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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.

Hai-Yan Du et al.

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Dear Editor,

We now submit the revised manuscript bg-2018-479R1 by Haiyan Du, Guanghui Yu, Fusheng Sun, Muhammad Usman, Bernard A Goodman, Wei Ran, Qirong Shen to Biogeosciences. Our point-by-point responses are detailed below, and all changes are highlighted in the revised manuscript. We are very grateful to Reviewer #1 for great comments, which have truly helped in the improvement of the article. Should you need

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to contact me, please use the above email or call me at [86-25-84396221].

Sincerely yours,

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Reply to the Comments

Anonymous Referee #1

The authors appreciate the report of Referee #1 and respond as follows.

General Comments: This study is a nice contribution to the analysis of mineral-microbe interactions in soils, and it presents some new evidence for the role of iron in diminishing certain bacterial populations. Of particular interest to me was the X-ray photoelectron spectroscopy data demonstrating the presence of Fe(II) on the ferrihydrite surface, suggesting that the microbe (J12) reduces mineral bound Fe(III). This is important, because Fe(II) is then available for Fenton reactions that can produce radicals that damage cell membranes allowing soluble metals to enter the bacterial cell. The work suggests that iron bearing minerals in soils contribute to the preservation of organic C by limiting the productivity of bacteria that degrade carbon.

Specific Comments:

-Line 108. Your description of the clay minerals used is not adequate for determining

the chemical composition and stability of the phyllosilicates. In particular montmorillonite has many chemical components in each crystallographic site that contribute to the mineral surface characteristics (charge distribution) and interlayer cations. Therefore, one must give the chemical formula for the mineral used. One montmorillonite might increase bacterial growth (if it provides nutrients) while another might decrease bacterial growth (if it provides toxins).

Response (R): In the revised manuscript, the chemical formulas for the clay mineral used were added and also listed as follows.

“Five minerals were selected in this study, including kaolinite (98%, Aladdin Reagent Company, Shanghai, China), montmorillonite (98%, Aladdin Reagent Company, Shanghai, China) and synthetic hematite, goethite and ferrihydrite.” (Pages 5-6, Lines 107-109 in the original manuscript)

was changed to

“Five minerals were selected in this study, including kaolinite (Al2O3âĂć2SiO2âĂć2H2O, 98%, Aladdin Reagent Company, Shanghai, China), montmorillonite ((Al2,Mg3)Si4O10(OH)2âĂćnH2O, 98%, Aladdin Reagent Company, Shanghai, China) and synthetic hematite, goethite and ferrihydrite.” (Pages 5-6, Lines 107-110 in the revised manuscript)

-Line 128. How were the concentrations of minerals (5, 10, 25 mg/ml) decided? Did you measure the minimum inhibitory and minimum bactericidal concentrations for the J12 bacteria under the pH conditions of the experiment? Were the minerals only hydrated by the growth media? If so, what is the speciation of soluble metals with the components of the media solution?

R: The concentrations of minerals (5, 10, 25 mg/ml) were referred to the previous literature (McMahon et al., 2016). According to McMahon’s results, negligible impacts of clays on bacteria growth when clays concentration is 5 mg/ml, while clays in suspen-

sions exceeding 10 mg/ml in concentration inhibit the growth of bacteria. In this study, we did not measure the minimum inhibitory and minimum bactericidal concentrations for the J12 bacteria. The minerals were hydrated by both the growth media and the J12 bacteria. Our results showed that the speciation of soluble metals was soluble Al in the kaolinite and montmorillonite treatments (Figure S9) but both Fe(II) and Fe(III) in hematite, goethite and ferrihydrite treatments (Figure 4).

-Line 135. It is unclear why the particle size distribution is presented before and after incubation with bacteria. The increase in particle size after incubation (which would be better demonstrated in a graph than a table) probably results from agglomeration of mineral-bacteria clusters rather than a crystal growth. Is this important to the conclusions? If anything, a measurement of specific surface area of the minerals would be more important to the chemical interactions.

R: Good comment! In the revised manuscript, we replaced table S1 by Figure S4 to show the particle size distribution. We agree with the comment that the increase in particle size after incubation probably results from agglomeration of mineral-bacteria clusters, which was added in the Caption of Figure S4 in the revised manuscript. We are sorry for not measure the specific surface area of the minerals. However, according to the data provided by manufacturers, the specific surface area of kaolinite and montmorillonite are ~40 and 800 m2 g-1, respectively. The synthetic hematite, goethite and ferrihydrite were referred to the method from Schwertmann and Cornell (2007), and their specific surface area are approximately 30, 20, 200-300 m2 g-1, respectively. In the revised manuscript, we added the specific surface area of minerals and also listed as follows.

"According to the data provided by manufacturers, the specific surface area of kaolinite and montmorillonite are ~40 and 800 m2 g-1, respectively. The synthetic hematite, goethite and ferrihydrite were referred to the method from Schwertmann and Cornell (2007), and their specific surface area are approximately 30, 20, 200-300 m2 g-1, respectively." was added in the revise manuscript (Page 7, Lines 160-164 in the revised

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Figure S4. The particle size distribution (% in volume) of both the applied raw minerals and the changes after 24 h of cultivation. 1-11 represent the particle size of < 0.1, 0.1-0.5, 0.5-1, 1-2, 2-5, 5-10, 10-20, 20-50, 50-100, 100-500 μm, respectively. (a) kaolinite; (b) montmorillonite; (c) hematite; (d) goethite; (e) ferrihydtire; (f) kaolinite + bacteria; (g) montmorillonite + bacteria; (h) hematite + bacteria; (i) goethite + bacteria; (j) ferrihydtire + bacteria. The increase in particle size after incubation probably results from agglomeration of mineral-bacteria clusters.

-Line 209. For chemical analysis you have filtered the mineral-microbe suspension through 0.45 μm membrane, which may remove the bacteria, but allows clay size particles through. This is then analyzed by ICP and results reported as 'soluble' Al and Fe. However, the clay particles in this fraction will contribute to the elemental analysis.

R: Yes. Here 'soluble' Al and Fe should include the nano-size mineral particles. Suspension filtered through 0.45 μm membrane can be used to chemical analysis in mineral-microbial and soil systems (Ahmed and Holmström 2015; Li et al., 2018). And elements in the obtained solution were considered as dissolved.

Ahmed, E., and Holmström, S.J.M.: Microbe–mineral interactions: The impact of surface attachment on mineral weathering and element selectivity by microorganisms. Chem. Geol. 403, 13-23, 2015.

Li, Z. B., Lu, X., Teng, H. H., Chen, Y., Zhao, L., Ji, J., Chen, J., and Liu, L.: Specificity of low molecular weight organic acids on the release of elements from lizardite during fungal weathering. Geochim. Cosmochim. Acta Doi:10.1016/j.gca. 2018, in press, 2018.

-Line 225. This analysis presumes that OD600 only reflects absorbance by bacteria, but what is the absorbance of the mineral suspension alone?

R: We added the absorbance of the mineral suspension alone in the different concen-

trations. The results were added as Table S1 in the revised manuscript and shown as follows.

"Then, 50 μL of the cultures were transferred to fresh medium (10 mL) so that the effects of minerals were negligible (Table S1)." (Pages 6-7, Lines 130-131 in the revised manuscript)

Table S1. Optical density at 600 nm (OD600) of the mineral suspension (n = 3)

Mineral Absorbance at 600 nm 5 mg/mL 10 mg/mL 25 mg/mL Kaolinite 0.005 $\pm$ 0.001 0.019 $\pm$ 0.004 0.035 $\pm$ 0.003 Montmorillonite 0.010 $\pm$ 0.005 0.005 $\pm$ 0.001 0.017 $\pm$ 0.003 Hematite 0.004 $\pm$ 0.001 0.004 $\pm$ 0.001 0.004 $\pm$ 0.001 Goethite 0.008 $\pm$ 0.001 0.023 $\pm$ 0.002 0.037 $\pm$ 0.002 Ferrihydrite 0.002 $\pm$ 0.000 0.007 $\pm$ 0.003 0.015 $\pm$ 0.002

-Line 245. Mn(II), being redox active, is more likely to produce hydroxyl radical than to scavenge (Zarate-Reyes et al., 2017 Appl Clay Sci; Shi et al, 2016 Nature Sci Rev.)

R: We agree with the comment. Mn(II) can not only act as a scavenger for hydroxyl radical but also likely to produce hydroxyl radical (Lemire et al., 2013; Barnese et al., 2012). Because enzymes are vulnerable to the Fenton reaction and the produced hydroxyl radical, the presence of manganese may prevent protein damage by radicals (Anjem et al., 2009). In the revised manuscript, we revised this sentence and also listed as follows.

“The Mn(II) in the montmorillonite was reported to act as a scavenger for any hydroxyl radical production (Garrido-Ramírez et al., 2010), which may explain the promotion of microbial growth.” (Page 12, Lines 244-246 in the original manuscript)

was changed to

“The Mn(II) in the montmorillonite was reported to act as a protective agent for enzymes (Anjem et al., 2009; Garrido-Ramírez et al., 2010), which may explain the promotion of microbial growth.” (Page 12, Lines 253-255 in the revised manuscript) The added references are also listed as follows.

Anjem, A., Varghese, S., and Imlay, J. A.: Manganese import is a key element of the OxyR response to hydrogen peroxide in Escherichia coli. Mol. Microbiol. 72, 844–858, 2009.

Barnese, K., Gralla, E. B., Valentine, J. S. & Cabelli, D. E.: Biologically relevant mechanism for catalytic superoxide removal by simple manganese compounds. Proc. Natl. Acad. Sci. 109, 6892–6897, 2012.

Lemire, J. A.; Harrison, J. J.; Turner, R. J., Antimicrobial activity of metals: mechanisms, molecular targets and applications. Nat. Rev. Microbiol., 11 (6), 371-384, 2013.

-Line 267. Replace expect with except.

R: Thanks! In this sentence "expect" was changed to "except" (Page 13, Line 280 in the revised manuscript).

-Line 270. The production of acids by bacteria is plausible, but the surface area of the minerals is far greater (in general) than that of the bacteria, thus you might consider that each mineral surface attracts or repels H+ and OH- which is the major factor in buffering the fluid pH. You should test this by monitoring the pH (and Eh) of the suspension of minerals alone, compared to the suspension of bacteria alone.

R: In the revised manuscript, we added the evidence about the production of acids by providing the mapping of 3344 cm-1 (OH from -COOH) and 1230 cm-1 (C-O from -COOH) in Fig. 5. Based on the suggestion of Reviewer 1, we added the Eh of the suspension of minerals alone and the suspension of bacteria alone as Table S2. All experiments were performed in triplicate. The results from Eh showed that the Eh of minerals or bacteria alone was markedly decreased after 12 h cultivation (Table S2), suggesting an increase of proton production or cation solubility. The revised part was colored in red in the revised manuscript and also listed as follows.

"The pH decline suggests the production of organic acids by Pseudomonas brassicacearum J12." (Page 13, Lines 268-269 in the original manuscript)

was changed to

“The pH decline suggests the production of organic acids by Pseudomonas brassicacearum J12, which was supported by the presence of -OH (3344 cm-1) and C-O (1230 cm-1) from -COOH (Fig. 5). The redox potential (Eh) of minerals or bacteria alone was markedly decreased after 12 h cultivation (Table S2), suggesting an increase of proton production or cation solubility.” (Page 13, Lines 281-286 in the revised manuscript)

Table S2. Redox potential (Eh) of the suspension of minerals alone, bacteria alone, and after incubation for 12 h (n = 3).

Treatment Eh (mV) Before incubation (mineral alone) After incubation (+ bacteria) Control 190.6 ± 20.18 110.3 ± 16.21 Kaolinite 135.1 ± 34.27 90.8 ± 10.84 Montmorillonite 142.5 ± 15.62 117.1 ± 25.17 Hematite 306.5 ± 43.74 189.5 ± 21.65 Goethite 199.4 ± 41.27 139.1 ± 11.17 Ferrihydrite 103.3 ± 5.88 79.8 ± 12.17

- Line 289. This requires H2O2, what is the source of that in the mineral-microbe suspension?

R: In this study, bacterium (i.e., Pseudomonas brassicacearum J12) is the source of H2O2.

-Line 295. Al3+ is not redox active, so why (or how) could it be correlated with production of hydroxyl radical? The toxicity of Al is from interactions with phospholipids, not production of radicals.

R: We agree with the comment. The toxicity of Al is from interactions with phospholipids, not production of radicals. Correlation between soluble Al and HOâĂć (R = -0.35, t = -3.36, p = 0.004) was found, because the volume of sample used by test is little, under this condition no correlation between soluble Al and HOâĂć is considered. In revised manuscript, we replaced “a weak” with “almost no” (Page 14, Line 311 in the revised manuscript).

-Line 305. This analysis is confusing to me. Did you add all of the minerals to this bacterial suspension and you are looking for which mineral dominates?

R: No. We did not add the minerals to this bacterial suspension. Here the sample was from the ferrihydrite treatment, i.e., cultivating 25 mg/mL ferrihydrite with Pseudomonas brassicacearum J12 for 12 h. During cultivation, iron oxides can be transformed into hematite, goethite, ferrihydrite, iron(II) oxalate, and iron(III) oxalate by the bacteria J12. Therefore, we used these samples to fit the component of iron oxides after cultivation.

-Line 320. This suggests that the bacteria reduce the mineral Fe, which in turn produces radicals that oxidize the bacteria. Why would bacteria not have a defense against such radicals?

R: Great comment! The production of ROS-scavenging enzymes, such as superoxide dismutase (SOD) and catalase (CAT), as well as other cellular antioxidants by bacteria may contribute to the protection of bacteria from such radicals. While excessive ROS in aerobic conditions causes the depletion of antioxidants and the inhibition of particular enzyme activities that are vital for cell growth (Lemire et al., 2013). Extracellular HOâĂć oxidizes cardiolipin (CL), the important component of cell membrane, which would facilitate soluble Fe2+ penetration into the cell that promotes intracellular HOâĂć production (Wang et al., 2017). Thus, we deduce that HOâĂć is continuously produced in the culture system and leads to oxidative damage of bacterial cells.

Lemire, J. A., Harrison, J. J., and Turner, R. J.: Antimicrobial activity of metals: mechanisms, molecular targets and applications. Nat. Rev. Microbiol., 11, 371-384, 2013.

Wang, X., Dong, H., Zeng, Q., Xia, Q., Zhang, L., and Zhou, Z.: Reduced iron-containing clay minerals as antibacterial agents, Environ. Sci. Technol., 51, 7639-7647, 2017.

- Line 341. A simple measurement of the OD600 on a mineral suspension should answer this definitively.

R: Thanks! We have added the absorbance of the mineral suspension alone as Table S1 in the revised manuscript. The revised part was colored in red and also listed as follows.

"As a result, the effect of mineral concentration may be minimal." (Page 16, Lines 340-341 in the original manuscript)

was changed to

“As a result, the effect of mineral concentration was minimal (Table S1)” (Page 14, Lines 366-367 in the revised manuscript)

Table S1. Optical density at 600 nm (OD600) of the mineral suspension (n = 3)

Mineral Absorbance at 600 nm 5 mg/mL 10 mg/mL 25 mg/mL Kaolinite 0.005 $\pm$ 0.001 0.019 $\pm$ 0.004 0.035 $\pm$ 0.003 Montmorillonite 0.010 $\pm$ 0.005 0.005 $\pm$ 0.001 0.017 $\pm$ 0.003 Hematite 0.004 $\pm$ 0.001 0.004 $\pm$ 0.001 0.004 $\pm$ 0.001 Goethite 0.008 $\pm$ 0.001 0.023 $\pm$ 0.002 0.037 $\pm$ 0.002 Ferrihydrite 0.002 $\pm$ 0.000 0.007 $\pm$ 0.003 0.015 $\pm$ 0.002

-Line 345. The inhibitory concentration of metals is pH dependent, so unless the work of Illmer and Schinner was at the same pH of your experiment (after 12 hrs) then this reported concentration may not be relevant. You need to determine the MIC and MBC concentrations at the pH of your mineral-microbe mixture (after incubation).

R: Thanks! In the revised manuscript, we deleted “A previous study showed that a 58 μM ($\sim$1.6 mg L-1) concentration of Al has toxicological effects on Pseudomonas sp. (Illmer and Schinner, 1999).” (Page 16, Lines 370 in the revised manuscript)

-Line 385. The phyllosilicates generally have a negative charge on their more extensive basal surfaces. Positive charges are limited to broken edges of the structure. This may not be true for ferrihydrite and goethite. But remember that hydroxyl radicals only exist for 1ns, so for them to interact with bacteria, there must be an attractions between the microbe and mineral surface where the radical is generated.

R: Great comment! In the revised manuscript, we revised this sentence and also listed as follows.

"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)." (Page 18, Lines 384-387 in the original manuscript)

was changed to

“we deduced that HOâĂć may mainly generate on the mineral surface, in view of the fact that superoxide does not diffuse far from the site of formation (Tang et al., 2013).” (Page 18, Lines 417-419 in the revised manuscript)

-Line 403. It is unclear what you mean by ’stabilization role’ here. What does the EPS do to stabilize the ferrihydrite?

R: Ferrihydrite is meta-stable and often aged to other crystalline minerals (e.g., hematite). The presence of EPS can enter the network structure of minerals and thus prevent the formation of crystalline minerals (Braunschweig et al., 2013; Li et al., 2016). In the revised manuscript, we added the corresponding explanation for this "stabilization role" and also listed as follows.

"The stabilization role of EPS was mainly identified as its combination into the network structure of minerals, which prevents the formation of crystalline minerals (Braunschweig et al., 2013)" (Page 19, Lines 441-443 in the revised manuscript).

Braunschweig J., Bosch J., and Meckenstock R. U.: Iron oxide nanoparticles in geomicrobiology: from biogeochemistry to bioremediation, New Biotechnol., 30, 793-802, 2013.

-Line 412. This is an important result of this study!

R: Thanks!

-Line 434. Do you suggest that the H2O2 required is generated by the bacteria or by dissolved O2 in solutions? Have you monitored the Eh of the solutions?

R: Yes. Our hypothesis in this study is that bacterium-initiated free-radical mechanism (i.e., Fenton-like reactions) by producing H2O2, which inhibits the growth of bacteria through. The paper published in Science by Diaz et al. (2013) had shown that taxonomically and ecologically diverse bacteria from terrestrial environments were a vast source of superoxide (O2âĂćïij■) and H2O2 (Diaz et al., 2013).

Diaz, J. M., Hansel, C. M., Voelker, B. M., Mendes, C. M., Andeer, P. F., and Zhang, T.: Widespread production of extracellular superoxide by heterotrophic bacteria, Science, 340, 1223-1226, 2013.

In the revised manuscript, we added Eh of the suspension of minerals alone and the suspension of bacteria-mineral mixture (after incubation) as Table S2. All experiments were performed in triplicate.

Table S2. Redox potential (Eh) of the suspension of minerals alone, bacteria alone, and after incubation for 12 h (n = 3).

Treatment Eh (mV) Before incubation (mineral alone) After incubation (+ bacteria) Control 190.6 $\pm$ 20.18 110.3 $\pm$ 16.21 Kaolinite 135.1 $\pm$ 34.27 90.8 $\pm$ 10.84 Montmorillonite 142.5 $\pm$ 15.62 117.1 $\pm$ 25.17 Hematite 306.5 $\pm$ 43.74 189.5 $\pm$ 21.65 Goethite 199.4 $\pm$ 41.27 139.1 $\pm$ 11.17 Ferrihydrite 103.3 $\pm$ 5.88 79.8 $\pm$ 12.17

-Supplementary Figures Figure S6. How do you explain the drop in pH from 7.2 even in the control suspension? Figure S8. Why does the control have soluble Al even though there are no minerals in it?

R: Thanks! The drop in pH is mainly attributable to the fact that bacteria can secrete amounts of organic acids during incubation as shown in Fig. 5 in the revised manuscript. The concentration of soluble Al in Figure S8 is almost zero.

Please also note the supplement to this comment:
https://www.biogeosciences-discuss.net/bg-2018-479/bg-2018-479-AC1-supplement.zip

Interactive comment on Biogeosciences Discuss., https://doi.org/10.5194/bg-2018-479, 2018.

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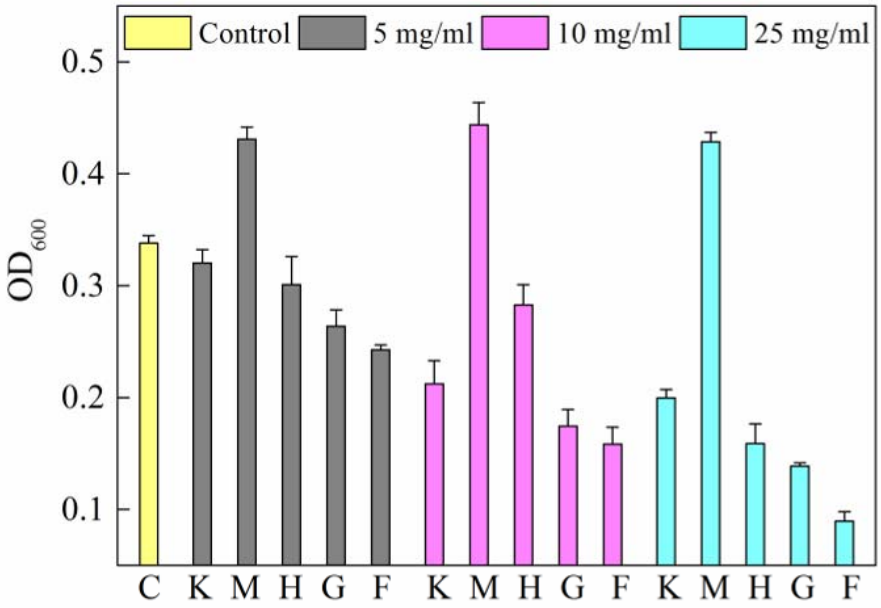

Fig. 1. Optical density at 600 nm (OD600) of 8-h-old Pseudomonas brassicacearum J12 subcultures taken after 12 h growth with different minerals and with no minerals (control). K, kaolinite; M, montm

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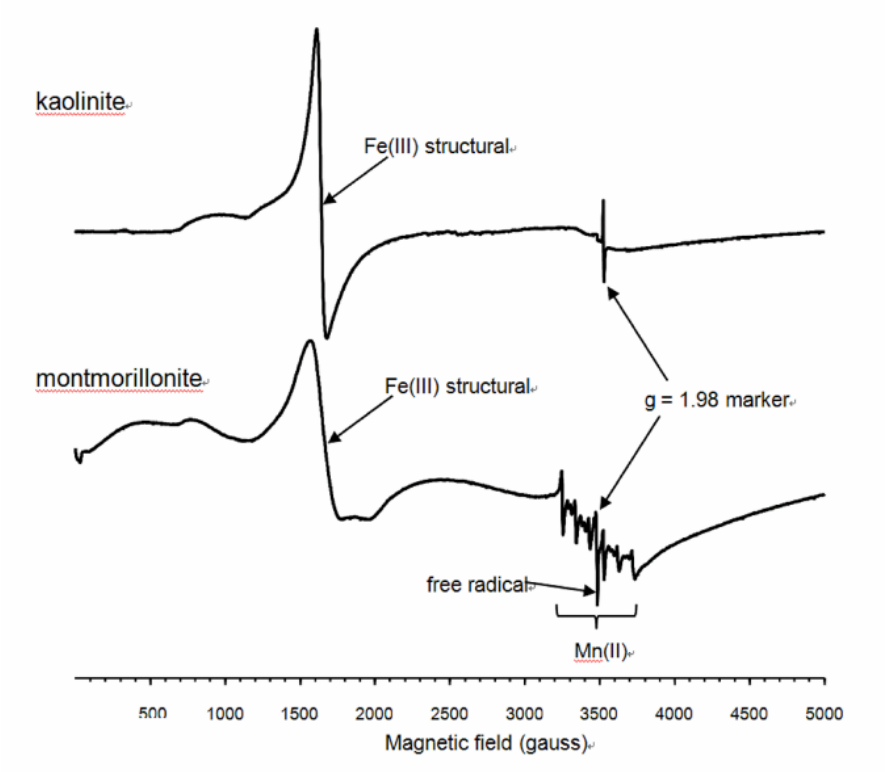

Fig. 2. Wide scan EPR spectra of both the kaolinite and montmorillonite.

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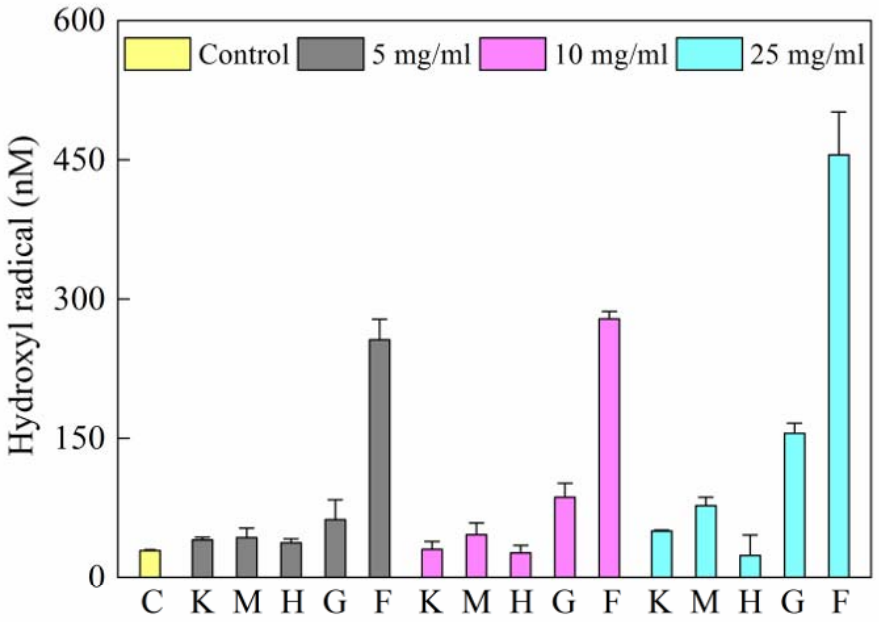

Fig. 3. Generation of hydroxyl radical (HOâĂć) after 12 h growth of Pseudomonas brassicacearum J12 with different minerals and with no minerals (control). K, kaolinite; M, montmorillonite; H, hematite; G, goeth

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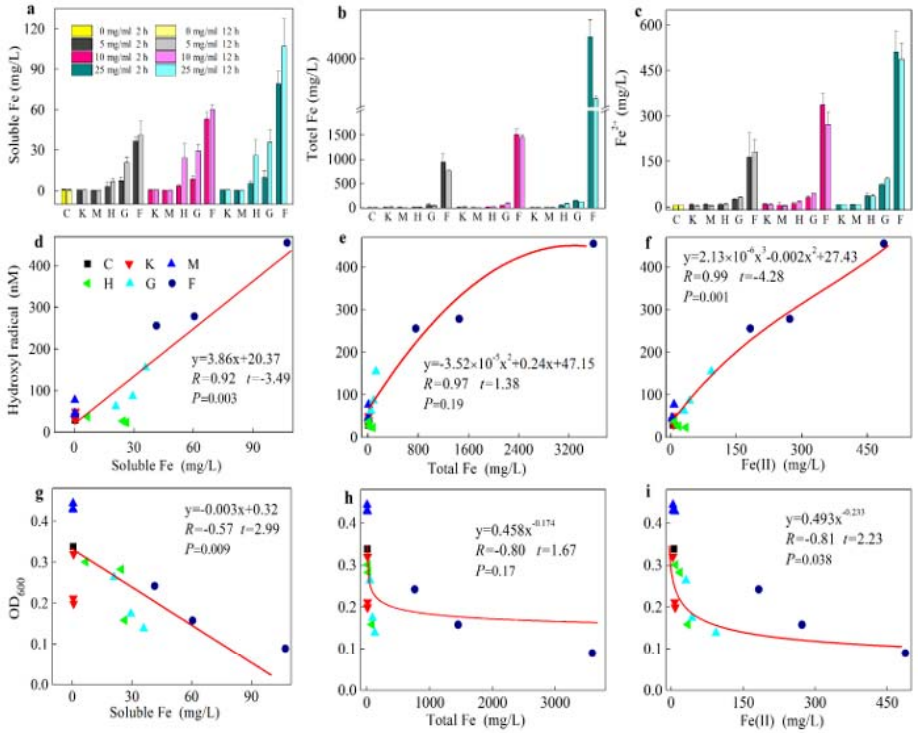

Fig. 4. Iron chemistry (a-c) and its correlation with hydroxyl radical (HOâĂć) (d-f) as well as optical density at 600 nm (OD600) (g-i). (a) soluble Fe. (b) total Fe. (c) Fe(II). (d) soluble Fe vs HOâĂć. (e) tota

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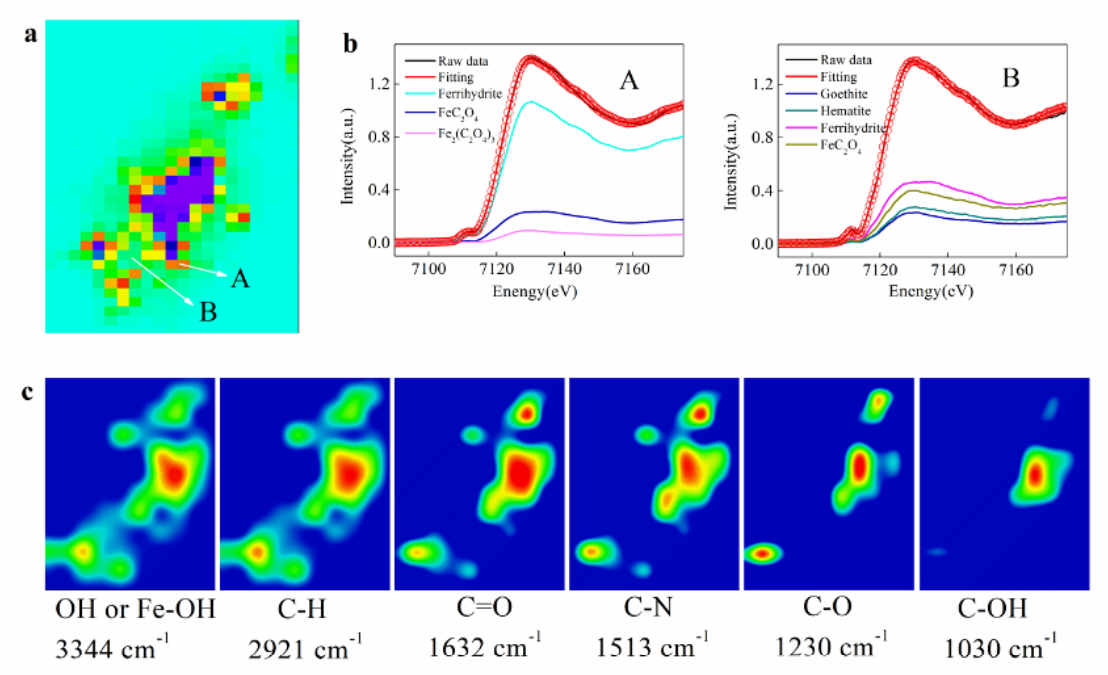

Fig. 5. Correlative micro X-ray fluorescence (μ-XRF) and synchrotron-based Fourier transform infrared (SR-FTIR) analysis of the thin section from the cultures of the 25 mg/mL ferrihydrite treatment after 12 h

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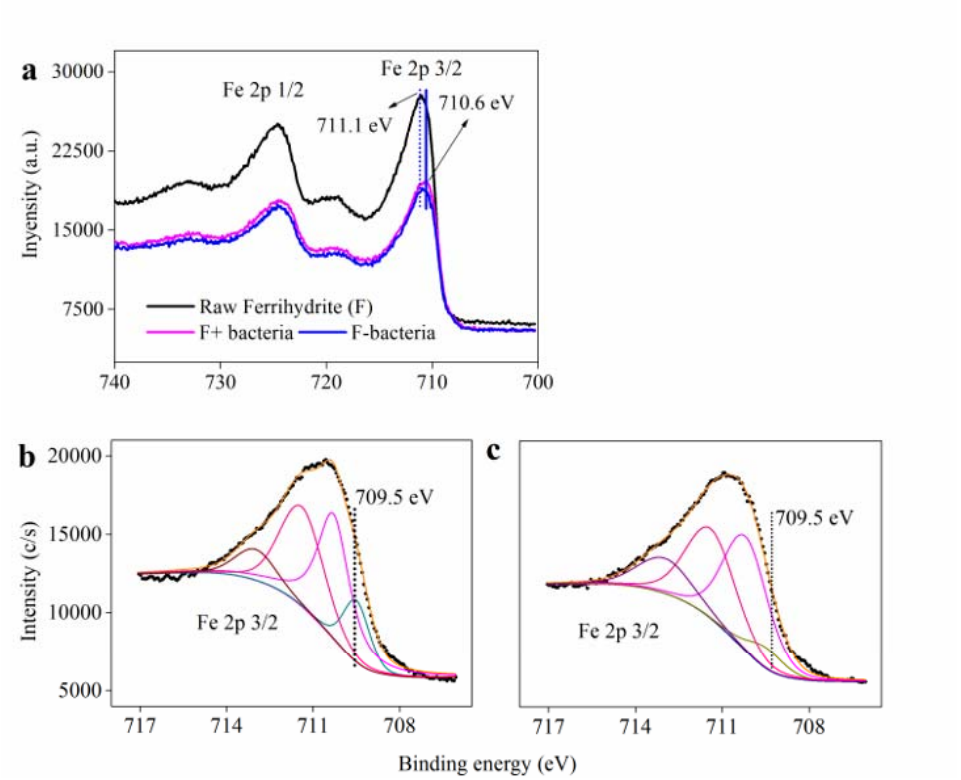

Fig. 6. (a) Fe 2p X-ray photoelectron spectroscopy (XPS) spectra of ferrihydrite samples, F+bacteria and F-bacteria; (b-c) Fe 2p 3/2 spectra of F+bacteria and F-bacteria, respectively, during the cultivation

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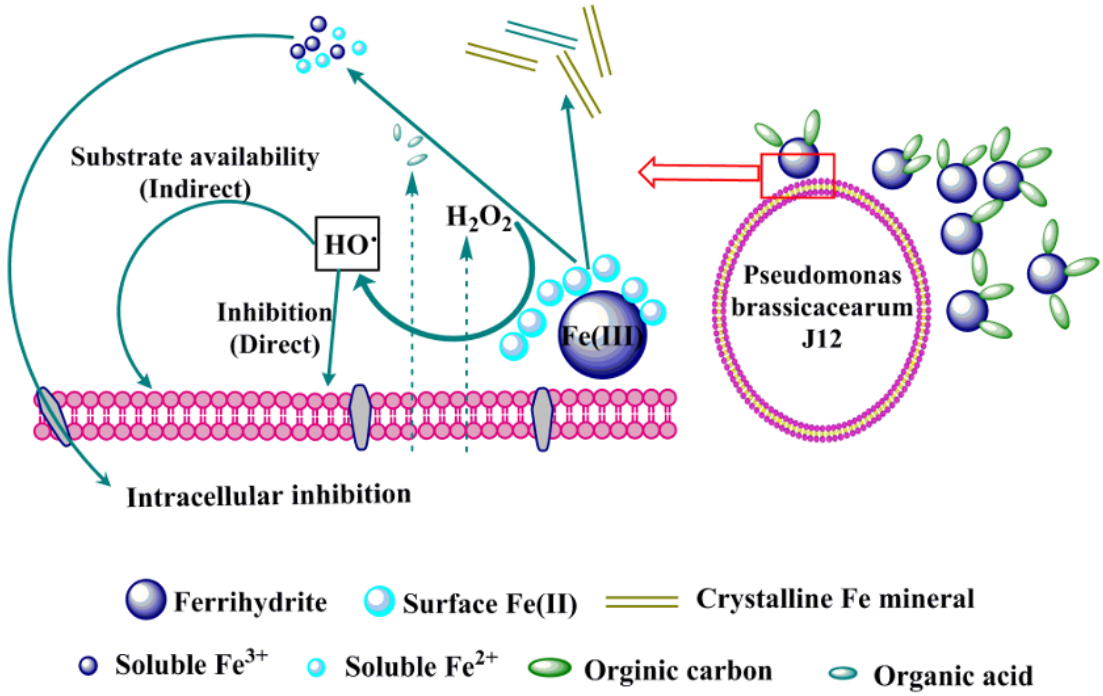

Fig. 7. Schematic of the bacterial inhibition by Fe(III)-containing minerals through a free-radical mechanism.

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