

Dear referee and PCI editors,

Thank you for the comments and suggestions. Most of the suggestions have been integrated in this revised manuscript. You may notice that the manuscript has changed beyond your suggestions as we received additional feedback on the manuscript during the preliminary PhD thesis defense of Shira Houwenhuysse. We made further improvements on the introduction and the discussion, but most importantly have also reanalyzed all our data. The variable 'stressor treatment' has been divided into two separate variables: infection (absent or present) and cyanobacterium (absent or present), both were incorporated as fixed factors in the model and new analyses were incorporated. We also updated the title to 'Microbiome-mediated tolerance to biotic stressors: a case study of the interaction between a toxic cyanobacterium and an oomycete-like infection in *Daphnia magna*' as now we have integrated the stressors in the title and made the general aim clearer. We also included Anna Krzynowek, Manon Coone, Silke Van den Wyngaert, Arne Sinnesael and Robby Stoks as co-authors as they gave a substantial contributor to this revision, which further improved the quality of the manuscript. We apologize for the fact that the resubmission has taken so long, but we had a lot of troubles with characterizing the infection. That was mainly due to the fact that we were using primers for fungal infections, whereas the infection is an oomycete infection. Going through all the original microscopic pictures and asking advice to Justyna Wolinska and Dieter Ebert, *Daphnia* parasites experts we conclude that the infection is an oomycete-like infection. Further sequencing will need to finally elucidate this, but given that this will take too much time to confirm, we wanted to submit the manuscript with indicating the infection as being oomycete-like. Microscopic pictures unravel proof for that and are integrated in the manuscript.

Kind regards,

Shira Houwenhuysse, Lore Bulteel and Ellen Decaestecker

Line 22: As it is, this part of the sentence is redundant with the previous sentence. I suppose you want to want to suggest a possible causality between the natural microbial community and the

absence of impact of the stressors. This result cannot be explained by the higher microbial diversity of the natural community, because you didn't find any effect of the diversity on the life history traits. It could however be mediated by?

Thank you for this remark. We removed this sentence as we indeed not find a correlation between microbial diversity and survival.

Line 38: Most of these references are related to plants. For me, it seems more appropriate to put references in relation to aquatic systems or at least on different organisms (animals, microorganisms,...) to do a link with the previous sentence.

We now also included references from aquatic environments and rewrote the introduction accordingly (line 40-43).

Line 47: Need some reference here

Due to rewriting of the introduction, this sentence was removed from the introduction.

Line 50: Again references on insects and plants

We added two extra references, one on *Daphnia* (Coors and De Meester, 2008) and one on damselflies (Janssens et al., 2015).

Line 51: Repetition with the previous sentence.

We removed this sentence.

Line 56: Less common because less studied?

No, in the review of Jackson et al. (2015) it is stated that meta-analysis of the marine literature shows that the net impact of multiple stressors is frequently either greater than (i.e., a synergistic interaction; Crain et al., 2008; Harvey et al., 2013) or equal to (i.e. an additive effect; Ban et al., 2017; Strain et al., 2014) the sum of their single effects. Net effects of two or more stressors that were less than the potential additive outcome (i.e., an antagonistic interaction) were less common (Crain et al., 2008; Harvey et al., 2013). We removed the comparison with the marine environment when rewriting as it did not contribute to the overall focus of the introduction.

Line 61: Model instead of study to avoid repetition

Repetition was deleted in the sentence (line 71-73).

Line 63: It might be good to add some percentages or numbers to justify that

In the revised introduction, this sentence was removed.

Line 64: Negative effect on...? Add an example. Maybe put this sentence at the end of the next one.

We clarified the sentence: "The negative effect of cyanobacteria on zooplankton fitness (e.g. survival, grazing,...) is well documented (Ferrão-Filho et al., 2000; Asselman et al., 2012; Lemaiere et al., 2012)." (line 76-77).

Line 72: For me, not necessary as in this paragraph the authors focused on *Daphnia*

We removed this sentence.

Line 75: Of the *Daphnia* I suppose?

It was indeed impacting the filtering process in zooplankton. We did remove this from the introduction as it appeared redundant in the current version to maintain a clear focus.

Line 82: Rephrase, it is obvious that parasites have negative impacts on their hosts.

We have rephrased this sentence (line 82-84).

Line 173: For me the introduction is too long. The authors tested both the stress responses and the differences between laboratory and natural inoculations, depending on the genotype. However, as the introduction progress, I am a little bit lost in all this information and do not quickly understand the purpose of the manuscript.

Based on the first revision, we generalized the introduction. We now restructured the introduction and removed redundant information to make the focus of the introduction clearer.

Line 265-272: Repetition with introduction. This information might be more relevant to use in the introduction when you make the link between *Microcystis* and *Daphnia*

We removed this detailed part from the introduction to avoid repetition and make the introduction clearer.

Line 367: SILVA v138 is available since December 2019 => it will not change the results a lot but referees in journals may ask you to start over with this database.

We have reanalyzed the data with the new training set and new model.

Line 367: If you used DADA2, the output is amplicon sequence variant table (ASV = the appropriate term to use) not OTU or you applied a threshold to groups sequences at this stage?

Thank you for this remark. We corrected throughout the manuscript.

Line 371: Why did you decide to group OTUs at the order level?

To visualize the data we grouped the OTUs at the order level to show the highest possible resolution of the bacteria present in the gut microbiome and still being comprehensible (at family level, too many families are represented). We did remove the figure in the newest version as it did not provide added value to the manuscript.

Line 407: By using raw sequencing data, you suppose that your data is quantitative but the amplification of sequences can vary a lot between samples and therefore may not represent “reality”.

We agree that because of the nested amplification some sequences might be amplified more than others, and thereby bias the real representation of the bacteria present in the gut microbiome. However, EdgeR and Deseq analysis are performed on raw sequencing data. First rarifying your data and then analyze it with EdgeR or Deseq will bias the results even more, as rarefaction can remove rare strains from the dataset.

Line 476: What is the life time of *Daphnia magna*? Why do you analyze the survival during 25 days? Maybe there are more mortality after?

Based on Macke et al. (2017) we expected differences in survival caused by *Microcystis* within 14 days. In addition, mortality by (virulent) parasites is often caused within two weeks and parasite studies on *Daphnia* often track survival for three weeks (e.g., Decaestecker et al., 2007; Overholt et al., 2020) Therefore, we choose to let the experiment run for three weeks after the initial stressor exposure at day 3.

Figure S7: It will be good to put asterisk on the figure (all figures representing boxplots?) between treatments that are significantly different, it will help for the interpretation.

We decided to not include this figure in the revised supplementary information due to reanalyzing all the data. We did indicate significant differences on Figures 4 in the revised manuscript.

Line 509-511: Very repetitive

Introductory sentences were introduced based on a suggestion by the other referee. We removed these introductory sentences in this revision.

Line 590: The beginning of this part can be more related to the first one (“Microbial composition”), as it is also very descriptive.

We restructured the result section and moved the section on the abundances to the top of the microbial composition paragraph. (Line 445-462)

Line 653: I would add one or two examples. Are some OTUs relevant in this analysis? (i.e., did someone already notice some OTUs differences in other model systems with those treatments?)

We chose not to list examples of the ASVs that differ between treatment as this broke the structure of the paragraph too much. A list of the different ASVs can be found in Table SI10 in the supplementary information.

Line 663, Figure S13: Write the p-value and correlations coefficient on Figures S13 and S14 in bigger letters.

We adjusted accordingly (Figure SI4 and Figure SI5).

Line 667: For me, the authors repeat the results too much and do not develop the discussion enough.

We removed repetitive elements from the discussion and elaborated on our possible explanations of the results.

Line 686-704: This paragraph could be shorter, it currently contains more reminders of the results than real elements of discussion. The sole impact of single stressor that is really discussed here is the absence of effect of the fungus treatment on the fecundity. If there is nothing to discuss about the effect of single stressors on survival and body size, you don't need to remind the results here.

We shortened this paragraph by removing repetitive elements from the result section.

Line 713: This sentence is not necessary, the expectations are reminded two sentences above.

We removed this sentence.

Line 720: The phrasing is not really clear

This sentence was rephrased in accordance with the suggestion and the restructuring of the discussion (lines 522-541).

Line 727: Are some bacteria/order/family known to be selected in a specific condition/treatment? I would further develop these two hypotheses.

We elaborated on the increase of Microbacteriaceae within the combination treatment under the lab conditions where the antagonistic interaction was found. (Lines 529-541).

Line 731: Rephrase to make one sentence

This line was removed due to restructuring and rephrasing of the discussion.

Line 736-740: In relation to the literature?

This part was removed in the revised version by restructuring and rephrasing the discussion.

Line 1261: Legend: write the entire word “combination” same for the other figures

We used combination in figures and legend whenever applicable.

Figure S8 and S9: Weighted bray-Curtis. It is either Bray Curtis or Weighted Unifrac but not the combination.

We ran analyses based on Bray-Curtis, weighted and unweighted Unifrac distance matrices and adjusted terminology.