

Interactive comment on “Microbial responses to chitin and chitosan in oxic and anoxic agricultural soil slurries” by A. S. Wieczorek et al.

A. S. Wieczorek et al.

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We appreciate the constructive comments of reviewer 2. You will find below a point-by-point rebutal. The uploaded manuscript version integrates changes due to comments of reviewer 1 and 2.

Interactive comment on “Microbial responses to chitin and chitosan in oxic and anoxic agriculturalsoil slurries” By A. S. Wieczorek et al. Anonymous Referee #2 Received and published: 2 April 2014

REVIEWER: [...] The authors identified previously unknown *chiA* genotypes and potential chitinolytic taxa in their soil slurry incubations. The main hypothesis '(i) that chitin in soil is not primarily hydrolyzed via deacetylation to chitosan' remains unveri-

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fied. This is mainly due to fact that the authors do not clearly address questions about the transferability of their results to in situ conditions or other soils.

RESPONSE: The authors do not think that the hypothesis was completely unverified. The authors agree that further studies are needed to address it in that generally worded form. However, our study revealed which pathway is the most likely one. In the revised manuscript version, we modified the wording '(i) that chitin in the investigated soil is not primarily hydrolyzed via deacetylation to chitosan'. Also, in the original version of the section final conclusion we chose a cautious interpretation of the relevance of our findings, i.e. 'The investigated soil microbial community likely degraded chitin via “direct” hydrolysis, and not by initial deacetylation to chitosan.'

REVIEWER: The applied methods are overall appropriate. However, the materials and methods section is missing some information, i.e. quantification of oxygen and ferrous iron.

RESPONSE: Information has been added. See below.

REVIEWER: The description of soil slurry incubations could be improved, e.g. better overview of the different treatments, information about unsupplemented controls, rationale behind the chosen concentrations. Latter is especially important for questions about their environmental relevance and if the amount of applied chitosan did result in (foreseeable) toxicity. A statement about this should be implemented in the text.

RESPONSE: Information has been added. See below.

REVIEWER: Lastly, I have concerns about the applied *chiA* genotype difference criterion of 50% amino acid dissimilarity. RESPONSE: This issue will be discussed in detail below.

REVIEWER: The paper makes a solid contribution but I definitely see room for improvement.

More specific comments below:

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REVIEWER: P.3 I.21: Toxic at which concentrations? In this context: is it environmentally relevant?

RESPONSE: Minimum inhibitory concentrations for *S. simulans* and *S. aureus* as determined by Raafat et al. (2008) were in the range of 2 to 750 $\mu\text{g/ml}$ depending on the culture medium. Such concentrations might be relevant, however almost no data on in situ concentrations of chitosan in soils exist. Note that Raafat and coworkers used a chitosan solution (1% [wt/vol] in 1% acetic acid) for their experiments.

In our experiments, soil slurries were supplemented with ground chitosan which is insoluble at neutral pH. The initial concentration was 2500 $\mu\text{g/ml}$. The biogeochemical interaction of the applied amount of chitosan with the soil matrix was not predictable for us due to the complex nature of a soil matrix. Therefore, also a toxic effect was not foreseeable. Before the experiment we could neither exclude nor predict a toxic effect. For example it can be assumed that anions compensate the polycationic nature of chitosan. The positive charge of chitosan has directly and/or indirectly a key function for the inhibitory effect of chitosan (Raafat et al., 2008). Notably, the nitrate concentration is decreased in the chitosan supplemented treatment under oxic conditions (Figure 1b) indicating that such ionic interactions indeed took place. Production of carbon dioxide in the supplemented oxic and anoxic treatments equals the values of the unsupplemented controls and therefore indicates no toxic effect.

REVIEWER: P.5 I.12: 'can differentially impact on the stimulation' change to 'can differentially impact the stimulation'

RESPONSE: Has been changed.

REVIEWER: P.5 I.1: I would avoid the term 'classic' in this context.

RESPONSE: Has been changed to 'well known'.

REVIEWER: P.6 I.11 Did the authors mean 'soil with oxic or anoxic water'?

RESPONSE: Yes. Soil was suspended in oxic water for oxic soil slurry incubations or
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in anoxic water for anoxic soil slurry incubations.

REVIEWER: P.6 I.24 Please specify? How large were these crystals? Why were these crystals not ground?

RESPONSE: The authors do not know the precise size. The ground material as inspected by eye contained distinguishable particles, suggesting that chitin crystals were much larger than single bacterial cells.

REVIEWER: P.7 I.7 Why did the authors choose these concentrations? Are they environmentally relevant? Would an inhibition of microbial activity by chitosan toxicity (at which concentration?) have been expected in this set up?

RESPONSE: Concentrations needed to be substantially higher than in situ to stimulate products and growth of responding organisms which would allow for detection by chiA-TRFLP. Soluble sugars were supplemented in equimolar (based on monomers) concentrations, i.e. 250 μM of monomer equivalents. Concentrations were in the lower range of used HPLC analytic. Detection limits for various sugars and products were in the range between 30 and 50 μM . To ensure sufficient amounts of formed products for quantification the aforementioned concentrations were chosen. Due to same reasons, knowing the chitin or chitosan degradation would be much slower, than that of soluble sugars high concentrations of biopolymers were supplemented to ensure detectability of products by HPLC.

REVIEWER: P.7 I.7-8 Concentrations in your treatments are not easily understandable, i.e. three treatments and two concentration levels. The reader has to go back in the text in order to understand. Consider rephrasing

RESPONSE: In the revised version of the manuscript the authors added a rationale for used concentrations.

REVIEWER: P.7-8 How were oxygen and ferrous iron quantified?

RESPONSE: This information has been added to the revised manuscript version.

Briefly, oxygen was measured with GC TCD and ferrous iron with a specific colorimetric assay employing phenantroline.

REVIEWER: P.10 I.24 I consider the chosen threshold value of 50% amino acid dissimilarity as too high - even for a functional marker gene. What is the (ecological) rationale behind this grouping? I don't see an incongruency to organismal phylogenies as a good reason here. For example, Cretoiu et al. (2012) chose a difference criterion of 20% which seems more appropriate for diversity estimations. Are the authors sure that they did not miss some important messages here?

RESPONSE: We are convinced that for a taxonomic grouping of OTUs on phylum level the chosen cutoff is suitable. The phylogenetic tree revealed a strong correlation of the OTUs with the phylogeny on phylum level. From our perspective that allows us to conclude when a given TRF responded, which phylum responded. The ecological meaning of alternative definition of cut offs to define OTUs (20% or 25% Beier et. al.2012, Cretoiu et al. 2012) would be difficult to interpret as there is not a guaranty that grouping on a lower taxonomical level (for example family or genus) is similar robust. Only few TRFs responded in our study and we wanted to identify which higher rank taxa responded.

REVIEWER: P.12 I.17 Please rephrase to 'unsupplemented controls' and add this information to your figures and materials and methods section.

RESPONSE: Done as suggested.

REVIEWER: P.15 I.17-20 I have doubts that rarefaction analysis at such OTU cut off values provide meaningful information about genotype richness.

RESPONSE: The authors are convinced (because on above described reasons) that the chosen cutoff was suitable to relate observed changes in TRFLP patterns with phylogenetic information. For sure, with means of a much larger dataset a more comprehensive and refined analysis of the detectable diversity of chi A would have been

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possible. Nonetheless, the taxonomic resolution of TRFLP would not improve. It was not the intention of our study to gain a comprehensive survey on detectable diversity of chiA in our soil but to have a reference dataset to relate TRF patterns to sequence information.

REVIEWER: P. 19 I.26 and P.21 I.17 The authors should be more precise about what they consider as 'high similarity' and 'distantly related'.

RESPONSE: We added concrete values to the text.

REVIEWER: P.21 I.25 Which experimental conditions?

RESPONSE: Slurry, i.e. liquid phase, and permanent shaking. We added this concrete information to the text.

REVIEWER: Fig. 4 Why did the authors choose significance levels of $p \leq 0.06$ and $p \leq 0.2$, instead of conservative values $p \leq 0.05$ and $p \leq 0.1$?

RESPONSE: $P \leq 0.05$ is a commonly used threshold. p values represent the likelihood with which the 0 hypotheses can be disproved. In addition, there are different levels of significance: weak significant $\leq 10\%$ (*), significant $\leq 5\%$ (**) and $\leq 1\%$ (***) highly significant. We have chosen the Mann-Whitney U Test because due to not normal distribution of data, it seemed to be more appropriate. However, if we would have applied the Student T test (which is more sensitive) TRF 54bp and TRF 264bp under oxic conditions would be significant ($p \leq 0.05$). Under anoxic conditions 264bp would even be highly significant ($p \leq 0.01$). TRF 114bp fails these criteria by the measures of the Mann-Whitney U Test because only 2 of the three replicates responded. However, with a p-value of 0.21 has to be regarded as somewhat significant.

Interactive comment on Biogeosciences Discuss., 11, 2155, 2014.

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