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***Interactive comment on “Effects of copper mineralogy and methanobactin on cell growth and sMMO activity in <i>Methylosinus trichosporium</i> OB3b” by E. Chi Fru et al.***

**E. Chi Fru et al.**

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Response to Reviewer 1 comments (Dr Kolb)

We thank Dr Kolb for his highly supportive comments and constructive criticism for manuscript improvement. Our response is divided into two sections. First, we respond to what we considered are the major scientific and technical issues. Second, we respond to comments related to presentation. Within each response, we provides our responses in the order of reviewer comments. Each comment is quoted in bold italics followed by our response. Our planned alterations to the revised manuscript are also described.

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## Scientific related comments

1. The major problem with current manuscript is lacking deep reaching conclusions. Overall conclusions (page 2852, lines 23-26; page 2864; lines 10-17) are weak. For example, why does methanobactin stimulate growth of *M. trichosporium* OB3b on tenorite, but did not repress the copper-independent sMMO activity in presence of the copper source Tenorite. .... what do you mean by 'This has implications to in situ bioremediation and other studies on methanotroph function in terrestrial systems.' What implications has your study for methanotrophs in terrestrial ecosystem; i.e., soils?

- We agree that the wider significance of the work was rather briefly articulated, especially in the abstract. However, we were reluctant to be too expansive in the work's implications because we are looking here at laboratory simulations, which are useful and suggestive but not conclusive. Regardless, in a revised manuscript, we will expand on a few of the specific issues raised by the reviewer, such as the role that methanotrophs with sMMO play in the degradation of organic contaminants such as chlorinated alkanes. Our results clearly suggest where in situ sMMO activity might dominate based on mineralogical conditions.

With respect to the reason for stimulation of growth of *M. trichosporium* by methanobactin when grown on Tenorite without suppression of sMMO we do not have an unequivocal explanation for this phenomenon; however, on page 2863 lines 21-25 of the manuscript we suggest this may result from 'subtle Cu toxicity suppression, enhanced Cu bioavailability after release, or something more speculative not yet known (e.g., quorum signalling seen with similar molecules; Miller and Bassler, 2001; D'Onofrio et al., 2010)'. More work is needed to differentiate between these possible causes.

2. Could methanobactin be also important for iron acquisition?

- While the properties of methanobactins are similar to those of some siderophores and it is also known that methanobactins can bind a variety of metals, methanobactins

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are unique because of their very high affinity for Cu (see El Ghazouani et al. 2011. Inorg. Chem. DOI: 10.1021/ic101965j.). This affinity is exemplified the observed selective acquisition of Cu from a Cu doped iron oxide (ferrihydrite). With respect to iron acquisition, it was suggested that *M. trichosporium* might produce siderophores (Yoon et al. 2010. Env. Microbiol.Rep. 2:295–303, although this is not yet proven because specific molecules have not been identified. Methanotrophs also have been shown to produce extracellular flavins (Balasubramanian et al. 2010. Appl. Environ. Microbiol., 76:7356–7358, 2010); however, the affinities for Cu and Fe of these molecules are many orders of magnitude lower than Cu for methanobactin. Therefore, strongly suspect methanobactins are not important to Fe uptake. In fact, this would be counter logical and atypical of most metabolic processing systems; i.e., if Fe is required for sMMO and Cu is required for pMMO then is highly unlikely that the same metal shuttling molecule would be used for both functions.

3. Page 2853 Lines 18-19, 'very high affinity'. Please, provide a concrete value, e.g. KM.

- In a revised manuscript, we will more explicitly include recent data indicating extraordinarily high Cu(I) affinity values ( $k$ ) of  $(6-7) \times 10^{20}$  litres per mole ( $M^{-1}$ ) (El Ghazouani et al. 2011). These values are the highest known Cu affinity values in biologically produced molecules (i.e., non-synthetic).

4. Page 2855 Line 11, Does dissolved S<sup>2-</sup> ions may be toxic for methanotrophs?

- In our experiments, we did not grow methanotrophs in the presence of sulfide minerals; however, the solubilities product of CuS and Cu<sub>2</sub>S are both very low (as shown in Table 1) and it is unlikely that these minerals will be toxic to Methanotrophs because soluble levels would so low. This is an interesting point and deserves further investigation.

5. Fig. 1, y-axis: remove the word 'level'. Error bars from duplicates, at least triplicates are needed to calculate fair errors. Thus, please, remove the error bars. Values are not 'relative values' the shown values are corrected by controls without methanobactin.

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- The reviewer (and also the other reviewer) criticised the quality and mode of data presentation in Figure 1 and we accept the criticisms. However, the reviewers are both reading more into the Figure than what was intended. This Figure and the underlying abiotic experiments were principally performed to assess how methanobactin generally solubilised different Cu minerals (in conjunction with Phreeqc modelling) to aid in choosing suitable minerals for subsequent growth experiments. For those that are not familiar with performing these growth and activity experiments, they are difficult and surprisingly time consuming, and we wanted to only test the most useful minerals for testing our general hypotheses.

Regardless, given reviewer concerns, we have decided to deemphasize this data (and Figure), and move it into the Supporting Information. Instead, we will more emphasize the modelled dissolution data in Table 1 for justifying  $\text{CuCO}_3$  and  $\text{Cu}(\text{OH})_2$  and CuO as the minerals for further study in the growth experiments.

#### Presentation comments

1. The text needs to be improved and the readability of figures should be enhanced (details below).

- We agree, and have identified and resolved the various small errors in the revised manuscript.

2. Use 'terrestrial environments' instead of 'terrestrial settings'.

We are happy to make this change in a revised manuscript.

3. void phrases as for example 'Figure 2 shows: :grew without lag phase' BETTER: ' :grew without lag phase (Fig. 2): :'. OR: ': : patterns shown in Table 1.' BETTER ': : patterns (Table 1).'

- We wholly agree with the reviewer have made appropriate changes in the revised version.

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4. Page 2852 Lines 1-26, the abstract is quite long and includes too much introductory information. Please, shorten it.

- This introductory information was aimed at the general reader to highlight the relevance of Cu and methanobactin to the pMMO and sMMO expression systems. We would like to retain most of this information in a revised manuscript. However, we must abbreviate a little because the reviewer also asked that we extend our implications. These issues will be resolved in a balanced manner.

5. Page 2854 Lines 8-18, Delete this part. This is not an optimal style. The whole part is redundant with the Material and Methods section and distracts from the story.

- A range of different experiments were carried out with subtle differences in setup designed to test different questions about methanobactin, Cu mineral and methanotroph interactions. On this basis, we included the section to help the reader navigate their way through the structure of the paper. This approach was suggested after getting non-expert colleagues to read the manuscript and we feel that this section adds to the clarity, although this section can be compressed if requested by the Editor.

6. Page 2859 Lines 12-13, exchange 'parallel' with 'agree with'

- This will be done in a revised manuscript.

7. Page 2860 Line 4, ': : and physical factors was not initially clear' Please, rephrase it in a concrete way. Which physical factors do you mean?

- The physical factor referred to was the level of contact between cells and minerals, which is properly described in the next section. In hindsight, this statement is not very clear and will be omitted in our revised version.

8. Line 21, replace 'be' with 'have been'

- The more correct past tense will be used in a revised manuscript.

9. Line 22, add after 'but' 'should have been'

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- This will be changed in the revised manuscript.

10. Page 2861 Line 27, ‘: : : and supplemental mb was provided to some flasks’ Which? How many? Cannot that not be clarified in Material and Methods section?

- In the methods section, we state that ‘Extra Mb was provided approximately at 1:1 mb:Cu molar ratio to half of the flasks (in duplicate) and no additional mb was provided to the others, and sMMO activity, CH<sub>4</sub> levels, and OD<sub>600</sub> were monitored over time’. In the revised manuscript, we will change the word ‘extra’ to ‘supplemental’ to reconcile the methods section with the later use of the word supplemental. We appreciate this comment because the terms were used in a rather sloppy manner.

11. Page 2863 Lines 14-16, too long sentence. Two thoughts = two sentences

- In the revised manuscript, we will break the one sentence into two sentences.

12. R1.15 Line 24, rephrase this statement in a concrete way.

- The statement is ‘Although mechanistic details for each mineral are still needed, these observations generally show that the nature of each mineral Cu influences how the cells obtain Cu and that the relative role of mb in that process varies from mineral to mineral.

In the revised manuscript, our rewording will be ‘Although mechanistic details for each mineral are still needed, these observations show that mineral type clearly influences Cu acquisition and that mb affects Cu acquisition differently among different minerals.’

13. Fig. 2, Remove the word ‘pattern’ from the legend text.

- This word will be removed in a revised manuscript

14. Fig. 3, Convert Panel B in a line-dot graph. It is much better readable and fits better to the temporal continuity of the data. Second last sentence in the figure legend: this is a result and should be mentioned in the text. Please, remove it from the legend text.

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- Both of these changes will be made in the revised manuscript.

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