Stressor interactions under differential exogenous microbial exposure in Daphnia magna

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10.31219/osf.io/mske8 version Stressor interactions under differential exogenous microbial exposure in Daphnia magna_version2
Submitted by Shira Houwenhuyse 2021-05-17 16:18

Abstract

Studies on stressor responses are often performed in controlled laboratory settings. The microbial communities in laboratory setting often differ from the natural environment, which could ultimately be reflected in different stress responses. In this study, we investigated how stressor responses differed between laboratory and natural conditions in *Daphnia magna* when exposed to single or multiple stressors. *Daphnia* individuals were exposed to the toxic cyanobacterium *Microcystis aeruginosa* and a fungal infection, *Aspergillus aculeatus* like type. Three genotypes were included to investigate genotype specific responses. Survival, reproduction and body size were monitored for three weeks and gut microbial communities were sampled and characterized at the end of the experiment. Our study shows that natural environments have a more diverse microbial community compared with laboratory conditions, which was ultimately reflected in the gut microbiomes after inoculation. Stressor responses in *Daphnia* were affected by their bacterial environment for survival, but not for fecundity and body size. Fecundity and body size did show a main stressor effect, which could possibly be linked with stressor-specific microbiomes (for *Microcystis* and the combined stressor treatment). In addition, genotype-specific responses were detected for survival and fecundity, which could be linked with the selective capabilities of the *Daphnia* genotypes to select beneficial or neutral microbial stains from the environment.

Keywords: Daphnia magna, multiple stressors, interactions, antagonistic, parasite, Microcystis, microbiome

Round #1

by Bertanne Visser, 2021-07-19 11:57
Manuscript: 10.31219/osf.io/mske8

Decision on your pre-print submitted to PCI Zoology.

Dear authors,

I have now received the reports of three reviewers. Overall, all reviewers were positive about your pre-print and I agree with their assessments. The reviewers made several suggestions that I think would improve your manuscript before recommendation. Please revise your manuscript accordingly and address each of the reviewer suggestions in your rebuttal.

I look forward to receiving your revision.

Yours sincerely,

Bertanne Visser

Reviews

Reviewed by Natacha Kremer, 2021-07-08 14:51

In this paper, the authors test the influence of water (laboratory vs. natural) used for the colonization of axenic *Daphnia magna* (3 different genotypes) on their response to two biotic stressors: a toxic cyanobacterium (*Microcystis aeruginosa*) and a pathogenic fungus (*Aspergillus aculeatus* like). They test how these biotic stressors, alone or in combination, impact host life-history traits and modify the gut microbiota composition. They show that gut microbiota diversity somehow mirrors the diversity of the bacterial composition of the 'inoculation' water (i.e., a more diverse community in *Daphnia* inoculated with water from natural pounds that also have a higher diversity). An interaction between the 'inoculation' water and the response against stressors occurs for survival, but not for the other tested life history traits (fecundity and body size). Bacterial composition was not strongly impacted by stressors.

This study is very interesting and points out how laboratory conditions can potentially bias the response to stressors. In addition, it considers the combination of stressors, which is generally lacking in this field. However, a few points need to be considered:

- The study is nicely designed but 3-factors analyses are complex. The presentation of results is complicated to follow and would benefit being clarified.

- For microbial analyses, various sampling efforts may bias the analyses and interpretation.

- Results should be interpreted and discussed more in light of the 'colonization then stress' experimental sequence and of the dynamics of colonization rather than a concomitant selective process.

- In addition, it would be interesting to address the question of bacterial density (and not only diversity) in this system (and its potential cost).

All these points are detailed bellow.

We thank Dr. Kremer for her constructive comments. We deal with these comments point by point below.

General suggestions for reorganization / clarification:

- Move the M&M before the results, otherwise, it is not possible to understand the experiments

We agree with the reviewer, M&M was placed before the results in order to improve readability of the results.

- Present *Aspergillus* (and potentially the last paragraph of the results) in the M&M (model system)

We introduced a new paragraph in the material and methods section in which we enclose information on the used stressor systems, among which *Aspergillus*, and their phenotypic effects. Additionally, we moved the section on the characterization of the fungus from the results to the M&M section (see lines 409-446).

- Restructure the part linked to the model system: 1) to combine information on feeding in the same paragraph, 2) present all stressor agents and their phenotypic effect together.

We have restructured the info in the M&M section and have pooled information on the model systems, the stressor treatments, the microbiome treatments and the timing of the experiment to further improve readability of the section.

- Add an extra scheme in the M&M indicating the sequence of the experiment (germ-free -> colonization -> stress).

A timeline was added to clarify the timing of the experiment (see line 312).

- Always use the same nomenclature for the experimental conditions (they are different between analyses of life-history traits and microbiota). In addition, 'infection' is misleading because both

the treatments (cyanobacterium and fungi) are biotic stressors. I would rather use 'control' (= Ctl), 'cyanobacterium' (= C)(or 'Microcystis'), 'fungus' (= F) (or 'Aspergillus'), 'combination' (=C+F)

We have made the nomenclature for the stressor treatments uniform throughout the text. We will now consistently use control or CTL, fungus or F, cyanobacterium or C, and combination or F+C.

- Maybe you could structure the result section with the scientific questions you are asking and not the measured traits.

We choose to discuss the results per measured trait to give a clear overview in the result section. We reorganized the discussion so that each research question is answered chronologically. To improve the clarity in the result section, we added introductory sentences in which we explain why a particular trait was investigated.

- Do not start the result section with the table of result

We have moved the table so that the result section does not start with a table.

- Union plots are difficult to read (for me who have never seen this type of graphs before). At minimum, it would be nice to see the categories better (with a cartoon for each stress?)

We clarified the Unionplot by making the nomenclature on the plots uniform with the rest of the manuscript. Unionplots are venn-like presentations of OTUs or ASV used in microbiome research (e.g. Gonzalez et al. 2019).

Met opmerkingen [ED1]: Ik zou hier misschien nog een paar referenties geven waar die Unionplots gebruikt worden. Als je die niet direct vindt, vraag dan aan Shinjini.

SH: Ik heb er één gevonden. Contacteer ik best Shinjini nog om er meer op te sommen?
EDC: je kan snel nog eens horen, misschien kan ze snel een paar refs oplijsten. Vergeet haar dan niet in acknowledgements te zetten misschien…

- I would start the description of bacterial diversity with OTU richness and beta-diversity (and not necessarily present the union plots)

We restructured the microbiome part of the results. This section now starts with OTU richness, followed by beta-diversity and ends with the microbiome composition, EdgeR analysis and Unionplots. We choose to represent the Unionplots as they help us to explain the effects of the microbiome and stressor treatments on *Daphnia* fitness and they are often used for visualization of microbiota/microbiome results (see references given above). We did move some figures to supplementary data as they were not essential for the main questions (see Figure S1, S7, S9, S10 and S11).

- L517-529: this part seems to be a 'result' part.

We agree with the reviewer, this part summarizes the obtained results of the genotype effects. We shortened this paragraph, now it is less repetitive and focusses more on explaining the results (lines 862 – 874).

- I did not have access to the supplementary tables

We uploaded the manuscript under “Restricted Access”. This means that you can see all data and supplementary information if we grant you permission. This is possible by clicking the button “Request access…”.

Introduction

-l22-24: This sentence is not very clear and does not seem to be supported by the results (no difference observed in the microbiome whatever the stressor condition)

We agree with the reviewer and rephrased the sentence based on the rewritten discussion: “Fecundity and body size were negatively impacted by exposure to stress, however, responses were here not microbiome-mediated.” (lines 24-25)

-l55: the production of proteases or genes involved in secondary metabolic transport and catabolism do not seem to be ‘defence’ mechanisms against *Microcystis* (as they do not participate to its elimination), but rather ‘tolerance’ mechanisms (as they limit the cost of their presence)

We agree with the reviewer and adjusted the sentence to “…anti-*Microcystis* tolerance mechanisms”. (Line 77)

- l88-90: Are the variation in gut communities reflecting a selective filtration process or just a random uptake of bacteria from the environment? Is there any order of colonization (with pioneer species)? Is the dynamic of colonization within *Daphnia* gut already known?

So far, not much is known about the dynamics of bacterial colonization within the *Daphnia* gut. What we do know (mostly from parasites) is that attachment to the gut epithelium is crucial. *Daphnia* has a peritrophal matrix (PTM), which makes it ideal for microbiota to establish in the gut epithelium. Ample microbial species are detected in the gut epithelial cells, e.g., microsporidians *Ordospora colligata* and *Mitosporidium daphniae* with prevalence up to 100% in the field. . A PTM can be relevant for bacteria to attach to, given that the chitin barrier is an ideal feeding source and an ideal place to express antimicrobial peptides (AMPs) and pathogen recognition receptors (some of which have been characterized in *Daphnia*, see wFleaBase). We expect that in a first stage, bacteria are taken up randomly and horizontally and is dependent of the environmental bacterioplankton. Throughout the colonization process, different competitive processes can influence the bacterial community. Besides competition between bacterial strains, it is suggested that the host (*Daphnia*) can select for certain strains, such as studied in Macke et al. 2017 and in Houwenhuyse et al. 2021. Studies on the gut microbiome of *Daphnia* have also indicated that the host genotype plays an important role in the present gut microbial community, mechanisms of selection, however, remain still unclear. One of these mechanisms, next to the expression of AMPs, could be vertically transmitted bacterial strains that structure the community through priority effects. But all these hypotheses are so far very speculative so far and need

further testing. We added some aspects on this in lines 116-121 of the revised version of the manuscript.

-l110: please define tolerance in this context

We added a definition on tolerance: "the ability to limit negative impact of a given stressor" (Lines 143 - 144)

-l123-125: Is the overall/specific bacterial load higher in natural pounds than in lab water? In colonized *Daphnia*, do we observe variation in bacterial load in the gut microbiota (and a cost associated with higher loads)?

We did not investigate the bacterial load of the bacterioplankton and *Daphnia* gut samples as we were interested in the bacterial richness and composition. In Callens et al. (2018) the bacterial load was investigated together with the bacterial richness and composition of the gut community and under exposure to different concentrations of the antibacterial oxytetracycline (OTC) In this paper, no correlation was detected between bacterial load and OTU richness (i.e. low bacterial load does not necessarily mean low OTU richness). In addition, Callens et al (2018) found a correlation between body size and bacterial richness, but no correlation between bacterial load and life history traits. As we are interested if *Daphnia* tolerance towards different stress situations is microbiome-mediated, we opted to examine bacterial richness rather than bacterial load as we suspected that tolerance would be more impacted by bacterial richness than bacterial load (as seen in Callens et al 2018).

Results:

- Table 1: I don't understand to what refers the 'sample type' in the table

Sample type refers to donor bacterioplankton vs recipient *Daphnia*. We added this clarification in the table and in M&M.

- Table 1 and all over the manuscript: 'Donor' and 'Recipient' are used in different ways. Sometimes it seems to be the water that has been used for inoculation (from ponds or water tanks where *Daphnia* were reared) but sometimes, it seems to be the *Daphnia* from the tanks (and not the water). In the table, I would be more explicitly using terms such as 'water used for inoculation', 'Daphnia + Microbiota', and throughout the text 'ex: l204, l261, l274, l281, l537), I would indicate that you refer to the water and not to donor *Daphnia*. By the way, is the community from donor *Daphnia* very different from the one in the water in which they are reared?

We apologize for the confusion. The donor microbial inoculum in this experiment is the bacterioplankton community present in pond water for the natural condition and the bacterioplankton community present in culture water from *Daphnia* jars for the laboratory condition. We clarified this throughout the manuscript. In general, "donor" is replaced by "donor

bacterioplankton" and "recipient" is replaced by "recipient *Daphnia*". We did not specifically investigate differences between inoculation of bacterioplankton and *Daphnia* gut microbiome. Our results show that *Daphnia* only take up a part of the present bacterial community in the water (lines 1203 - 1217 and Figure S3, OTU richness). We did an additional analysis to investigate how many OTUs are unique or shared between the donor bacterioplankton and recipient *Daphnia*. The results showed that when *Daphnia* received a laboratory donor inoculum, they take up 35.8% of the donor inoculum. In the control treatment, 41.0% of the gut microbiomes consists of OTUs present in the donor laboratory bacterioplankton, while in the stressor treatments, only 34.5% of the gut microbiomes consists of OTUs present in the donor laboratory bacterioplankton. When *Daphnia* received a natural donor inoculum, they take up 31.4% of the donor inoculum. The difference in uptake between *Daphnia* that received a control or stressor treatment is smaller when they received a natural donor inoculum than when they received a laboratory donor inoculum. In the control treatment, 32.5% of the gut microbiomes consists of OTUs present in the donor natural bacterioplankton, and in the stressor treatments, 31.6% of the gut microbiomes consists of OTUs present in the donor natural bacterioplankton. This additional analysis is now included in the supplementary information in the revised version of the manuscript (lines 1203 - 1217).

- Fig 1: As 3 replicates are used, could you indicate the variance associated with these curves?

We added 95% confidence intervals, represented as dotted lines, on the survival plots (see Figure 3).

- Fig 1: Condition microbiome control / genotypes KNO and T8: the control treatment exhibits a lower survival than the stressors treatments. Can we thus talk about 'stressors'? Is it a cost of harboring gut symbionts (at a higher density?)?

Microcystis and *Aspergillus* are known stressors for *D. magna* (Macke et al., 2017, Houwenhuyse et al., 2021, Boudry et al., 2020). It is possible that the KNO and T8 genotype had a lower inherent survival when they received a natural bacterioplankton microbiome. These genotypes had a lower fecundity under *Microcystis* and combi stress, and in general the body size was higher in the control treatments. So, these genotypes still responded to the stressor treatments, only not in terms of survival. It is possible that the KNO and T8 genotype had difficulty with taking up bacteria from a bacteria rich environment, however, we do not have proof for this.

- Fig 2: how do you explain such variance in control treatments?

These variations are not unusual, this is because of inherent variation within the individuals. This is also observed in Macke et al. (2017 and 2020) in *Daphnia* and Boyen et al. (2020) in copepods. It is possible that variance in the control treatments is higher than in the stressor treatments as no selection was present which could reduce variance. In the stressor treatments *Daphnia* that do not have defense or tolerance mechanisms for that stressor will die. In the control treatment death of the *Daphnia* is because of inherent variation in the individuals.

- L216: Union plots do not indicate the link between gut microbial communities and life-history traits but between gut microbial communities and stressors. In addition, why to go into detail since the stressors did not impact the bacterial community in daphnia (table 1: OTU richness and Beta diversity)? Finally, what is the significance between the numbers in each class?

As OTU richness and Beta diversity didn't give us clarity on how the microbiome influences the life history traits, we investigated the gut microbiomes at the OTU level, with EdgeR analysis and Unionplots. EdgeR analysis revealed that the relative abundance of certain OTUs significantly differed between stressor treatments, microbiome treatments, stressor x microbiome interaction and stressor x microbiome x genotype interaction. The significant differences between the OTUs are found in table S3.

The Unionplots are a visual representation of what is happening at the OTU level. They helped us to interpret some of the effects we saw of the treatments on the life history traits. For example, genotype T8 had a lower survival compared with KNO and OM2 when they received a natural microbiome and control treatment, this lower survival could possible be explained by the higher number of unique OTUs in the T8 genotype as shown in the Unionplots (see Figure 9).

- Fig 4: please indicate the meaning of the letters (and homogenize the nomenclature). Why is the number of replicates different in the different combination genotype x stressor * environment? How do you explain the high variability in some samples (KNO/Lab/M2 or OM2/lab/F2)? Please also indicate the composition of the water used for inoculation.

We now made the nomenclature on the figure uniform (now Figure S9) with the nomenclature used throughout the manuscript and explained them in the caption. In addition, we added the bacterial composition of the donor inoculation bacterioplankton used in this experiment, and the number of gut samples per treatment. During the sequencing process, some samples got lost due to low quantity or quality, which explains why the number of replicates per treatment differed.

High variability in the gut microbiome of some samples is not unusual (Macke et al., 2017; Callens et al., 2018; Callens et al., 2020; Bulteel et al., 2021; Houwenhuyse et al., 2021). In a first stage each individual takes op random bacteria from the environment. This first line of bacteria, will be bacteria that are able to settle in the gut and have a high reproducing capability (i.e. conform the priority effect, see Debray et al. 2021). In a later stage other bacteria that can settle in the gut and have higher competitive ability, will outcompete this first line of bacteria. In each *Daphnia* individual this process is happening, the chance that different bacteria settle in each gut is thus very high.

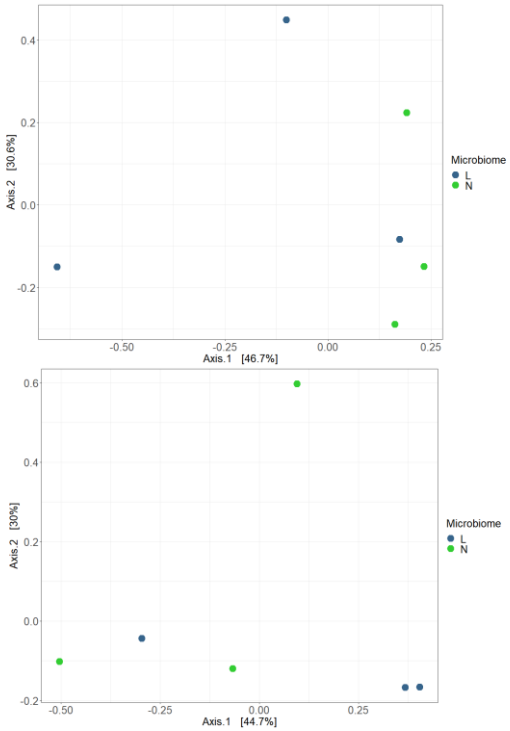
- Fig5: please make these graphs more easily readable. Do they represent the combination of the 3 replicates?

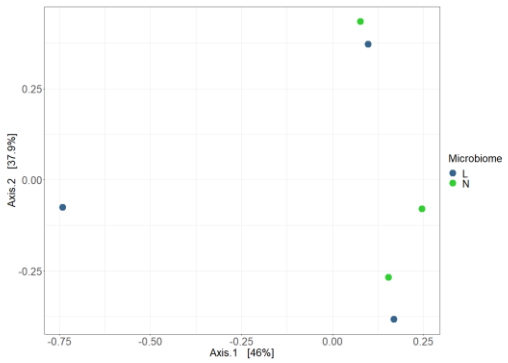
We made the nomenclature on the figure uniform with the nomenclature used throughout the manuscript and increased the text size. The Unionplots represent the combination of the three

replicates. Some explanation on the interpretation of the Unionplots is given in the M&M section (Lines 402 - 403).

- Fig S5: The number or samples is very different between donor and recipient communities. Could the sampling effort bias the analysis? (e.g., if we pick randomly 3 green dots and 3 blue dots from the PCA from recipient communities, there is a high chance that they structure separately as they do for the donor community).

We ran a simulation where 3 samples were randomly picked from the recipient pool of *Daphnia* exposed to a (i) laboratory inoculum and (ii) a natural inoculum. From this simulation we learned that the sampling effort did not bias the analysis as each time the laboratory and natural dots showed overlap (some examples of the simulation shown below).





- Fig 11: the number of replicates seems different between conditions (as in fig4). Could this impact the interpretation of the structuration of bacterial communities?

Due to low quantity or quality of the samples, the number of replicates per condition differs. This could probably impact the structuration of the bacterial communities. However, per replicate we took a pool of gut samples and thereby increased the variation per replicate and in that way decreased possible bias due to a different amount of replica.

- The characterization of the infection by *Aspergillus* does not really fit to the general question, but more to the characterization of the stressor (method)

We agree with the reviewer. We moved the paragraph on the characterization of the fungus to the Materials and methods of the manuscript.

Discussion:

- L362: Is the difference in one OTU when all replicate samples are merged per condition?

The relative abundance of one OTU differs between the donor bacterioplankton inocula. The three laboratory donor inocula were pooled and the three natural donor inocula were pooled to investigate the difference in OTUs via EdgeR analysis.

- L366: 'selection of bacterial groups was stressor-dependent' -> If I well understood, colonization occurs during the first 2 days and only then the stressor is added. It would be interesting to modulate this sentence with the way the establishment of the colonization occurs (selection by the host might be done at 2 days, but resistance of bacteria could be different depending on the presence/absence of stressors)

We thank the reviewer for this remark and agree. We have rewritten large parts of the discussion and integrated your suggestion (see lines 739 - 746).

- L367-368: suggest an explanation?

We have rewritten large parts of the discussion and have now integrated a possible explanation for this selection as they could possibly select strains with a phytoremediating effect against *Microcystis*. These particular strains were possibly not present in the lab bacterioplankton environment (see lines 739 - 746).

- It would be interesting to discuss more why survival (and indirectly fecundity) are impacted by the microbiome, while bacterial diversity and richness did not change much in response to stressors.

We thank the reviewer for this remark. We have rewritten parts of our discussion and have taken into account this remark (see lines 713 – 746). In the revised version of the manuscript, we discuss that the bacterial diversity and richness is more the result of a random and general uptake from the environment (reflected in the higher diversity in the guts of the *Daphnia* receiving the natural bacterioplankton which have a more diverse environmental community). Responses to stressors, however, can also be modulated through the presence of specific strains (through selection) which are not necessarily reflected in the microbial diversity.

Methods:

- Fig12: please add 10µM before 'filtered', otherwise we think that the water is sterile

We adjusted accordingly. (lines 222 - 223)

- Fig13: please indicate the scale bar on the image (not only the magnification)

A scale bar was already present on the figures, however, probably not directly visible due to the smaller size of the figure. As we moved this figure to supplementary information, we did not add another larger scale bar.

- L630: after a first event of colonization of the gut, is a colonization by other bacteria still possible?

Yes, we postulate that the first colonization event is ad random. We expect that different competitional process will take place and that priority effects will be of importance. We assume that bacteria that have a high reproductive capacity and are able to settle in the *Daphnia* gut, will dominate. Over time, other bacteria that are able to settle in the gut, with lower reproductive capacity, but higher competitive abilities, will be able to outcompete this first line of bacteria and take over the *Daphnia* gut microbiome. In addition, we assume that *Daphnia* are able to select bacteria with specific bacterial functions that will help the *Daphnia* to increase their fitness in certain circumstances. We would also like to refer to Macke et al. (2017) where they found that

colonization in an initial phase is dependent on the environment, but is taken over by the host genotype 14 days after inoculation.

- Is the same 'inoculation water' used for the 3 replicates?

No, each replicate received a different bacterioplankton inoculum. We clarified this in the revised version of the manuscript (Lines 231 - 239).

- L652: please indicate on fig4 how many guts were dissected per samples

We thank the reviewer for this remark and added the number of guts per sample on the figure (see figure S9).

- L655: How were designed the nested primers? Could they bias the amplification and/or limit the number of orders amplified?

In the first round of replication the 16S fragment is amplified to increase the amount of bacterial DNA. In the second round the V4 fragment of the 16S sequence is amplified. In this second round the barcodes are pasted onto the sequences to be able to identify the sequences after pooling. This universal set of primers is used in many microbiome experiments: Macke et al. 2017, 2020, Callens et al. 2016, 2018, 2020, Bulteel et al. 2021, Houwenhuyse et al. 2021, Motiei et al. 2020, Frankel-Bricker et al. 2020, Mushegian et al. 2017, Sison-Mangus et al. 2018, …

It is possible that this nested PCR creates a bias by amplifying wrongly annealed fragments. However, due to the low amount of bacterial DNA in the initial samples, we are forced to first amplify the complete 16S fragment before focusing on the V4 region. In addition, we are looking at shifts in microbiome composition, if there would be bias, this would be in the same direction for the donor and recipient samples.

- L660: why did you choose the V4 region?

The hypervariable V4 region of the 16S genome is a frequently used fragment for microbiome analysis (papers cited in previous remark). The main reason for using the V4 region is that in the majority of the cases this region contains the maximum nucleotide heterogeneity and displays the maximum discriminatory power. However, it should be noted to that no single region can differentiate among all bacteria.

- L671: why n=6, n=2 and n=1?

We had six donor bacterioplankton inocula (three natural and three laboratory), two stressor treatments (fungus and cyanobacterium) and one food sample (*Chlorella vulgaris*).

- L699: which samples have less than 1000 reads?

Following samples had less than 1000 reads:

Sample name	Number of reads	Clarification sample
K1 L F	89	KNO replica 1 lab inoculum fugus treatment
K1 N MF	10	KNO replica 1 natural inoculum combi treatment
K2 L F	203	KNO replica 2 lab inoculum fugus treatment
K2 L MF	178	KNO replica 2 lab inoculum combi treatment
K2 N C	66	KNO replica 2 natural inoculum control treatment
K2 N F	33	KNO replica 2 natural inoculum fugus treatment
K2 N M	72	KNO replica 2 natural inoculum cyanobacterium treatment
KP 2	12	Natural inoculum: Kennedypark replica 2
M5 2	4	Laboratory inoculum: culture water from M5 replica 2
MA 2	8	Natural inoculum: Marionnetten replica 2
O1 L MF	25	OM2 replica 1 lab inoculum combi treatment
O1 N M	193	OM2 replica 1 natural inoculum cyanobacterium treatment
O1 N MF	44	OM2 replica 1 natural inoculum combi treatment
O2 L C	98	OM2 replica 2 lab inoculum control treatment
O2 L M	82	OM2 replica 2 lab inoculum cyanobacterium treatment
O2 N C	35	OM2 replica 2 natural inoculum control treatment
O2 N F	107	OM2 replica 2 natural inoculum fungus treatment
O2 N M	151	OM2 replica 2 natural inoculum cyanobacterium treatment
O2 N MF	43	OM2 replica 2 natural inoculum combi treatment
O3 N MF	451	OM2 replica 3 natural inoculum combi treatment
T1 L C	65	T8 replica 1 lab inoculum control treatment
T1 L F	29	T8 replica 1 lab inoculum fungal treatment
T1 L MF	96	T8 replica 1 lab inoculum combi treatment
T1 N C	182	T8 replica 1 natural inoculum control treatment
T1 N M	103	T8 replica 1 natural inoculum cyanobacterium treatment
T1 N MF	128	T8 replica 1 natural inoculum combi treatment
T2 L F	216	T8 replica 2 lab inoculum fungal treatment
T2 L MF	64	T8 replica 2 lab inoculum combi treatment
T2 N F	37	T8 replica 2 natural inoculum fungal treatment
T2 N M	142	T8 replica 2 natural inoculum cyanobacterium treatment
T7 2	0	Laboratory inoculum: culture water from T7 replica 2
ZWE 2B 2	10	Laboratory inoculum: culture water from ZWE 2B replica 2

- L703: why to choose 1000 reads? Please show the rarefaction curves.

Analysis of the number of reads per sample showed that most of our samples have more than 1000 reads per sample. The rarefaction curve confirms this by reaching a plateau around 1000

reads per sample. Rarefaction curves are now represented in supplementary information (Figure S2)

Typos / grammar:

-l22: fecundity

Sentence was reformulated and typo was corrected (see line 24).

-l120-125: please cut your sentence in two

We changed our paragraph on our research questions and hypotheses. We made sure to not include sentences that were too lengthy.

-l128: please remove the comma after individuals

We changed our paragraph on our research questions and hypotheses as such that this remark does not apply to the text anymore.

-l240: Fig 6B

Figure 6A was corrected to Figure 6B (now Figure 9)

-l241: fig 6A

Figure 6B was corrected to Figure 6A (now Figure 9)

-l529: fig please specify to what refer 'this'

We added clarification to this (see line 867).

-l733: characterization of the infection

Adjusted to 'characterization of the fungus' (see line 409).

-l741: please specify what are the LSU (=large subunit) and SSU (=small subunit) regions

We added the full name of the abbreviation as 'large subunit (LSU) and small subunit (SSU) region' (see line 417).

Reviewed by anonymous reviewer, 2021-07-07 16:40

Download the review (PDF file)
Reviewed by anonymous reviewer, 2021-07-08 14:43

This paper aims to compare the effect of multiple versus single stressors on aquatic invertebrates (*Daphnia magna*). Emphasizing that studies on stressors are often performed under laboratory conditions, the authors contrasted *Daphnia* with natural and laboratory microbiota to examine their response to the stressors. They also investigated the effect of genotype. The two stressors used, in combination and individually, are a toxic cyanobacterium (*Microcystis aeruginosa*) and a fungal infection (*Aspergillus aculeatus*-like type). The effects of those stressors were determined by measuring survival, reproduction, body size, as well as the correlation with microbial diversity. This paper includes a lot of measurements and the results are thoroughly described.

We thank the reviewer for the constructive comments. We deal with these comments point by point below.

Major comments:

The paper would become more interesting for a more general audience if the authors would put their work in a broader framework, considering also the work of others and the work on different systems.

We put the first part of the introduction in a broader framework as we added some more information on stress systems and its different types. However, we choose to focus in the second part on cyanobacteria and fungal parasites in aquatic systems as they are the focus of the manuscript and are fundamental for the understanding of this paper. We did give some more detail on why we focus on freshwater systems and its stressors as we focus on *Daphnia magna*, a keystone grazer in aquatic systems and an important model system for research on stressors.

The main hypotheses and the goal of the study do not become very clear from the introduction (see minor comments). Was the initial goal of the study to investigate stress responses or to know the differences between lab and natural settings or both? And why is this relevant for your system or of interest in general?

We are indeed interested in both. We are interested in how *Daphnia* individuals deal with stress when exposed to two stressors simultaneously compared with only one of the two. In addition, we are interested in the role of the microbiome in relation with stress responses, as we expect that tolerance in *Daphnia* is microbiome-mediated (as described for *M. aeruginosa* in Macke et al. 2017). Most experiments are performed in laboratory environments (and accompanying bacterioplankton community. The bacterioplankton of natural and laboratory environments, however differs in diversity and composition, and as such does the gut microbiome of the *Daphnia*

exposed to these bacterioplankton communities as *Daphnia* individuals take up bacteria from their environment to establish in their gut. Laboratory experiments involving stressors can't just be extrapolated to natural situations. And finally, we were also interested in genotype-specific responses. We rephrased the paragraph on the research questions so that the main hypotheses become clearer (see lines 153 – 173).

Your explicit expectations do not become very clear for the stress responses. In what direction do you expect to see differences in life history traits or microbiome diversity?

We rephrased the paragraph on the research questions. We expected to see a negative impact on the life history traits for both single stressors compared with the control (no stressor treatment). In addition, we expected to see an antagonistic interaction for survival under the multiple stressor treatment compared with the single stressor treatments (as seen in Boudry et al. 2020). In addition, we expected an increase in bacterial diversity in the natural gut microbial community compared with the lab community, resulting in a higher tolerance (see lines 153 - 173).

When introducing stress responses, it would be useful if the authors could add a paragraph with more concrete examples and findings in *Daphnia* (also to explain why this model was chosen compared to other model systems in this context). While a lot of papers on *Daphnia* are cited, how these works differ from the current work does not become very clear. It might be worth to mention explicitly what the novelty of this paper is compared to the broader literature (and perhaps include a full paragraph on stress responses in other organisms and why it is important).

We added some lines on the importance of *Daphnia* in aquatic systems, as in 1) they are keystone grazers, and 2) they are an important model system to study stressor interactions (see lines 59 - 62). We also added a line on the novelty of the paper (see lines 121 - 125). We also added some lines on the broader literature (see lines 35 – 53), however, we opted not to go too much into detail as the introduction would lose focus in this manner.

In the introduction, you emphasize that the effect of stressors can be synergetic, additive or antagonist and that it varies according to the ecosystem (freshwater or marine), yet you never mention that *Daphnia* is a freshwater organism.

We specified in the introduction that we focus on freshwater ecosystems and on *Daphnia*, which is a keystone genus in freshwater systems (see line 59).

The results section is currently placed before the materials and methods, but as it stands, the materials and methods must be read before the results can be understood and interpreted correctly. I suggest that the authors either rewrite the results and state more clearly what was done and why for each section or that the materials and methods are placed before the results section.

We agree with the reviewer. We moved the material and methods section in front of the result section. We also added some introductory lines in the result section.

Throughout the paper, there is some redundancy and repetitiveness. I have highlighted several places in the minor comments. The discussion is also largely a repetition of the main results. As mentioned above for the introduction, I think that this requires some rewriting to put the work into a broader context.

We addressed the minor comments (see below). We have included some research in the introduction to include a broader context, but still kept our focus on the aquatic stressors. In addition, we rewrote our discussion to avoid repetition.

There are many figures, which reduces the coherence and readability of the paper. It would be a good idea to provide some basic stats (means, standard deviations) for the different traits measured in a table (this would already reduce the number of figures). For the remaining figures, please also add the relevant statistics and sample sizes.

We moved some figures to the supplementary information to reduce the numbers of figures in the main manuscript. We decided not to give a table with an overview of the basic stats for the different traits, as due to are numerous results, a resulting table would be too lengthy and bulky. We did incorporate relevant statistics and sample sizes on figures when possible.

Please find some minor comments in the provided PDF. Download the review (PDF file)

Minor comments mentioned in the PDF:

- Line 22: remove “Fecundity”

We have rewritten this paragraph and this is no longer applicable.

- Line 29: This sentence is rather strange. Exposure to biotic and abiotic environments does not necessarily lead to diversity

We added the verb ‘can’ as it indeed does not necessarily lead to diversity, but it is a possibility (see line 30).

- Line 31: Biotic stressors are also environmental stressors

We agree with the reviewer, we wanted to emphasize on biotic stressors. We rephrased and added more information on the types of stressors (see lines 34 – 47).

- Line 35: Not clear what is meant here. Do you mean that single stress exposures often occur, but that these are different types of stressors? Or that a similar stressor can occur repeatedly during the lifetime of an organism? Please explain more clearly.

We meant that different single stressor can co-occur and interact with each other. We added "different" to the sentence to make this clearer (Line 47).

- Line 49: If it is toxic, it already implies that there is a negative effect. In this case, the effect is a reduction in survival and health. Rephrase.

We rephrased this part in the manuscript, see next remark (Lines 71 - 72).

- Line 51: Why is this important?

Microcystis is detrimental for zooplankton in different ways: by producing toxins, by being poor food quality and by forming colonies. We wanted to mention every detrimental aspect of *Microcystis* as *Daphnia* might respond to one aspect, but not to another aspect of the cyanobacteria. We rephrased this part in the manuscript to make this clearer (see lines 70 - 79).

- Line 55: For readers unfamiliar with this system, the relevance of these molecules is not clear.

We opted to keep this sentence in the introduction as it gives some notions on the capability of *Daphnia* to increase their tolerance/resistance towards exposure to *M. aeruginosa.*

- Line 69: The model system appears here out of the blue. Please provide a more thorough introduction of your system (and why it is studied here).

We thank the reviewer for this remark and added some extra lines to introduce the model organism used in this study (see lines 59 – 62).

- Line 98: What is the relevance of the inflammatory stimuli.

The stressor used in the research on mice was specified to inflammatory diseases. We changed inflammatory stimuli to environmental stress to clarify the relevance (see line 128)

- Line 104: Switching to the soil is confusing. Why is what is happening in the soil relevant for what is happening in the aquatic environment. In any case, this paper provides a rather weak argument for the conclusive sentence thereafter because the control is an absence of invasion and not the invasion of a non-pathogen.

We opt to keep the reference to the soil community as we focus on the general concept of obtaining bacteria from the environment and as you can compare the bacterioplankton of an aquatic environment to the bacterial community in the soil. In combination with the reference to Booth (2002), this gives some insights on why bacterial diversity can be a codetermining factor in terms of tolerance. We did include another possible explanation as the host community does not necessarily has to differ in richness, but that the selection process in a more diverse environment could also lead to an adapted host microbiome (and thus have different strains).

- Line 105: What is the system studied here? There is not enough detail to determine the relevance compare to your system.

Booth (2002) is a review on how bacterial diversity is a mechanism to deal with stress. The system studied in this review are bacteria. We added "bacterial" to make clear that this review is on bacteria (lines 135 – 137).

- Line 116: The use of infection is rather confusing. Why not just refer to Aspergillus?

We agree with the reviewer. We changed the nomenclature throughout the manuscript. For *Microcystis* we refer to cyanobacterium or C, for *Aspergillus* we refer to fungus or F, for the combination of both stressors we refer to combination or F+C and for the control, we refer to control or CTL.

- Line 118: Why was this species chosen? Is this the normal, non-toxic environment? Also: It seems here that the other treatments were fed only with *Microcystis,* while they were fed with both *Chlorella* and *Microcystis*.

Chlorella vulgaris is known as good quality food for *Daphnia* culturing. This is mentioned in the materials and method section of the manuscript (Lines 196 - 197). We changed the sentence to "fed with only *Chlorella vulgaris* instead of a mixture of *C. vulgaris* and *Microcystis*…" to make this clearer (Line 151).

- Line 123: Are you here talking about your own system or in general? This statement need references. I would suggest mentioning this before your hypothesis.

In this paragraph we refer to our study (we added this in the sentence), but it can also be extrapolated to natural environmental communities in general. We added two references (Rosshart et al. 2017, Callens et al. 2020).

- Line 126: This is not very clear. What exactly are your expectations here for the specific treatments. And Line 127: This is also not very clear within the context of your different treatments. Explicitly mention what you expect for your treatments.

We thank the reviewer for this remark. Based on this and previous remarks, we rephrased our hypothesis section (see lines 153 – 173).

- Line 131: remove "present"

We removed the word present from this sentence.

- Line 132: It would be very interesting to go into these studies in more detail, because it is very relevant for the work described here. This will also allow you to make more explicit expectation based on the different treatments that you are using.

We do not fully agree and opted not to go into more detail as a description of all these papers would not give any additional relevant insight for our hypotheses. The model system *Daphnia* is known for its genotype-dependent reactions and we do not aim in this paper to formulate expectations depending on the used genotypes. To our knowledge, no other studies have been undertaken on our genotypes for the *Aspergillus* infection and *Microcystis* exposure in single and multiple stressor exposures.

- Line 137 (Table 1): As your material and methods follows after the results section, I would suggest including some more detail here as to what the donors and recipients are. What does the sample type refer to?

We moved the material and methods section before the results section. In addition, we added a clarification on the term in the legend of the table and in the manuscript to explain 'sample type'.

- Line 142: I would start with a brief sentence to reiterate what was done (as the M&M follows later).

We moved the material and methods section in front of the results and added some introductory sentences for each part of the result section.

- Line 153: In which direction did you see the change? Please include that info here.

We rephrased this part, the direction of the change is explained in the following sentences (see lines 463 - 468).

- Figure 1: The figure is not really clear, because there is a lot of overlap in some of the graphs. Could you change the dimensions of the graphs so that the trajectory for each treatment become more clear (reduce width and increase height)?

We thank the reviewer for this remark. As the survival of genotype T8 in the natural, control treatment (lower, right plot) drops to 0.7, we chose to set the minimum limit of the y-axis at 0.65. Changing the height of the other plots will lead to misinterpretation of the survival plots (it will seem like the difference in survival is higher in the upper plots, than it actually is). Therefore, we choose to set the minimum limit of the y-axis the same for all plots.

- Line 165: How was total fecundity measured? This is not clear to me, also after reading the material and methods.

We thank the reviewer for this remark and clarified by adding "measured as the total number of hatched eggs per *Daphnia* individual" to the manuscript (Line 478 - 479).

- Figure 2: It would be useful to see the individual datapoints here as well.

We agree with the reviewer and adjusted accordingly (see Figure 4).

- Line 222: These sample sizes should be mentioned in the material and methods. It would also be useful to include the sample sizes in figures/tables.

These numbers do not refer to sample sizes, but to the total number of OTUs present in that particular treatment. We removed “n=” to avoid confusion.

- Figure 4: This could be become a supplementary figure to reduce the total number of figures in the main text. The main results are explained in the written part of the manuscript.

We agree with the reviewer and moved this figure to the supplementary information.

- Figure 5: These figures are not very easy to read. Perhaps also move to the supplementary figures and highlight the most important points in the written results section.

We thank the reviewer for this remark. However, we choose to keep these figures in the main text as part of our discussion is based on these figures. We clarified the figures by changing the nomenclature and increasing the text size.

- Line 261: This transition between this and the previous paragraph is not very clear. The previous paragraph concerns a count of OTU, while this paragraph is about the quantification of individuals for each OTU. Please make the transition and the differences between these paragraph more explicit.

We thank the reviewer for this remark. An introductory sentence was added to make a transition between the paragraphs (see lines 590 – 593, 607 -609, 625 – 628, 644 – 645).

- Figure 7: The datapoints/lines within the figure are not very clear. Please increase the resolution/size of the actual plots (perhaps use abbreviations for he bacterial names).

We adjusted accordingly.

- Figure 8: Also here the lines and dots are not very clear.

We adjusted accordingly.

- Line 275: This variable needs to be explained also in the materials and methods.

We agree with the reviewer and explained ‘sample type’ throughout the manuscript.

- Line 331: Remove first sentence. Not necessary, same content as next sentence, but with less information.

We adjusted accordingly.

- Line 338: Is not very clear what you mean here.

We have rewritten this paragraph and made sure that research questions and hypotheses were clear (see lines 671 – 674).

- Line 339: Higher tolerance in which sense?

We have rewritten this paragraph and this sentence is no longer present.

- Line 341: How, in which direction?

The direction of the response caused by the genotype is not predictable. Some genotypes can be more tolerant to a certain stressor. We didn't have prior information on how the genotypes would respond to the stressors.

- Line 342: The numbering here is not in line with the numbering of the hypotheses above, so this is rather confusing. Rather just list which results substantiate hypothesis (i) and (ii).

We adjusted accordingly (see lines 671 – 684).

- Line 347: It would be useful to also write what your original expectation was.

We have rewritten our discussion and have now included our hypotheses (see lines 667 – 674).

- Line 359: This would suggest that this is an intentional and voluntary process. How can you determine this? It is more likely that some strains are better able to persist in competition than others, rather than that *Daphnia* is selecting strains.

The dynamics of bacterial colonization in the *Daphnia* gut are not known yet. See our response above on this issue with Reviewer 1 and lines 724 -727 and 739 - 746 in the revised version of the manuscript. We expect that in a first stage there is a random uptake of bacteria and will be followed by competitional processes between bacteria but also by selection by the host. E.g. studies have shown that the genotype mediated the gut microbiome and as such selects for a certain gut community. We assume that bacteria that are beneficial for host fitness will be able to settle in the gut. These bacteria, with specific bacterial functions will help the host to deal with certain stressors. As such, we expect it will be a combination of random and intentional processes.

- Line 361: That the relative abundance of only one OTU differed.

We thank the reviewer for this remark and adjusted accordingly.

- Line 366: Do you have an explanation as to why this would be the case.

We thank the reviewer for this remark and added a possible explanation for this obtained result: "But the differently structured gut community could hint at a possible microbiome-mitigated defense mechanism. Especially as ordinations show a possible cyanobacterium-associated microbiome (as suggested in Macke et al. 2017, 2020 and Houwenhuyse et al. 2021). No specific fungus-associated microbiome was observed. One possibility is that the physical presence of *Microcystis* cells in the gut (through digestion) trigger selection responses, which directly influence the bacterial gut communities (e.g., through production of bacterial peptides), whereas the fungus probably infects the hemolymph instead of the gut cavity. Another possibility is that selection processes were not triggered by the fungal infection as the impact was minimal compared with the *Microcystis*-induced effects on life history. Combined with our results on survival, it is possible that after initial colonization two processes are determining the *Daphnia* gut community: (a) the general take-up of more strains which could possibly include strains which have a positive effect on defence mechanisms linked with survival, and (b) selection of the *Daphnia* host of particular microbial strains that have a phytoremediating effect against *Microcystis*. These particular strains were possibly not present in the lab bacterioplankton environment. The high amount of differing bacterial strains (as shown in EdgeR analysis and union plots) between stressor treatments could also reflect a stressor-specific selection." (see lines 730 - 746).

- Line 375: If survival is higher when both stressors act together than the interaction is synergistic, not antagonistic.

We use the definition of antagonistic used in stressor studies (as formulated in the introduction), i.e."…the net impact of multiple stressors are frequently either greater than (i.e. a synergistic interaction) or equal to (i.e. an additive effect) the sum of their single effects. Net effects of two or more stressors that were less effective than the potential additive outcome (i.e., antagonistic interaction)". The interaction between the stressors, based on these definitions, is thus antagonistic as the net effect of the stressors is lower than the potential additive outcome. So, if the survival is higher (i.e., the stressor had a lower effect) under the multiple stressor treatment, this means that the net effect of the stressors was lower than their additive effect, and the stressors thus acted antagonistically.

- Line 505: This is redundant with the previous sentence.

We rewrote this paragraph, so this remark is no longer applicable.

- Line 560: Include a reference for these procedures/conditions.

A reference was included.

- Line 578: I suggest repeating this in the result section if the M&M follows after.

We moved the material and methods in front of the results and added some introductory sentences in each part of the result sections.

- Line 582: As there are many figures in this manuscript, I suggest you use the pictures of Figure 13 for the overview of the experiment in Figure 12.

We thank the reviewer for this suggestion. We added the figures from Figure 13 (now Figure 1) to Figure 12 (now Figure S1). We moved Figure 13 (now Figure S1 to the supplementary information.

- Line 587: Which means that only some genotypes are susceptible?

We indeed refer to a differential susceptibility. This has been added to the manuscript for clarification (see lines 259 -260).

- Figure 12: The arrows are a bit confusing in this figure, as it seems that only some genotypes receive some treatments (e.g., KNO->C, etc…).

We thank the reviewer for this remark and adjusted the figure to make it clearer (now Figure 1).

- Line 614: Where were these located?

We removed table 2 and added this information in text form in the manuscript (Lines 231 - 239).

- Line 632: And was this necessary? And if so, how was this correction done?

We compared gut samples with previous experiments and did not observe notable contaminations and as such did not need a correction. We took these samples as a potential buffer, but assumed this would not be necessary as we expected little possible contamination.

- Line 636: First mention of the type of tube used. I would suggest writing: “the tube volume was gradually… etc”

We adjusted the sentence as follows: “the medium volume in the falcon tubes…” (Line 297).

- Line 638: What do you mean here? What is the rationale for having a lower food concentration? Please explain.

The lower food concentration in the first days was to ensure the stressors could have a sufficient effect on the *Daphnia*. In addition, young *Daphnia* are very small and are sufficiently fed with this lower food concentration.

- Line 640: How was this measured? Were the amount of *Daphnia* standardized? Please explain more clearly.

For survival, the number of surviving *Daphnia* was followed each day for 21 days. For fecundity, the number of eggs and hatched eggs was counted each day for 21 days. For body size the size of the *Daphnia* was measured from top of the head to the base of the tail on the 21st day of the experiment. All *Daphnia* were followed, meaning 10 *Daphnia* per treatment combination.

- Line 641: How was this measured? Add a reference or explain how this was done, i.e., micrometer, pictures with software?

We added a reference (Telesh et al. 2009). In short, the *Daphnia* was measured by taking a picture of the *Daphnia* under a stereomicroscope, with a piece of millimeter paper as reference. The size of the *Daphnia* was then measured with ImageJ, the length of the top of the head to the base of the tail was measured.

- Line 643: How many individuals were pooled together?

The number of individuals pooled per treatment can be found in table S9. We added "number of individuals per unique combination can be found in table S9" to the manuscript (Line 305 - 306).

- Line 644: How were these obtained? Was just the mater tested? Does n=6 mean that each environment was tested one? Please clarify.

From each donor inoculum the bacterioplankton was sampled once. We adjusted this in the manuscript (Lines 310 -311).

- Table 2: I suggest including this information in the text (to reduce the number of figures/tables).

We thank the reviewer for this remark. We removed table 2 and added this information in text form in the manuscript (Lines 231 - 239).

- Replicate in table 2: this is not really a replicate, but rather a different environment obtained from the lab or nature. I would refer to this as "environment" because a replicate implies to me that the same environment would be tested multiple times.

With replicate we meant that the *Daphnia* from replicate 1 received that line of microbial inocula. In the revised manuscript table 2 is removed and the information from that table is integrated in the material and methods section of the manuscript (Lines 231 - 239).

- Line 649: This should be included in the paragraph above.

We thank the reviewer for this remark and adjusted accordingly.

- Line 677: Explain what this does for those not familiar with R.

The ggqqplot function was used to make quantile-quantile plots. We added this information in the manuscript (Line 345 - 346).

- Line 679: Here you mean the isofemale line. This should indeed be a random factor (you have no a priori prediction as to how one isofemale line will respond compared to another).

We indeed use the synonym of isofemale line, i.e. maternal line.

- Line 681: So based on the AIC criterium?

Indeed, we clarify in the revised version of the manuscript by adding “according to the AIC” (Line 350).

- Line 686: Why would you keep all the different types of interactions? It would be better to predict which interactions are biologically meaningful to test and only test those.

For this experiment we were interested in all possible interactions. All interactions were biologically meaningful.

- Line 699: But then you actually lose the information available at the family level.

This was only done for the visualization of the data. At family level there are too many different families to visualize, that is why we chose to group them at the order level.

- Line 706: So then maternal line was included as a fixed factor?

No, maternal line was only included in the model as a random factor, not as a fixed factor. The AIC showed that the model without inclusion of maternal line as random factor was the best predictive model of the data. Maternal line was not included as a fixed factor as testing this was not biologically meaningful. We were not interested in how the different maternal lines respond to the microbiome and stressor treatments.

- Line 731: Do you mean that there is no normalization?

With raw sequence data, we mean the sequence data before rarefaction. After rarefaction some OTUs are lost, leading to misinterpretation of the data if you analyze at the OTU level, as is done for EdgeR analysis and Unionplots.