fig. 4a)": Please give some values.

- "The solubility of Fe is closely related to pH value.": Are you sure about that? The pH of goethite solution is equivalent to the pH of kaolinite, montmorillonite, hematite and

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goethite but the solubility of Fe in solution containing hematite and goethite seems to be more important. You should draw the graph showing the correlation between pH and the soluble Fe.

- Fig.4.b: You have a surprising result: the significant decrease of Total Fe in solutions containing 25 mg.mL-1 ferrihydrite between 2h and 12h. How do you explain that?

- "For all of the examined minerals, the trends of total Fe and Fe(II) were similar in the following order: ferrihydrite » goethite > hematite > montmorillonite ≈ kaolinite ≈ control (Fig. 4b-4c)": Please give some values. What do you mean with » and ≈? Is there a relation with a statistical analysis?

- [l.274-245] "Furthermore, a positive correlation exists between OD600 and soluble Fe content 275 (R = 0.92, t = -3.49, p = 0.003) and Fe(II) (R = 0.98, t = -4.28, p =0.001) (Fig. 4d and 4f, Table S2).": I think that you wanted to say "a positive correlation between Hydroxyl radical content and soluble Fe content".

- The interpretation of "R" and "t" should appear in the Material and Methods.

- [l.277]: "R=-0.75" and "t= 2.27" do not correspond to the values in the Fig.4.

- Fig.S7 should appear in Fig.4

- Fig.S7: Can you explain why the inhibition of Pseudomonas is more important in Fe(III) 100mg.L-1 than in Fe(III) 50mg.L-1 while hydroxyl radical production still the same between those two concentrations? Is that not the sign of the existence of another process involved in the Pseudomonas growth inhibition? I find this result very important, it should be considered in your discussion.

Paragraph [l.296-328]:

For non-chemist, this part is difficult to understand. Maybe the next comments will allow you to make it more accessible for biologists.

- Fig.5.a: What do the colors mean?

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- Why did you select those regions of the spectra for XANES?

- Fig.5.c: What do the colors mean?

- Conserve the same colors between Fig.5.b Spot A and B.

- [l.309]: Spot A or Spot B?

- [l.307-309]: why don't you speak about FeC2O4 (25.9%) in spot B?

- Why is there goethite and hematite in sample which only contain ferrihydrite?

- You should give different title to the paragraph [l.298-309], [l.310-320]

- Paragraph [l.321-328] should be describe in paragraph [l.298-309]: it is the same figure and consequently the same idea.

- [l.317-320] "Interestingly, the area of the peak at 709.5 eV was bigger in the F + bacteria treatment than that in F - bacteria treatment (Fig. 6b-6c), suggesting that Fe(II) was generated on the surface of ferrihydrite during the cultivation with bacteria. Based on the reaction 1, HO2 âĂć should be the oxidant products.": Is that reproducible between samples? Is that spectrum the mean representation of several spectra?

- Fig.6 b and c: what do the colors mean?

- Fig.6 a: Correct "Inyensity" by "Intensity"

- [l.325]: "good", can you precise this term please?

New paragraph:

- Given the importance of Al in your discussion (half of the discussion), the Fig.S8 should appear in the main manuscript (not in SupMat) with the results presented in Fig.4.

- In Fig.1, Fig.3, Fig.4, Fig.S6, Fig.S8: you should distinguish Al- from Fe- containing minerals.

Another time: I disapprove the use of the term “bacteria” which may refer to the domain of bacteria (see main comment 1).

4.1. Effect of Al(III)-containing minerals on the inhibition of bacterial growth

- [l.336-343]: “It should be noted that the presence of minerals may potentially interfere with the measurement of cell numbers in Fig. 1. In this study, we subsampled the experimental cultures and diluted them in fresh medium so that both clay particles and bacteria were 200× less concentrated (Fig. S3), following the protocol of McMahon et al. (2016). As a result, the effect of mineral concentration may be minimal. In addition, plating the bacteria by evaluating populations by counting colonies may act as a complementary method for OD600 and needs to be investigated in the future.”: I am not waiting for a response to that comment: In your case, I would have chosen the association of a cell labeling with DAPI and a count of labeled cells with flow cytometry (or fluorescence microscopy).

- [l.355-357]: “Furthermore, the formation of some Al intermediates by the decreasing pH, such as Al13O4(OH)24 7+, is also suggested to be more toxic for bacterial growth (Amonette et al., 2003; Liu et al., 2016).”: what pH are you referring to? Is that in accordance with the pH measured in your study?

- The information given at [l.357-359] should appear after [l.345-349]. Then, you can discuss (i) on the results that you expected to observe and (ii) on the interpretation of the results that you obtained.

4.2. Inhibition of bacteria by Fe(III)-containing minerals via a free-radical mechanism

- If we take the two equations cited in your introduction: (1) ≡ Fe(III)-OH + H2O2 ≡ Fe(II) + H2O + HO2 (2) ≡ Fe(II) + H2O2 ≡ Fe(III)-OH + HOâĂć Where does H2O2 come from? Pseudomonas? If it come from the bacteria, the reduction of it development should induce a decrease of HOâĂć production in LB medium containing Fe

minerals (if H2O2 is the limiting compound in the reaction, and it should be the case here), am I wrong?

- Correct the sentence [l.383-387]: “In line with other studies (Kwan and Voelker, 2003; Wang et al., 2017b), we deduced that HOâĂć may mainly generate on the mineral surface, partly due to the positive charge of mineral surface (Tombácz and Szekeres, 2006) but the negative charge of microbes (Jucket et al., 1996).”

- [l.401-404]: “High percentage of the less stable ferrihydrite (Table S3) may be attributable to the stabilization role of produced EPS (Fig. 5c) by bacteria to minerals, which had been shown during the cultivation of fungi with minerals (Li et al., 2016).Âż please, divide this sentence into two sentences in order to distinguish your contribution from the contribution of Li et al. (2016). Can you precise your idea on the role of EPS on stabilization process please?

- [l.414]: suppress “cellular”. I do not understand the difference between cellular and free reductant? Free reductant such as FADH2 are intracellular, no? I think that you want to separate (i) cellular from (ii) non-cellular reactions, am I wrong?

- [l.413-418]: Are those reactions linked to HOâĂć production?

- [l.424]: Given that results in Fig.1 and Fig.S7 are produce by different experimental device, are you sur that you can give this interpretation to your results?

- [l.427]: “simultaneously”?

4.3. Inhibition of bacterial growth by a free-radical mechanism and its implications for soil carbon storage

- Fig.7: Can you please explain the figure in the caption?

- Can you please go further in the processes through which soluble Fe3+ and Fe2+ will have an inhibition effect on Pseudomonas?

- Can you please go further in processes through which HOâĂć will have a “direct”

inhibition power on Pseudomonas (modification of cell membrane physico-chemical properties?)

- [l.436-437]: “Oxidative damage of extracellular HOâĂć may lead to bacterial inactivation, and protection of carbon from microbial degradation.” Please go further in this interpretation: HOâĂć have a role on Pseudomonas growth (it is your study), but HOâĂć can have other impacts in soils. What are they? How can HOâĂć and Fe act (i) on the soil C storage and (ii) on the soil C degradation-mineralization?

- [l.439-442]: “In addition, the generation of free radicals may also have indirect effects on bacterial growth via substrate availability (Table S4). Substrate availability is improved in the presence of radicals, owing to the following two facts: 1) the depolymerization role of radicals on the complex substrates; 2) the inhibition role of radicals on bacteria indirectly increasing the amounts of available substrates.”: Do you think that we can see an inhibition of bacterial growth through the reduction of nutrient availability induced by free radicals in a medium where nutrients are in excess?

- What about the role of minerals on the “stabilization-adsorption” of organic compounds of the NB medium?

- [l.445-448]: Fe is one of the numerous processes regulating carbon cycle in soils. I suggest something like: “In this study, we suggest that soil carbon cycle is partly regulated by Fe minerals (i) by the formation of organo-mineral complexes and (ii) by the bacterial development inhibition (specify the processes).”

- l.451 replace “but” by “and”

Conclusions

- [l. 467-458]: “effects on bacterial growth and the presence of minerals may potentially interfere with the measurement of cell numbers”: I do not think that it is necessary to speak about that here.

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