

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

**A. S. Wiczorek et al.**

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We appreciate the constructive comments of reviewer 1. 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 agricultural soil slurries” by A. S. Wiczorek et al. Anonymous Referee #1 Received and published: 21 March 2014

REVIEWER: General comments.

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REVIEWER: . . .a) Only a single soil was sampled, there is no guarantee that different environmental conditions don't favor different degradation mechanisms.

RESPONSE: We agree that different environmental conditions could have favored different degradation mechanisms. A general or final conclusion about the preferential degradation mechanism is indeed only possible when more soils with different characteristics would have been compared. Therefore, we avoided a general or final conclusion (e.g. P2171 L 9 or P2175 L6).

REVIEWER: . . .b) Experiments were done in 1:2.5 slurries, not in natural soil and on an overhead shaker. This introduces substantially different conditions than found in natural soil (more akin to wetlands or sediment. These limitations should be mentioned in the discussion.

RESPONSE: Slurry format was chosen, to guarantee homogenous experimental conditions and to allow for sampling small aliquots of the slurry for chemical analytics. We are aware of the fact that soil slurries feature different conditions compared to natural soil. Therefore, we do not over interpret the data and talked of changes we observed in agricultural soils slurries.

REVIEWER: The study uses TRFLP and classic clone libraries. While this is no longer the latest technology, I think it is sufficient for the purpose here. I am a bit doubtful of the association of OTU with T-RFLP presented by the paper– I think this is a bit tenuous as the clone library may not represent the full diversity, and thus doing this could lead to wrong interpretations. In some cases the association is anyway equivocal (see below). I would like to see this discussed briefly.

RESPONSE: Please, see below.

REVIEWER: The authors conducted additional short term experiments with GlcN additions, but not much is reported on these. In general it would have been interesting to measure GlcN, especially also in the GlcN treated samples.

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RESPONSE: Information on the short term experiments with GlcN can be found on P2167 L18 and following and in figure 2 except the data on GlcN, which we unfortunately were not able to measure with the used HPLC method. We agree that measuring GlcN would have added value to our study. However, it is was evident that GlcN was metabolized to carbon dioxide under oxic conditions and carbon dioxide and acetate under anoxic conditions for the GlcN supplemented soil slurries.

REVIEWER: The methods section actually mentions analysis of sugars, but no data are reported – it is noted once that no GlcN was measured, which I think is unusual for soil - what happened there?

RESPONSE: Sugars were measured. Data on [GlcNAc]<sub>2</sub>, GlcNAc and GlcN are reported. Please, refer to figure 2 and P1267 L18 and the following lines. Although GlcN was not measured by the used HPLC method, we have convincing indirect evidence that it was metabolized, since net CO<sub>2</sub> production occurred in both oxic and anoxic GlcN supplemented slurries. GlcN is an essential part of soil organic matter and a substantial source for carbon and nitrogen. However, it occurs predominantly in a polymeric form for example in the cell wall of prokaryotes or in structural biopolymers such as chitin and chitosan. The proportion of soluble single molecules of GlcN in soil is likely very low due to efficient consumption by pro- and eukaryotes. [GlcNAc]<sub>2</sub>, GlcNAc, or Glc were also not measured in any of the controls. Although, it can be assumed that the investigated soil contains these compounds in relative high amounts. The steady state concentrations of soluble and free available [GlcNAc]<sub>2</sub>, GlcNAc, or GlcN likely were below our limit of detection (<50 μM). Many of the studies that have measured the content of amino sugars in soil often use hot water extraction or hydrolysis with HCl or H<sub>2</sub>SO<sub>4</sub> to solubilize and hydrolyse monomer sugar containing polymers. Thus, datasets often not distinguished between free available amino sugars and the polymeric form but report the total amount, which is not the pool of monomers that is directly available to saccharolytic microorganisms.

REVIEWER: It seems a bit of a missed opportunity that these experiments weren't

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performed and analyzed a bit more in-depth, and that potential chitin degradation intermediates like GlcN were not measured. Language wise the manuscript is solid, a few issues I noted are mentioned below. Figures are likewise OK, but could be improved, a few specific comments are also found below. Overall I think this is a valid, if somewhat limited contribution. Specific issues: The methods section seems in parts a bit incomplete: Oxygen was measured, but the method is missing from the materials and methods section.

RESPONSE: Missing information has been included.

REVIEWER: The results report on ferrous iron, materials and methods mentions only iron. Typically determination of Ferrous iron requires special precautions against rapid re-oxidation and it cannot be analyzed in an IC.

RESPONSE: A specific protocol for ferrous iron measurement had been applied and missing information has been included in the materials and methods section.

REVIEWER: Methane is mentioned with two different GC methods.

RESPONSE: This is correct. The setup of the GC used, splits the injected gas sample and loads them on two columns. Both are able to separate methane. One column is connected to a thermo conductivity detector (TCD) and the other to a helium ionization detector (HID). Both are able to detect methane whereby the HID is more sensitive. We did not detect methane within 41 days either with the TCD or the HID.

REVIEWER: A somewhat obscure but potentially relevant paper that was not discussed in this manuscript is the study by Makarios-Laham and Lee 1995, especially because in contrast to this study they found good degradability of chitosan (chitosan PE films, to be precise) in soil incubations.

RESPONSE: We did not state that chitosan was not biodegradable. We did not detect any potential degradation products within 41 days. However, after 156 days we detected an increased CO<sub>2</sub> production in the chitosan supplemented soil slurries and

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thus, concluded that chitosan degradation was hampered compared to chitin degradation in soil slurries (P2166 L22) because of an adaptation of the soil microbial community towards chitin rather than chitosan (which is less abundant in such environment). In the study by Makarios-Laham and Lee (1995) the first time point to determine the weight loss of the samples was after 3 months. Thereby, these authors observed good degradation of chitosan whereby chitin still was more pronounced. After six months both substrates were totally degraded. Under the assumption that chitosan was fully degraded, 54% of the total supplemented carbon were recovered as CO<sub>2</sub> after 156 days (~5 months) in our study. That fits quite well with what is known from literature for microbial metabolization of sugars under oxic conditions (50% dissimilation, 50% assimilation).

REVIEWER: P2164 I think a cutoff value of 50% for OTUs is extremely low, and groups chiA from ecologically very different groups together, making such a definition largely meaning-less. Beier et al. 2012 e.g. used 75%, which seems more sensible. While OTUs thus defined may not correspond to the 16S rRNA phylogeny, they may nonetheless have ecological meaning.

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 allowing 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.

Technical Comments:

REVIEWER: P2157 L5. And in addition some algae.

RESPONSE: Information has been included as suggested. Rephrased.

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REVIEWER: P2159 L 25 gene marker -> marker gene

RESPONSE: Has been changed as suggested.

REVIEWER: P2160 grounded -> ground

RESPONSE: Has been changed as suggested.

REVIEWER: P2161 L5 “Therefore” seems to be misplaced here. L 11 – not clear for a reader what products refers to, at this point. L21 – delete “i.e., “

RESPONSE: Has been changed as suggested.

REVIEWER: P2163 L25+ I think that is questionable. Did you verify this against your clones what exactly is in the "chiA like gene dataset"?

RESPONSE: Our wording might have been imprecise and misleading. Sentence was rephrased for more clarity.

REVIEWER: P2166 L20 “:::within >the< same period:::”

RESPONSE: Has been changed as suggested.

REVIEWER: Figure 1: Hard to make out what is what, not clear where most control values lies. Maybe split this into more panels.

RESPONSE: Figure 1 has been improved as suggested.

REVIEWER: P2167 L 8-9 It is not clear what the percentage values given here refer to (% of what?)

RESPONSE: Has been specified as suggested. Percentage values indicated the recovery of supplemented carbon as acetate, propionate, butyrate and carbon dioxide and electrons as molecular hydrogen under the assumption of a complete degradation of the supplemented chitin. The percentage values are therefore indeed misleading and we have rephrased the sentence and given the gross values.

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REVIEWER: P2169 L18 As noted above, I think the 50% cutoff is too low. Therefore, conclusions regarding the coverage should also be reevaluated.

RESPONSE: We think that for the taxonomic grouping of OTUs on phylum level the cutoff is suitable – see comment above and also rebuttal letter to Reviewer 2.

REVIEWER: P2170 L15: could not be detected – what's your LOD? Did you detect them in the GlcN treated samples? To my knowledge, GlcN is regularly detected even in untreated soils and sediments.

RESPONSE: For GlcNAc2 and GlcNAc our LOD and LOQ are  $1\mu\text{M}$  and  $30\mu\text{M}$ . Nevertheless, we are not sure which page and line you are referring to. P2170 L15 seems unlikely. Did you mean P2171 L16? If this section was meant, we are confused as in this section we concluded that the degradation products likely were consumed after being released by enzymatic hydrolysis. Why should we have detected GlcNAc2 and GlcNAc in GlcN treated slurries?

REVIEWER: L 19 do you maybe mean compared to that observed in? The rest of the sentence implies that you observed faster degradation than Sato et al., but the first part of the sentence suggests that the faster RESPONSE was observed by Sato et al.;

RESPONSE: We indeed meant what you suggested. Sentence has been rephrased.

REVIEWER: L21 grounded -> ground.

RESPONSE: Has been changed as suggested.

REVIEWER: L25 “That is likely ::: ” maybe consider “This is in agreement with our expectations, as:::”;

RESPONSE: Has been changed to: “This observation was in agreement with our expectations”

REVIEWER: L25 “...due >to< high...”

RESPONSE: Corrected.

REVIEWER: P2172 L1f – in this section it's not always clear if you refer to results in the oxic or anoxic treatment. Please, revise.

RESPONSE: Has been revised as suggested.

REVIEWER: L10 Under anoxic conditions accumulation of NH<sub>4</sub> is pretty much inevitable, as far as I know

RESPONSE: Sentence has been rephrased as suggested previously. However, you are right. NH<sub>4</sub><sup>+</sup> is stable under anoxic conditions if no anammox occurs (depending on the environment). However, ammonium released by ammonification can be assimilated by organisms. In addition, degradation of chitin under anoxic conditions, and thereby the release of NH<sub>4</sub><sup>+</sup>, seemed to be slower than under oxic conditions.

REVIEWER: L 10-13 “At the community level. . .” I am not sure this sentence is correct, the metabolism will certainly be different – you observed similar degradation products. You also show no data on N-sugars.

RESPONSE: Sentence has been rephrased. However, we show data on N-sugars in figure 2.

REVIEWER: P2172 L21 According to Figure 5 OTU 3 corresponds to several TRFs, including, again, to 264 – so this association of OTU 3 with TRF 188 appears tenuous. See also OTU 1 and 2 in Fig. 5.

RESPONSE: TRF 188 is without a doubt associated with OTU 3. To be more precise: certain sequences of OTU 3, which likely represent a taxonomic group we could not identify are associated with TRF 188bp. The fact that other TRFs are also associated with this OTU does not weaken the association of OTU 3. OTU 3 is not associated with TRF 264. It is associated with TRF 268.

REVIEWER: L21 According to Fig. 3, TRF 54 also increased in the anoxic chitin treat-

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ment – or maybe it's 223? The colors are hard to distinguish.

RESPONSE: TRF 54 indeed increased also in the anoxic treatment. Due to the high error bars and the low relative fluorescence we decided not to discuss this TRF for anoxic treatment. However, it is not unlikely that TRF 54 responded also under anoxic conditions. TRF 54 is associated with Planctomycetes like *chiA* sequences. Members of Planctomycetes are known to be facultative aerobic. Nonetheless, we improved the figure in a way that the TRFs can be better distinguished.

REVIEWER: Figure 2 I think the inset (panel b) is mostly redundant, the information is already available in panel a. If somewhat smarter placement of the labels can be achieved, panel a would suffice. The significance levels given are strange, however.

RESPONSE: Panel b has been removed as suggested. The significance levels of CCA are not strange in our opinion. They can also be read as: 32% and 43% of total variance which is appropriate for a high explanatory power of each axis.

REVIEWER:  $P \leq 0.06$  is not a typical value, and  $p \leq 0.2$  is not significant. I assume  $p \leq 0.05$  and  $p \leq 0.01$  might be meant?

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.

REVIEWER: Figure 3 – legend “were such small” -> “were so small”; “symbol”: there

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are no symbol in this figure.

RESPONSE: This sentence has been corrected as suggested and was placed in legend 2 as it was supposed to be. The same mistake was corrected in the legend of figure 1.

REVIEWER: Figure 5 legend “...and the numbers of organismal ... and is given in parenthesis.” Check this sentence, something seems wrong.

RESPONSE: “... and is given in...” was changed to “are given in”.

REVIEWER: P2174 L22f It might be noteworthy in this respect that Beier et al. 2011 also concluded that Actinobacteria in aquatic environments appear to rather use chitin degradation products than chitin itself.

RESPONSE: We are thankful for this point and included it in our discussion

REVIEWER: P2175 L8 “Proteobacterial:::”

RESPONSE: Has been changed as suggested.

REVIEWER: P2175 Final conclusions: I think these should relate directly to the initially given hypotheses – the connection with the previous cellulose experiments is not really the topic of this paper.

RESPONSE: We think that the first three sentences directly relate to the hypotheses. Although it is not the main topic the cellulose section adds some valuable additional information that links the issue of the study to the more general issue of biopolymer degradation.

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

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