Stressor interactions under differential exogenous microbial exposure in *Daphnia magna*

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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. ~~Fecuntiy~~ and body size did show

23 a main stressor effect, which could possibly be linked with stessor-specific microbiomes (for
24 *Microcystis* and the combined stressor treatment). In addition, genotype-specific responses were
25 detected for survival and fecundity, which could be linked with the selective capabilities of the
26 *Daphnia* genotypes to select beneficial or neutral microbial stains from the environment.

27

28 **<u>Introduction</u>**

29 Organisms are constantly involved in biotic and abiotic interactions that lead to the flourishment
30 and diversity of life (Bøhn and Amundsen 2004, Ratzke et al. 2020). Interactions with
31 environmental and biotic stressors have an important role in shaping co-evolutionary dynamics
32 by altering the strength of and response to selection and/or population dynamics (Theodosiou et
33 al. 2019, Thompson and Cunningham 2002). We can fairly say that all organisms in their natural
34 settings are forced to cope with environmental stress (Holmstrup et al. 2010). There is, however,
35 increasing evidence that single stressors generally co-occur and interact (Jackson et al. 2016) and
36 generate complex effects on natural populations (Piggot et al. 2015). A meta-analysis by Jackson
37 et al. (2016) of the marine literature shows that the net impact of multiple stressors are frequently
38 either greater than (i.e. a synergistic interaction) or equal to (i.e. an additive effect) the sum of their
39 single effects. Net effects of two or more stressors that were less effective than the potential
40 additive outcome (i.e. antagonistic interaction) are less common in marine systems, but occur
41 frequently in freshwater systems (Crain et al. 2008, Holmstrup et al. 2010, Jackson et al. 2016). One
42 important aquatic stressor ~~is~~ cyanobacteria which ~~is~~ becoming increasingly dominant in aquatic
43 ecosystems (Visser et al. 2016). The negative effect of cyanobacteria on zooplankton is well

44 documented (Ferrão-Filho et al. 2000, Asselman et al. 2012, Lemaire et al. 2012). Cyanobacteria are
45 known to produce a wide range of toxic, secondary metabolites, classified as cyanotoxins among
46 which hepatotoxins, neurotoxins, dermatotoxins, and general cyanotoxins (De Figueiredo et al.
47 2004, Bittner et al. 2021). A commonly occurring and well-studied cyanobacterium is *Microcystis*
48 *sp.* (von Elert et al. 2003), which is known to produce various toxins, such as the most-frequently
49 occurring hepatotoxic microcystin (Van appeldoorn et al. 2007). The toxic effects of microcystins
50 are detrimental for the survival and health of aquatic organisms such as zooplankton and fish
51 (Penaloza et al. 1990). *Microcystis* is also of low food quality due to the absence of essential
52 polyunsaturated fatty acids and sterols (von Elert et al. 2003, Martin-Creuzburg et al. 2008).
53 Additionally, *Microcystis* is known for its colony formation which interferes with the filtering
54 process (DeMott et al. 2001), which further negatively impacts zooplankton fitness. In response,
55 zooplankton has developed multiple anti-*Microcystis* defences in e.g., production of proteases or
56 increased gene expression of genes associated with secondary metabolite transport and
57 catabolism (Schwarzenberger et al. 2014).

58 Not only cyanobacteria pose a severe threat to the zooplankton communities, also parasites are
59 an increasing threat, especially upon global change. Fungal parasitism received increasing
60 scientific interest in the last years (for zooplankton: e.g. Decaestecker et al. 2005, Civitello et al.
61 2015, Banos et al. 2020; for cyanobacteria: e.g. Gerphagnon et al. 2015, Gleason et al. 2015) and are
62 omnipresent and diverse in morphology, phylogeny and ecological functions. Fungal parasites,
63 however, are still poorly understood in their role in vital interactions and ecosystem functions in
64 most aquatic ecosystems (Grossart et al. 2019). Combined with the increasing abundance of
65 cyanobacterial blooms, this sparked some studies to examine potential interactions between fungi

66 and cyanobacteria impacting aquatic food webs. Some research focussed on altered predator-prey
67 interactions by fungal infections of cyanobacterial blooms (e.g. Kagami et al. 2007, Tao et al. 2020).
68 Agha et al. (2016) focussed on chytrid infection of cyanobacterial populations, revealing a positive
69 impact on the zooplankter *Daphnia* by improving food quality. Other research focussed on altered
70 host-parasite interactions by feeding infected *Daphnia* populations with cyanobacteria (Coopman
71 et al. 2014, Boudry et al. 2020). Boudry et al. (2020) revealed an antagonistic interaction between a
72 fungal parasite and *Microcystis* as a higher survival was obtained in infected *Daphnia* compared
73 with non-infected *Daphnia* when fed on *M. aeruginosa*. Other studies have also revealed
74 antagonistic interactions using other parasitic systems in *Daphnia* (e.g. predation x bacterium:
75 Coors and De Meester 2008, pesticide x bacterium: De Coninck et al. 2013, salinity x bacterium:
76 Hall et al. 2013, cyanobacteria x iridovirus: Coopman et al. 2014, microsporidium x bacterium:
77 Lange et al. 2014).

78 The last decade, studies have shown that it is not just the host's genome that determines host
79 fitness and reaction towards stressors, but rather a complex interplay of the host genome and
80 microbiome (McFall-Ngai et al. 2013). Especially the gut microbiome, the genetic material of all
81 microorganisms present in the host's gut, plays a key mediating role in host physiology (e.g. organ
82 development: McFall-Ngai et al. 2013, immunoregulation: Renz et al. 2011, metabolism:
83 Turnbaugh et al. 2006). Research has shown that the microbial community in *Daphnia* is structured
84 by diet (Callens et al. 2016), host genetics (Macke et al. 2017, 2020, Bulteel et al. 2021), antibiotics
85 (Callens et al. 2018, Motiei et al. 2020), temperature (Sullam et al. 2018, Frankel-Bricker et al. 2020)
86 and cyanobacterial exposure (Macke et al. 2017). Mushegian et al. (2018) suggested that *Daphnia*
87 functioning is largely determined by environmental bacteria, suggesting a strong role of

88 horizontally transmitted symbionts. Callens et al. (2020) showed that exogenous exposure to
89 different environmental pools of bacteria, resulted in different gut microbial communities,
90 reflected in both community composition and community structure. These results show an
91 important role of the bacterioplankton community in structuring the gut microbial community in
92 *Daphnia*. As *D. magna* is a well-established and key study system, many studies have been
93 performed on this model organism, but mostly under laboratory conditions. The bacterioplankton
94 community under laboratory conditions, however, differs from communities in natural
95 conditions, among which a reduced species richness in the laboratory communities (Callens et al.
96 2020). Similar studies on fish and mice have shown that the gut microbiome from hosts in
97 laboratory conditions are to some extent the same, but also differ from its free-roaming
98 counterpart under natural conditions, which may modulate a different response to inflammatory
99 stimuli (Roeselers et al. 2011, Adamovsky et al. 2018, Rosshart et al. 2017, 2019).

100 In general, exposure to different bacterial environments could impact the strength and specifity
101 of stressor responses (e.g. host-parasite: Wolinska and King 2009). Host organisms under
102 laboratory conditions encounter fewer microbes compared with their free-roaming counterparts,
103 which should ultimately be reflected in a less diverse laboratory host microbiome. Previously, it
104 has been shown that invasion of pathogens decreases when soil bacterial diversity is high (van
105 Elsas et al. 2012). Booth (2002) has also shown that bacterial heterogeneity could aid in the survival
106 of the host, whereby a small fraction of the bacterial population would be able to survive the
107 exposure to single or multiple stressors that kill the majority of the population. These studies
108 indicate that high bacterial diversity is a codetermining factor in protecting the host against single
109 or multiple stressors. As the host microbiome plays a crucial role in immune responses, exogenous

110 exposure to laboratory microbiota could potentially not mirror expected tolerances as occurring
111 in natural populations (Greyson-Gaito et al. 2020). With this experiment we aim to investigate the
112 response of *D. magna* individuals to single or multiple stressors when exposed to different
113 exogenous microbial inocula. Individuals, inoculated with either a natural or a laboratory
114 microbial community, were exposed to one of ~~the~~ four stressor treatments; the toxic
115 cyanobacterium *M. aeruginosa* (further referred to as *Microcystis*), infection with the fungus
116 *Aspergillus aculeatus* (further referred to as *Aspergillus* or infection) , the combination of both *M.*
117 *aeruginosa* and the infection (further referred to as Combi), and a control treatment (fed with
118 *Chlorella vulgaris* instead of *Microcystis* and no exposure to the infection).

119 Firstly, we expect an impact of the microbial exposure on *Daphnia* life history responses when
120 comparing the stressor treatments. We hypothesize that individuals receiving the natural
121 microbial inoculum will have a higher tolerance to particular stressors (i.e., have a higher survival,
122 fecundity and body size) compared with individuals that receive a laboratory microbial
123 community as natural bacterioplankton communities are generally associated with a more diverse
124 microbial community and provide a broader pool of microbiota for the host to select beneficial
125 strains from. In addition, we hypothesize that exposure to the natural microbial community will
126 result in a lower additive outcome of the antagonistic interaction (antagonistic, as described in
127 Boudry et al 2020) compared with exposure to a lab microbial community. A reduced effect of the
128 single stressors on the individuals, will consequently result in a lower net effect of the multiple
129 stressor as we assume a higher tolerance towards single stressors when exposed to a natural
130 microbial community. Finally, we include the role of the host genotype as previous research has
131 revealed a strong genotype-effect on the ~~present~~ gut microbial community and genotype x

132 microbiome interactions with respect to stress tolerance (Macke et al. 2017, 2020, Callens et al.

133 2020, Massol et al. 2020, Bulteel et al. 2021, Houwenhuyse et al. 2021), so we expect intraspecific

134 differences within *D. magna* responses to the stressors under the different exogenous microbial

135 exposures.

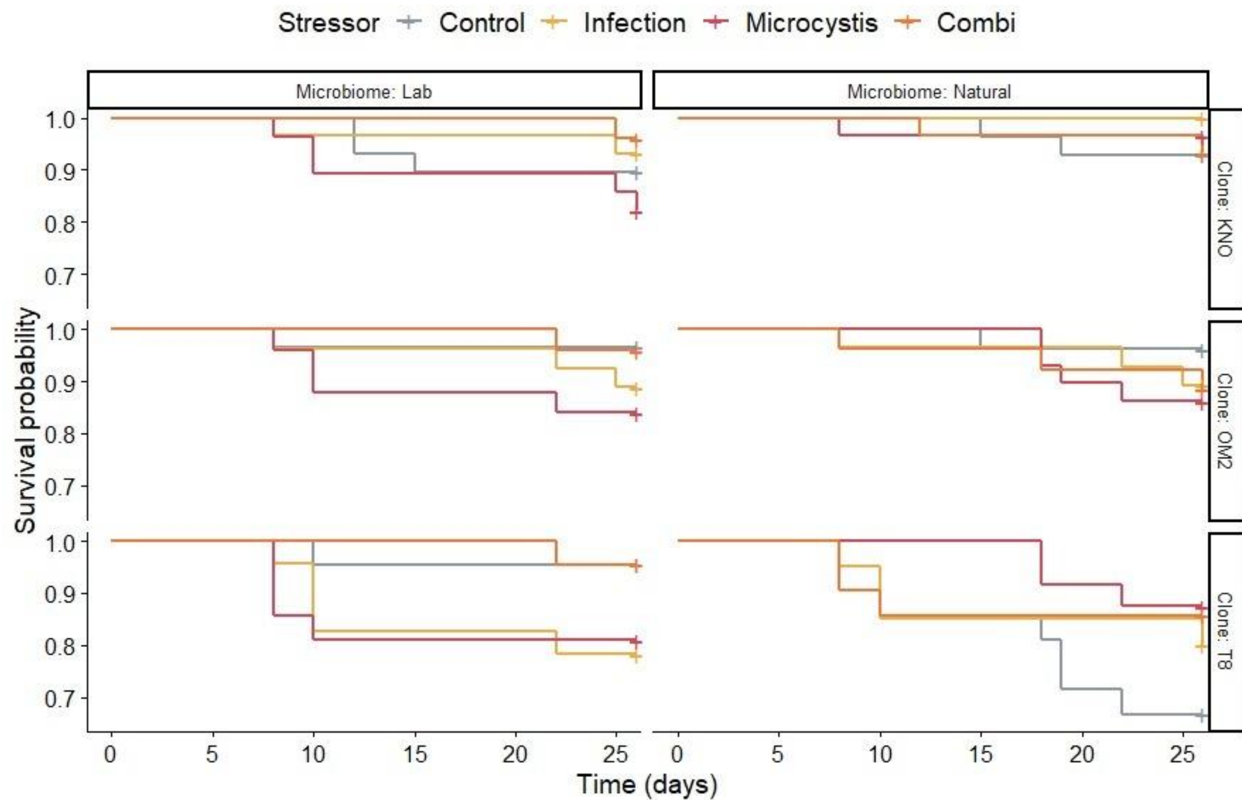
Results

Table 1: Overview results LMER for life history traits for the recipients and amplicon sequencing for the combination of donors and recipients, the donors separately and the recipients separately. Significant results (p<0.05) are indicated with *. Highly significant results (p<0.001) are indicated with ***. df = degrees of freedom.

	df	Survival		Fecundity		Body Size		OTU richness		Beta diversity	
		p-value	Chi^2	p-value	F	p-value	F	p-value	res. dev.	p-value	R2
Donor + Recipients											
Microbiome	1							1.434e-06***	179.85	0.003*	0.081
Sample type	1							1.113e-14***	255.77	0.006*	0.069
Microbiome x Sample type	1							0.001778*	147.94	0.022*	0.042
Donors											
Microbiome	1							0.007906*	46.797	0.09	0.34698
Recipients											
Stressor	3	0.4	3.3	<0.001***	42.7744	<2e-16***	36.8367	0.142041	102.227	0.428	0.076
Microbiome	1	0.8	0	0.067	3.3736	0.5035	0.4492	0.005648*	84.395	0.021*	0.091
Genotype	2	0.009*	9.4	<0.001***	30.2822	0.3985	0.9247	0.163476	114.901	0.371	0.067
Microbiome x Genotype	2	0.02*	13.9	0.076	2.5936	0.2500	1.3965	0.956061	68.304	0.428	0.052
Stressor x Genotype	6	0.07	18.8	<0.001***	4.0359	0.3944	1.0502	0.337666	68.513	0.825	0.111
Stressor x Microbiome	3	0.2	10.2	0.461	0.8615	0.5370	0.7271	0.732375	65.309	0.428	0.076
Stressor x Microbiome x Genotype	5	0.04*	36.3	0.014*	2.7011	0.7396	0.5880	0.232264	49.368	0.825	0.085

141 ***Survival***

142 The survival analysis revealed a main genotype effect, microbiome x genotype interaction, and
143 stressor x microbiome x genotype interaction on *Daphnia* survival. No stressor x microbiome
144 interaction was present. Separate analyses per microbiome treatment, however, revealed a
145 significant main effect of the stressor treatment in *Daphnia* individuals that received a laboratory
146 microbial inoculum (X^2=9.5, df=3, p=0.02), but not in *Daphnia* that received a natural microbial
147 inoculum (X^2=0.8, df=3, p=0.9). In the lab microbial treatment, *Daphnia* that received a *Microcystis*
148 treatment had a significant lower survival than *Daphnia* individuals that received the combi (X^2=
149 6.9, df=1, p=0.009; Figure S1) and the control treatment (X^2=4.9, df=1, p=0.03; Figure S1). Genotype,
150 additionally, determined survival as our analyses revealed a significant stressor x microbiome x
151 genotype interaction (Table 1, Figure 1). When tested separately per genotype, no significant
152 stressor x microbiome interaction was revealed for KNO, OM2 or T8 individuals (Table S1). We
153 did, however, find a significant stressor x genotype interaction in *Daphnia* receiving a natural
154 microbial inoculum (X^2=22, df=11, p=0.02), but not in *Daphnia* receiving a laboratory microbial
155 inoculum (X^2=14, df=11, p=0.2). Within the natural microbial treatment, the survival probability of
156 the T8 individuals was significantly lower compared with KNO individuals (X^2=5.6, df=1, p=0.02;
157 Figure 1) and OM2 individuals (X^2=7, df=1, p=0.008; Figure 1) for the control treatment. For all the
158 other stressor treatments within the natural microbial treatment, no significant differences
159 between the genotypes were observed (Table S1).



160

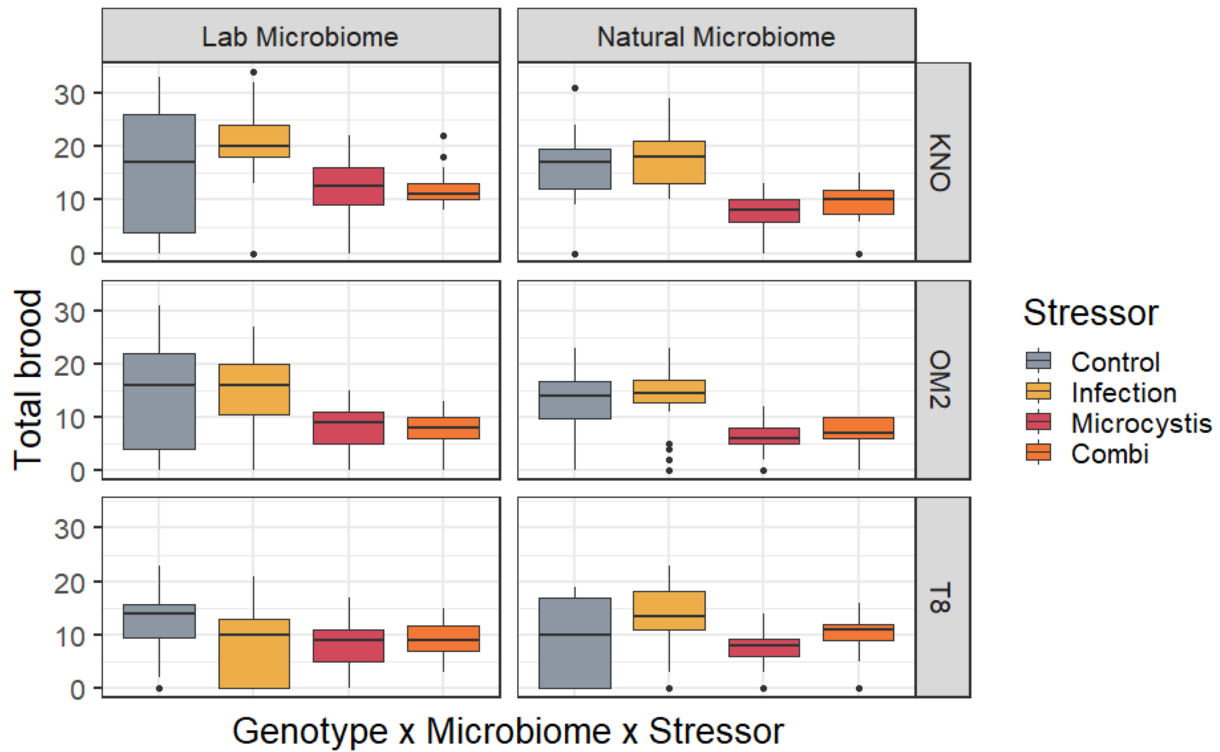
161 Figure 1: Survival plots of recipient *Daphnia* between the stressors treatments for the different
162 microbial inocula (columns) and genotypes (rows). Colors indicate the different stressor
163 treatments.

164

165 ***Total fecundity***

166 The fecundity analysis revealed a significant main effect of the stressor treatment and genotype,
167 a significant stressor x genotype interaction, and stressor x microbiome x genotype interaction on
168 total fecundity (Table 1). Analyses revealed no significant effect of stressor x microbiome on total
169 fecundity (Table 1, Figure S3). Separate analysis per microbiome treatment, revealed a significant
170 main effect of the stressor treatment in *Daphnia* individuals that received a natural ($F=47.36$, $df=3$,
171 $p<0.0001$) and laboratory microbial inoculum ($F=15.53$, $df=3$, $p<0.001$), with total fecundity

172 significantly differing between the control treatment and the *Microcystis,* and control and combi
173 treatment. On average, *Daphnia* had a lower reproduction when they received *Microcystis* (both as
174 a single stressor and in the combi treatment) compared with the control and infection treatment
175 (Figure S2). Genotype co-determined total fecundity as our analyses revealed a significant stressor
176 x microbiome x genotype interaction (Table 1, Figure 2). The KNO genotype revealed significant
177 differences between the infection and *Microcysti*s treatment, and the infection and combi
178 treatment within both microbial inocula (Table S1, Figure 2). The OM2 genotype revealed
179 significant differences for fecundity between the control and *Microcystis*, infection and *Microcystis*,
180 and infection and combi when exposed to the natural microbial inocula (Table S1, Figure 2). A
181 similar pattern was observed within the laboratory microbial inocula for OM2 with an additional
182 significant difference between the control and combi treatment (Table S1, Figure 2). The T8
183 genotype revealed no significant differences between the stressor treatments within both
184 microbial inocula (Figure 2).



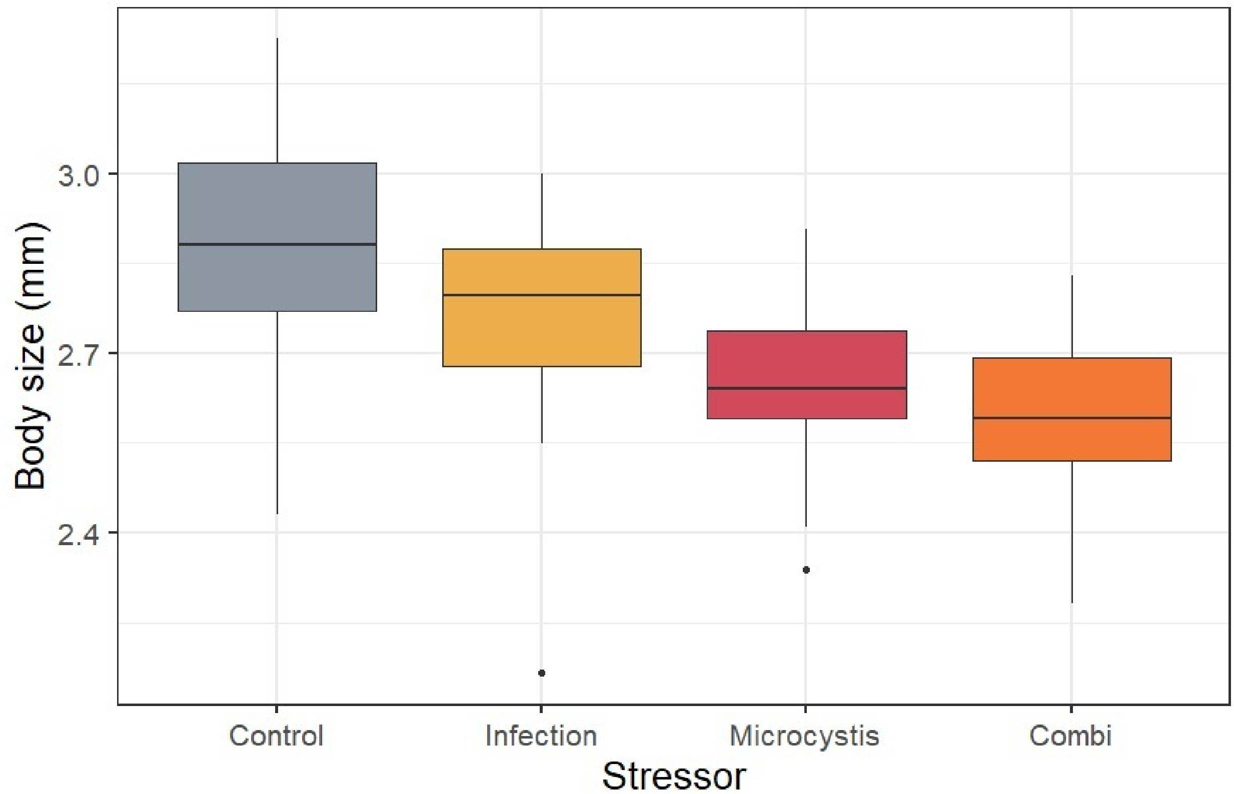
185

186 Figure 2: Box plots of the total brood for the different stressor treatments for the three-way
187 interaction (stressor x microbiome x genotype). Box plots are given for the two microbiome
188 treatments (columns) and the three genotypes (rows). Colors indicate the different stressor
189 treatments.

190

191 ***Body size***

192 Analyses on *Daphnia* body size revealed a significant main effect of the stressor treatment (Table
193 1, Figure 3). Post hoc analyses showed a significant difference between all stressor treatments,
194 except between the single stressor *Microcystis* and the combi treatment (Table S1). Individuals in
195 the control treatment had the highest body size, followed by, in decreasing order of body size,
196 individuals exposed to the infection, *Microcystis* and the combi treatment (Figure 3). No impact of
197 microbiome treatment or genotype were detected for *Daphnia* body size (Table 1).



198

199 Figure 3: Box plots of the recipient body size at the end of the experiment per stressor treatment.
200 Colors indicate the different stressor treatments.

201

202 ***Microbial composition***

203 Microbial community

204 Combined donor and recipient microbial communities were dominated by Betaproteobacteriales
205 (mean=46.484%, sd=26.554%), Pseudomonadales (mean=20.005%, sd=23.323%) and
206 Verrucomicrobiales (mean=5.388%, sd=7.092%). Donor microbial communities, analyzed
207 separately, were dominated by Betaproteobacteriales (mean=33.092%, sd=22.393%), Micrococcales
208 (mean=21.3728%, sd=31.5502%) and Chitinophagales (mean=11.525%, sd=17.676%), whereas

209 recipient microbial communities were dominated by Betaproteobacteriales (mean=48.397%, sd=26.780%), Pseudomonadales (mean=22.464%, sd=23.944%) and Verrucomicrobiales (mean=5.811%, sd=7.317%, Figure 4). A similar top 3 was observed for all recipient groups, whether they were exposed to the laboratory or natural microbial inoculum. Additionally, the same top 3 was observed for recipient *Daphnia*, indifferently of the stressor treatment, except for *Daphnia* exposed to the control, whereby the third most abundant order was Rhizobiales instead of Verrucomicrobiales (Table S2).

216 To examine a possible link between the gut microbial community and the life history traits, Union plots were performed for the stressor x microbiome interaction. When comparing the total number of OTUs from *Daphnia* exposed to the control treatment with the single stressor treatments (Figure 5A and 5B), a higher number of OTUs in the single stressor treatments (Infection: n=153, *Microcystis*: n=156) was observed compared with the control treatment (n=134) within the laboratory microbial inocula. The opposite was observed within the natural microbial inocula, whereby *Daphnia* exposed to the single stressor treatments (Infection: n=196, *Microcystis*: n=183) had a lower total number of OTUs compared with the control treatment (n=202). Union plots comparing the single and multiple stressor treatments (Figure 5C and 5D) showed that the total number of OTUs was lower in the combi treatment (lab: n=138, natural: n=167) compared with the infection treatment (lab: n=154; natural: n=186) and the *Microcystis* treatment (lab: n=161; natural: n=170) for both microbial inocula.

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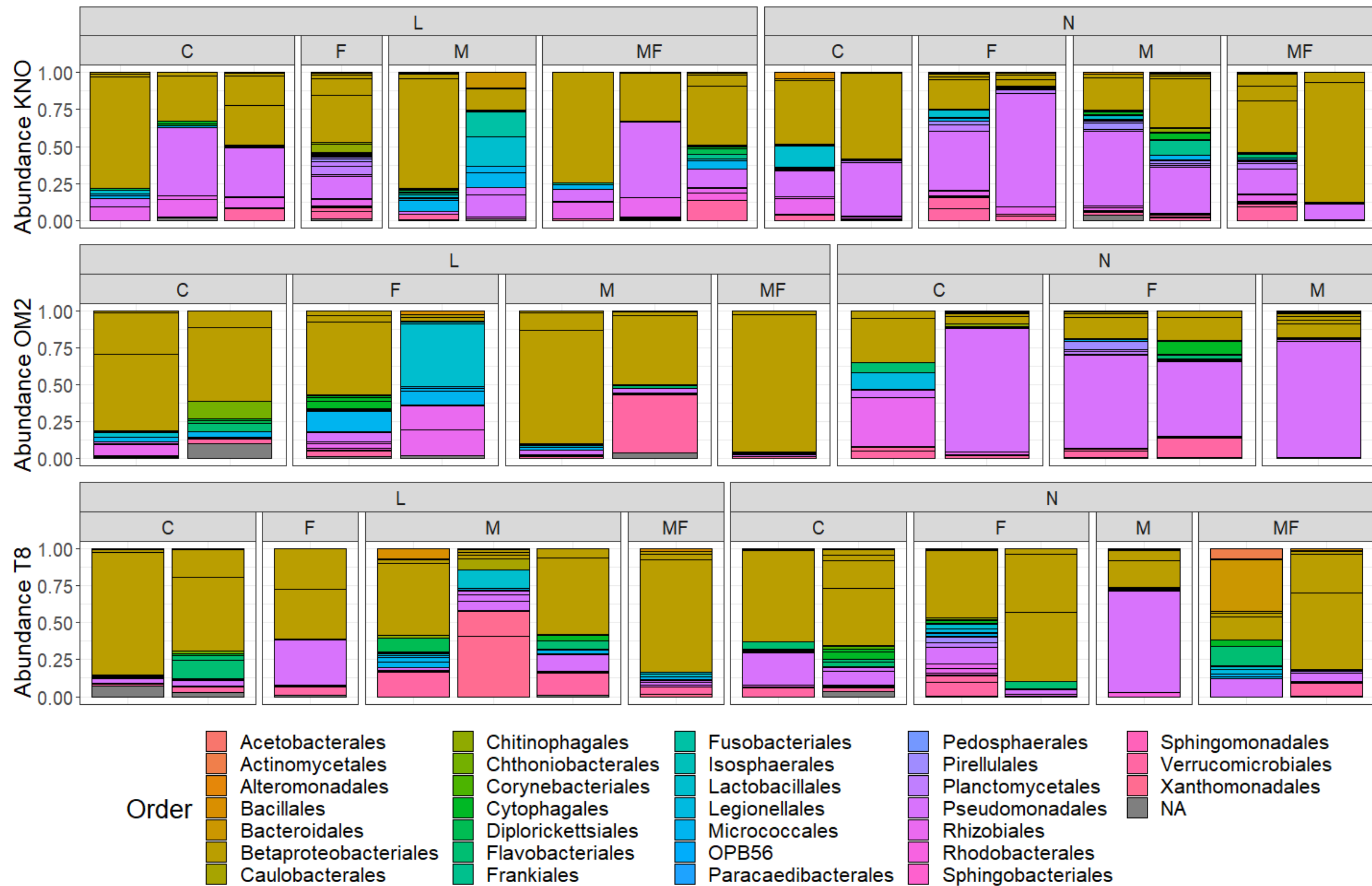

229 Figure 4: Relative abundance of the gut microbial composition of the recipient population grouped per genotype x microbiome x
230 stressor interaction. Colors indicate the bacterial order. OTUs with a relative abundance lower than 1% are not included. Analyses are
231 performed on rarefied data.

232 Figure 5: Union plots representing the OTUs that are unique within and shared between stressor
233 treatments when exposed to the lab (A and C) or natural microbiome inocula (B and D). OTUs
234 illustrated in A and B are: control (C), infection (I) and *Microcystis* treatment (M). OTUs illustrated
235 in C and D are: infection (I), *Microcystis* (M) and combi treatment (MI). Numbers between brackets
236 indicate the total number of OTUs. Colors indicate OTUs grouped per class.

237 Genotype T8 displayed a lower survival probability compared with KNO and OM2, when
238 receiving a natural microbial inoculum and control treatment. To examine a possible link between
239 survival and the gut microbial community, a Union plot for the three clones within the natural
240 microbial inocula and control treatment was made (Figure 6A). The complementary Union plot
241 for the laboratory microbial inocula and control treatment (Figure 6B) was also portrayed as T8
242 did not show this reduced survival probability under laboratory conditions. T8 had a higher
243 number of unique OTUs (n=58) and total number of OTUs (n=117), compared with KNO (unique:
244 n=30, total: n=85) and OM2 (unique: n=35, total: n=95) when receiving the natural inocula. When
245 we examined the present OTUs after receiving the laboratory inocula, T8 (unique: n=32, total:
246 n=82) had the same number of unique OTUs as KNO (unique: n=32, total: n=84) and a higher
247 number of unique OTUs compared with OM2 (unique: n=21, total: n=70).

248 When comparing main stressor treatment effects, the highest number of total OTUs was observed
249 in the gut community of individuals which received the infection treatment (n=251), followed by
250 the control treatment (n=246) and the *Microcystis* and combi treatment (both n=243, Figure S4).

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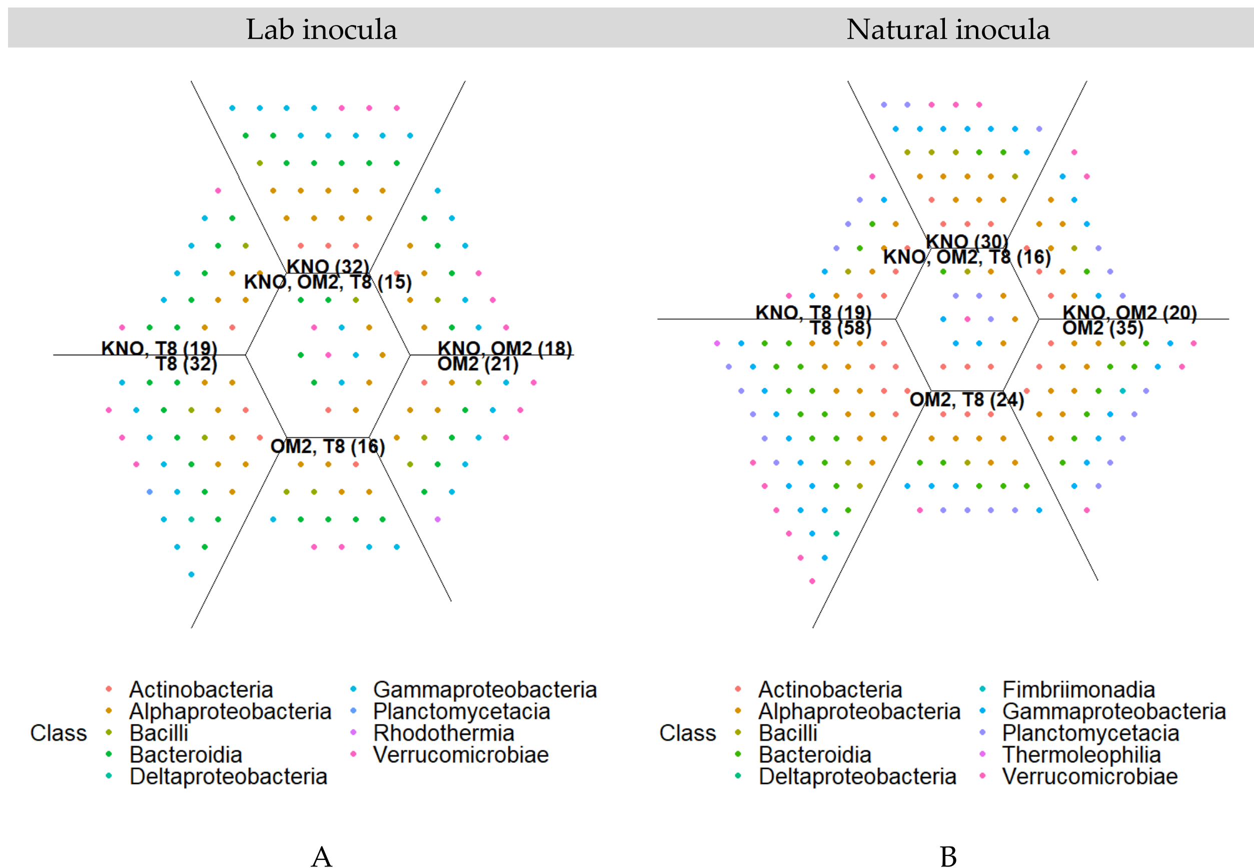


257 Figure 6: Union plots representing the OTUs that are unique within and shared between the
258 genotypes in the control treatment when exposed to the (A) lab and (B) natural microbiome
259 inoculum. Numbers between brackets indicate the total number of OTUs present in that
260 compartment. Colors indicate the OTUs grouped per class.

261 EdgeR analysis revealed highly significant differences for 213 OTUs between the donor and
262 recipient *Daphnia* (Table S3). Within the donors, only one OTU was highly significantly different
263 between the laboratory and natural microbial inocula (Table S3). Within the recipients 141 OTUs
264 were significantly different between the four stressor treatments, 285 OTUs between the
265 microbiome treatments, 34 OTUs within the stressor x microbiome interaction and 5 OTUs were
266 significantly different within the stressor x microbiome x genotype interaction (Table S3; Figure
267 7). Analysis per microbiome treatment revealed significant differences between the stressor

268 treatments for 12 OTUs within the lab microbiome treatment and for 24 OTUs within the natural

269 microbiome treatment (Figure 8, Table S3).

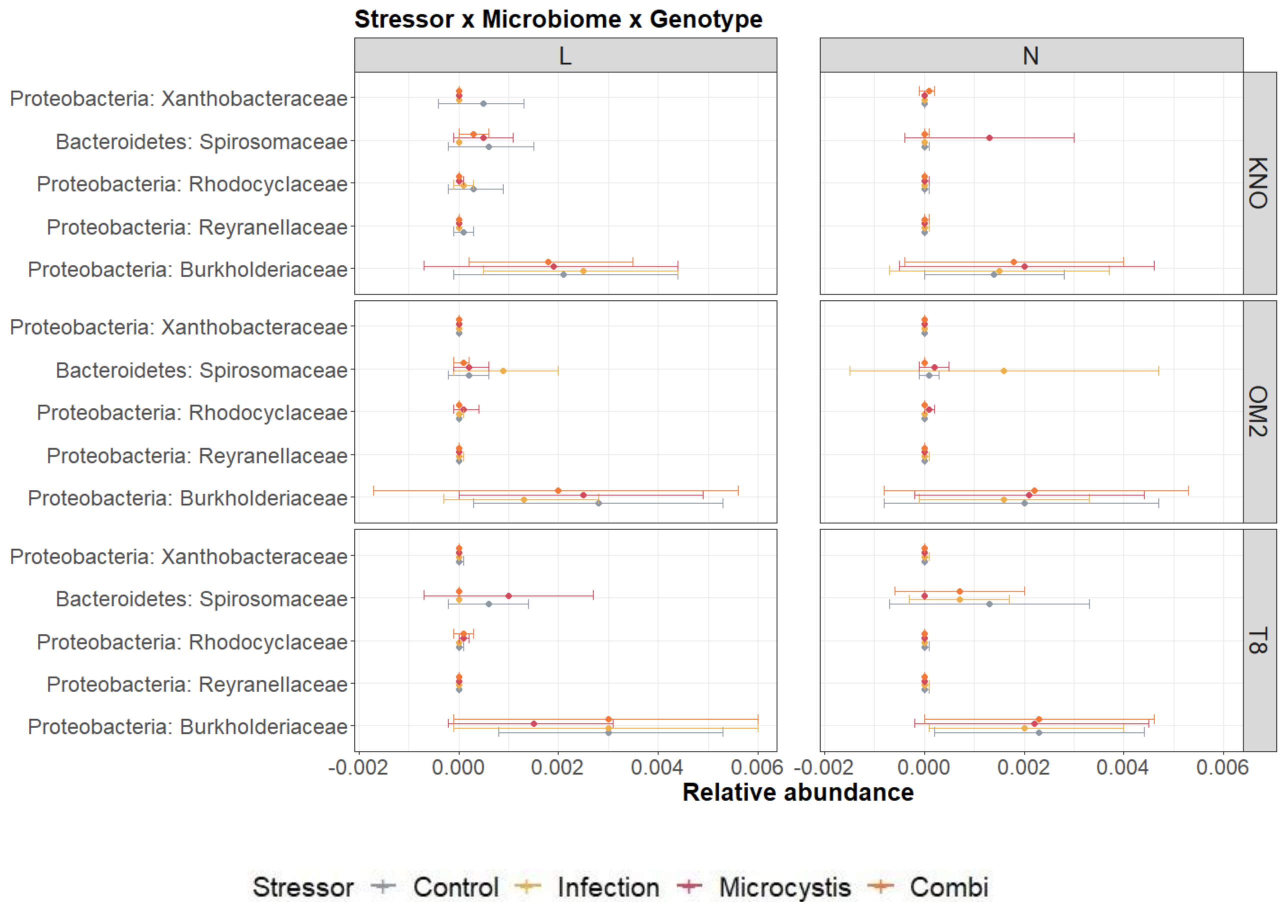
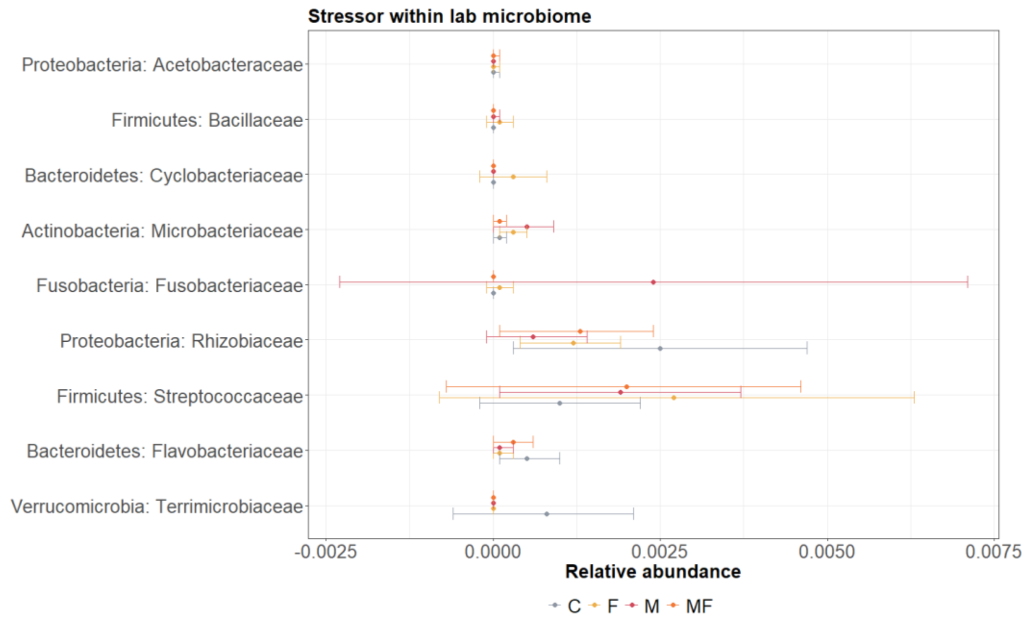


Figure 7: ggplot representing the OTUs at family level that were significantly different between the stressor x microbiome x genotype interaction. Colors indicate the stressor treatments.



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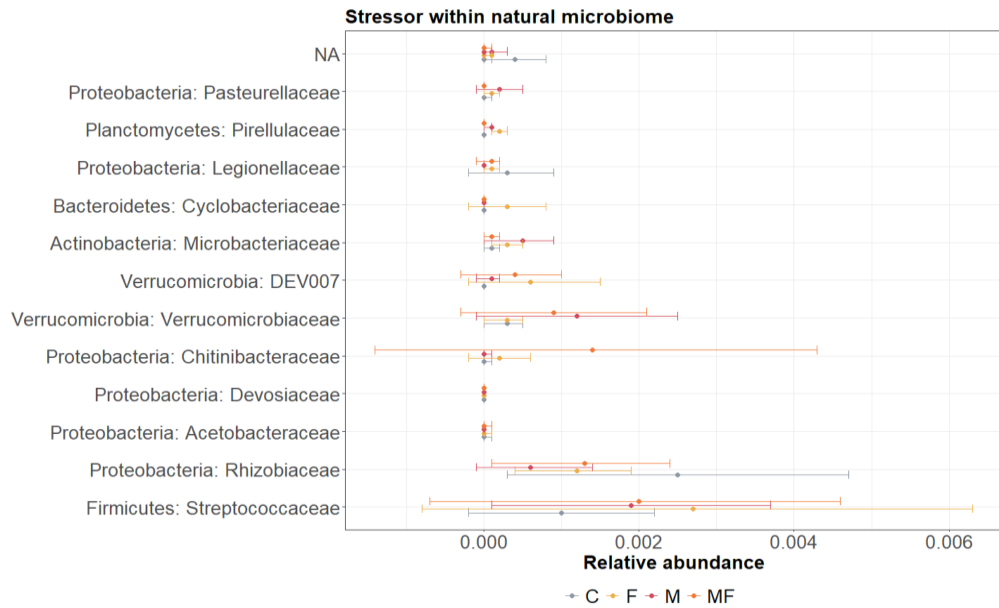


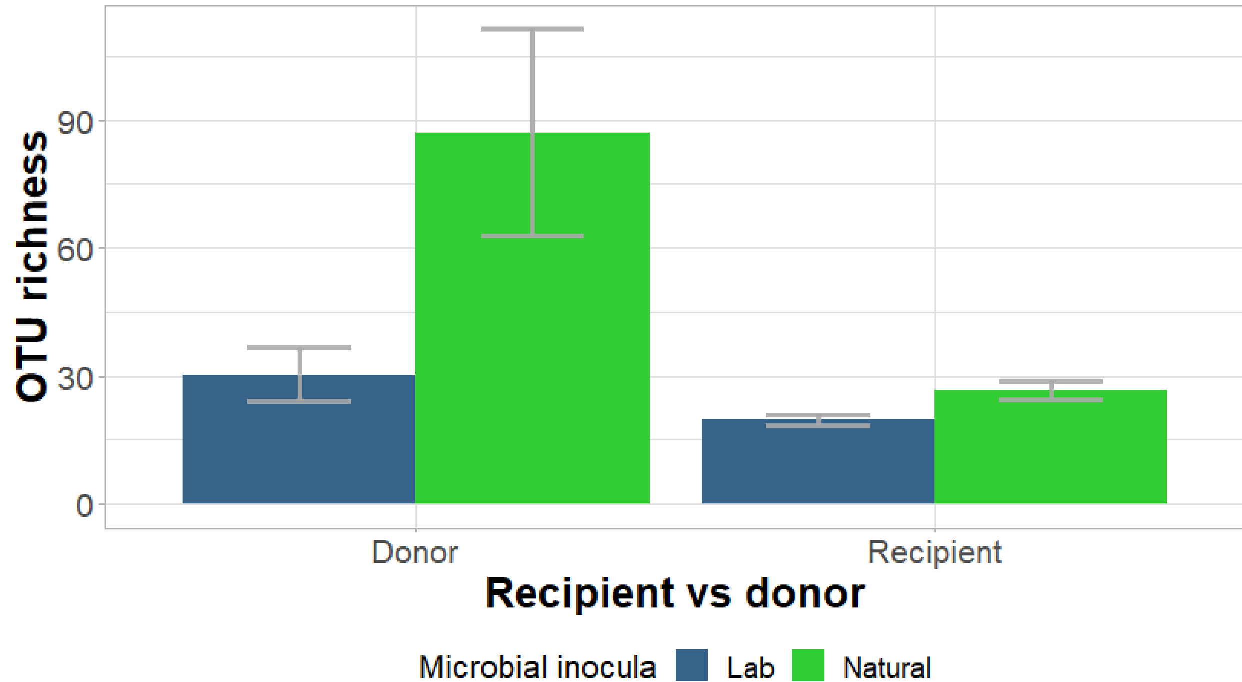
Stressor Control Infection Microcystis Combi

B

Figure 8: ggplot representing the OTUs at family level that were significantly different between the different stressor treatments within the (A) lab and (B) natural microbiome treatment. Colors indicate the stressor treatments.

273 OTU richness

274 Analysis of the data set containing both the microbial donor inocula and the recipient gut
275 microbiomes revealed a significant sample type (donor vs recipient) x microbiome interaction
276 (Table 1). Post hoc analysis revealed significant differences between all combinations, except
277 between the laboratory donors and the natural recipients (Table S4). In both donor inocula and
278 recipient microbiomes, OTU richness was significantly higher in the natural conditions (donor:
279 mean= 87.000, sd= 42.036, recipient: mean= 26.550, sd= 9.556) compared with the laboratory
280 conditions (donor: mean= 30.333, sd= 10.970, recipient: mean= 19.652, sd= 5.441, Table S4, Figure
281 9). OTU richness was also significantly higher in the donors (mean= 58.667, sd= 41.452) compared
282 with the recipients (mean= 22.860, sd= 8.303; $p<0.001$, z-value=-12.13, Figure 9). Analysis of the
283 recipients revealed a significant microbiome effect on OTU richness (Table 1). No stressor, stressor
284 x microbiome interaction or stressor x microbiome x genotype interaction was observed (Table 1).
285 A separate analysis per microbiome treatment did not reveal a significant main effect of the
286 stressor treatment in both *Daphnia* individuals that received a laboratory microbial inoculum (Res.
287 Dev.= 23.174, df=3, p= 0.129) or a natural microbial inoculum (Res. Dev.= 57.756, df=3, p= 0.056).



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289 Figure 9: Bar plots of OTU richness of donor and recipient samples which are grouped per sample
290 type and microbial inocula. Colors indicate the different microbial inocula Error bars indicate
291 standard error.

292

293 Beta diversity

294 Analysis on beta diversity of the donor and recipient samples revealed a significant sample type
295 x microbiome treatment interaction (Table 1, Figure 10), and a significant main effect of both the
296 sample type and microbiome treatment (Table 1). All pairwise comparisons for the main effects
297 and the interaction effect on the combined data of recipients and donors were significantly
298 different, except for the difference between the laboratory and natural inoculum treatment within
299 the donors (Table S4). The analyses on beta diversity on the microbial donor inocula separately
300 revealed no significant difference between the different inocula or microbiome treatments (Table

301 1). Bray-Curtis ordinations, however, demonstrated a complete separation between the natural
302 and laboratory microbial donor inocula, indicating that the bacterial community of the inocula
303 were differently structured (Figure S5). Analyses on beta diversity on the recipient's gut microbial
304 composition revealed that most of the variation was explained by the microbiome (lab versus
305 natural) treatment (Table 1). The bacterial composition in recipients receiving the natural
306 microbial inoculum differed significantly from those receiving the lab microbial inoculum (Table
307 1). Stressor, stressor x microbiome interaction and stressor x microbiome x genotype showed no
308 significant contribution to the differences in beta diversity (Table 1). Ordinations based on Bray-
309 Curtis, however, demonstrated an overlap between individuals exposed to natural and laboratory
310 bacterial inoculum, indicating that the bacterial community of these communities were similarly
311 structured (Figure 10). Separate analyses per microbiome treatment did not reveal a significant
312 main effect of the stressor treatment in both *Daphnia* individuals that received a laboratory (R2=
313 0.11379, df=3, p= 0.694) or a natural microbial inoculum (R2= 0.20147, df=3, p=0.18). Ordinations
314 based on Bray-Curtis for *Daphnia* individuals that received the laboratory inoculum showed an
315 overlap between all stressor treatments (Figure 11A). Ordinations based on Bray-Curtis for
316 *Daphnia* individuals that received the natural inoculum, however, demonstrated a complete
317 separation between the *Microcystis* and combi treatment, both showing small overlap with the
318 control and infection treatment (Figure 11B) reflecting a specific *Microcystis* associated
319 microbiome.

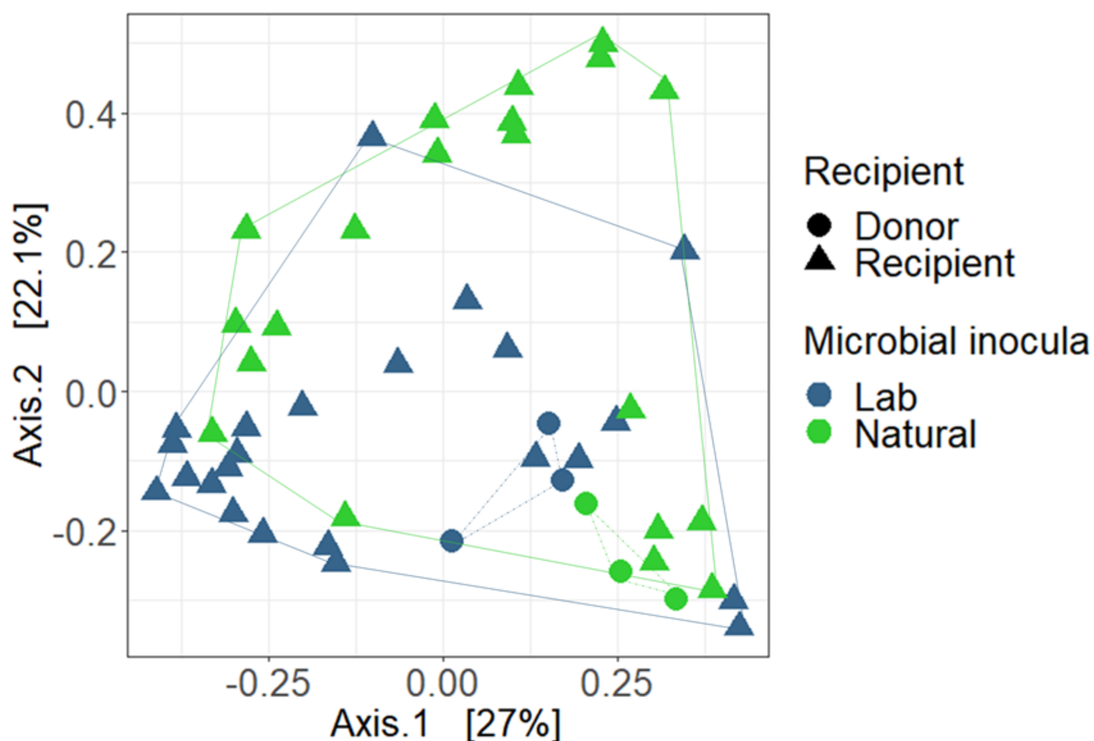
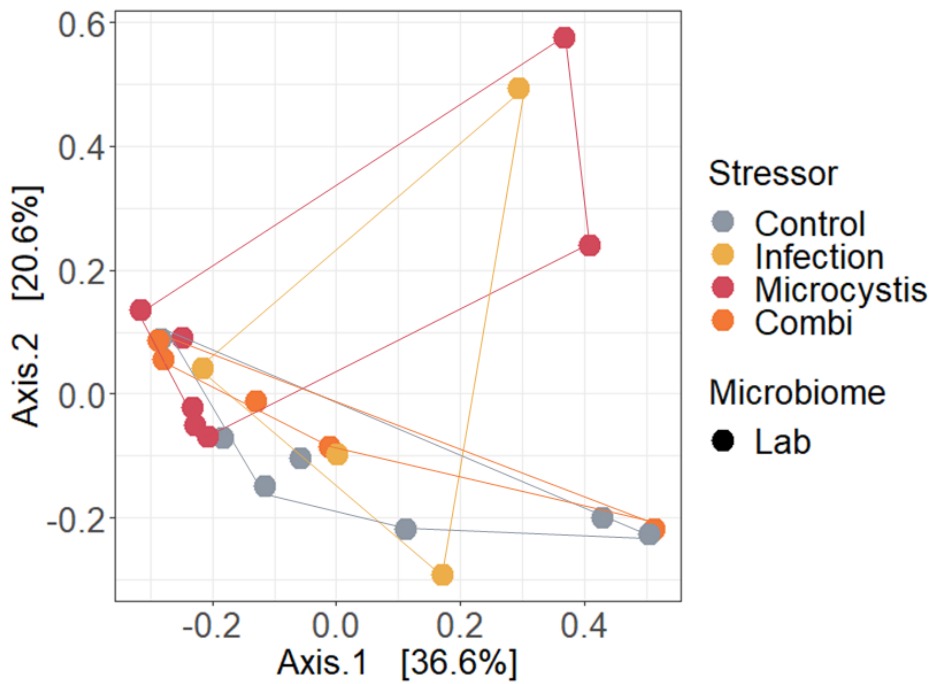


Figure 10: PCA of the gut microbial communities using weighted Bray-Curtis distance for donor and recipient data. Colors indicate microbiome treatment. Symbols and line type indicate sample type.

Correlations

Correlation tests were performed between percentage of survived *D. magna*, total brood, body size and OTU richness. No correlation was observed between the life history traits and OTU richness of the gut microbial community (Table S7, Figure S6). We did observe a positive correlation between survival and fecundity (cor=0.32, t= 2.84, df=70, p-adj=0.017; Table S5, Figure S7), and fecundity and body size (cor=0.33, t= 2.96, df=70, p-adj=0.017; Table S5, Figure S7).



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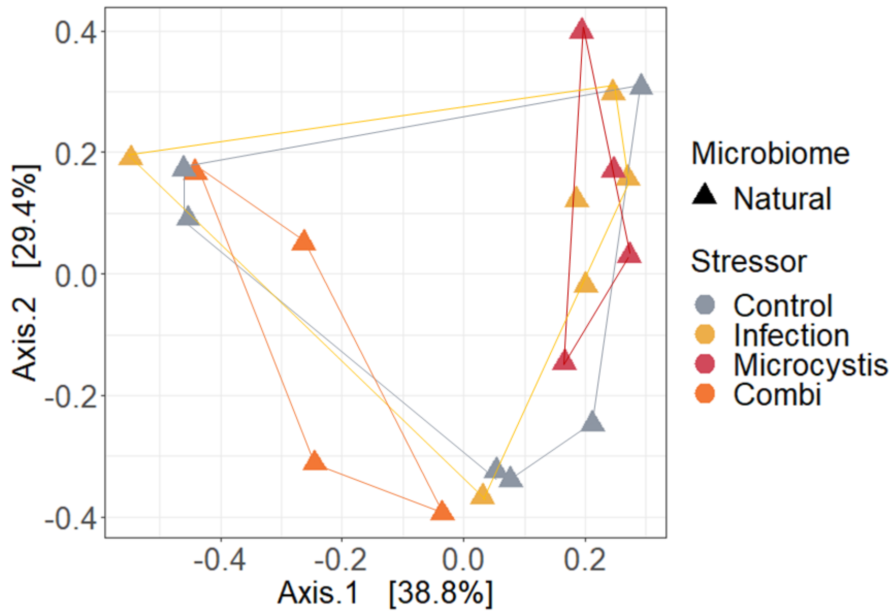


B

320 Figure 11: PCA of the gut microbial communities using weighted Bray-Curtis distance for
321 recipients exposed to (A) the lab microbial inocula, and (B) the natural microbial inocula. Colors
322 indicate stressor treatment. Symbols indicate microbial inoculum.

323 ***Characterization of infection***

324 After obtaining sequencing results (see Table S8), *Daphnia* with visible or non-visible infection
325 showed the highest match with *Aspergillus aculeatus* and *Aspergillus niger*. Multiple sequence
326 alignment further revealed a highly specific match with nucleotides 1 to 1900 for *Aspergillus*
327 *aculeatus* KV879170 (strain: ATCC 16872, Figure S8). No specific match with *Aspergillus niger* was
328 found in the multiple sequence alignment. Based on these results, we conclude that the fungal
329 infection is related to *Aspergillus aculeatus* ATCC 16872.

330 **Discussion**

331 ~~The aim of this experiment was to investigate the response of *Daphnia* to either a single stressor~~
332 ~~or combination of both stressors when exposed to either a laboratory or a natural microbial~~
333 ~~inoculum.~~ We inoculated germ-free *Daphnia* to either a laboratory or natural microbial community
334 and compared host responses to a parasitic fungus, an *Aspergillus aculeatus* like strain, and the
335 toxic cyanobacterium *M. aeruginosa* in single and combined exposures. Additionally, we examined
336 intraspecific responses by including three *Daphnia* genotypes in our study. We expected that the
337 gut microbiome (obtained after colonization by the inoculated bacterioplankton community),
338 would affect stressor responses and alter interactions under multiple stressor conditions. We
339 hypothesized that (i) *Daphnia* would obtain a higher tolerance to the stressors when receiving the
340 natural microbial community compared with the laboratory microbial community and (ii)
341 *Daphnia* genotype would shape responses to both single as multiple stressor exposures. Our
342 results showed that (i) laboratory and natural microbiome communities differ in OTU richness,
343 with a higher OTU richness in natural microbial communities, (ii) microbial exposure can alter
344 stress responses for survival, but not for fecundity and body size, (iii) *Daphnia* responses are
345 genotype dependent for survival and fecundity, but not for body size, and (iv) stressor specific
346 microbiomes (*Microcystis* and combi) can be detected. Not all responses were in agreement with
347 what we originally expected. More in particular, we only found an antagonistic response between
348 both stressor treatments on *Daphnia* survival, and only when *Daphnia* were exposed to a laboratory
349 and not to a natural microbial inoculum.

350 The results on OTU richness confirmed our assumption that natural microbial communities
351 (obtained from natural freshwater waterbodies) are more diverse in number of strains compared
352 with laboratory microbial communities (obtained from *Daphnia* medium). This more diverse
353 community was also reflected in the gut of the *Daphnia* as the gut community of *Daphnia*
354 individuals inoculated with a natural microbial inoculum had a higher OTU richness compared
355 with those inoculated with a lab microbial inoculum. This is in accordance with other study
356 systems (e.g. *Drosophila*: Chandler et al. 2011, *Limulus polyphemus:* Friel et al. 2020, zebrafish:
357 Roeselers et al. 2011, mice: Rosshart et al. 2017). In addition, the OTU richness between donors
358 and recipients significantly differed in the microbiome treatments. This suggests that *Daphnia*,
359 when exposed to a rich bacterial community, selects certain bacterial strains from the
360 bacterioplankton community, as suggested in Macke et al. (2017) and Mushegian et al. (2018) and
361 shown in Callens et al. (2020). EdgeR analysis revealed that only one OTU differed between the
362 laboratory and natural donor inocula. Combined with the lower OTU richness in the laboratory
363 donor inocula compared with the natural ones, it appears that the laboratory microbial inocula
364 contain a subset of the natural microbial inocula. The sampled laboratory donor inocula, thus,
365 contained selected particular groups of bacteria from a diverse (natural) microbial inocula.
366 Analysis of the recipient microbiomes showed that selection of bacterial groups was also stressor
367 dependent, with a stronger selection of bacterial strains in the *Microcystis* and combi treatment in
368 *Daphnia* that received a natural microbial inoculum, as shown in the beta diversity analysis. This
369 is conform as suggested in Macke et al. (2017, 2020) and Houwenhuyse et al. (2021).

370 The microbial environment plays a role in stress responses when looking at *Daphnia* survival.
371 Survival analysis revealed a significant difference in the survival probability between the stressor

372 treatments when *Daphnia* received a laboratory microbial inoculum, but not when they received
373 a natural microbial inoculum. When receiving the laboratory microbial inoculum, survival
374 probability was higher when exposed to both stressors simultaneously compared with exposure
375 to these stressors separately. This suggests occurrence of an antagonistic interaction between the
376 two stressors in relation to survival, resulting in a higher survival probability when exposed to
377 both stressors compared with single stressor exposure. This is in accordance with the recent study
378 of Boudry et al. (2020) who also observed an antagonistic interaction between a fungal infection
379 (most likely the same as in this study) and *Microcystis* on *Daphnia* survival. Boudry et al. (2020)
380 described this antagonistic interaction as a potential protective effect of the *Aspergillus* infection
381 on *Daphnia* towards *Microcystis* exposure through a parasite-mediated reduction in toxicity of
382 *Microcystis.* Alternatively, *Daphnia* can be boosted through an increase in general tolerance levels
383 by ingestion of the produced zoospores. Cross-tolerance could be initated as zoospores could
384 function as an additional food source, which is in accordance with Frenken et al. (2017), Kagami
385 et al. (2007) and Agha et al. (2016), indicating that fungal parasites can transfer energy and
386 nutrients from otherwise inedible algae to *Daphnia*, and thereby increase *Daphnia* growth and
387 survival. These studies, however, used fungal parasites that are obligate parasites from inedible
388 diatoms and cyanobacteria. In this study, *Aspergillus* infects the *Daphnia* host, resulting in reduced
389 body size and a genotype dependent reduction in fecundity, as well a high mortality in juveniles
390 (L. Bulteel and S. Houwenhuyse, personal observations). It is, however, not yet examined whether
391 this specific *Aspergillus* can also infect cyanobacteria. The *Aspergillus* genus is diverse and wide-
392 spread containing up to 339 species (Samson et al. 2014), which consist of several pathogenic
393 species, significantly impacting food production (e.g. Alshannaq et al. 2018), and animal and

394 human health (e.g. Kousha et al. 2011, Seyedmousavi 2013). *Aspergillus aculeatus* exposure, on the
395 other hand, has been described to be associated with phytoremediation and detoxification in
396 plants (Xie et al. 2019).

487 The antagonistic interaction on *Daphnia* survival was only observed when exposed to the less
488 diverse laboratory microbial community, which could imply a dependency on a particular,
489 selected and more effective (non-diluted in terms of number of strains present) microbiome. We
490 originally expected more positive effects on life history traits in the natural (e.g. Booth 2002, Van
491 Overbeek et al. 2010, van Elsas et al. 2012), more diverse microbial community, given the higher
492 potential for redundant effects, but here tend to find the opposite response. We here do not find
493 an antagonistic interaction in the multiple stressor treatment in the natural microbial treatment.
494 Given that the antagonistic interaction was only present in the laboratory treatment, it could
495 reflect that the presence of particular microbial strains that were more strongly selected for or the
496 phytoremediating effect of the fungus against *Microcystis* was not diluted by the presence of other
497 strains in the laboratory conditions. In this study, however, we found no possible correlation
498 between gut microbial diversity and the observed life history traits. We did find a differently
499 structured gut community within the laboratory treatment as *Daphnia* gut microbiomes exposed
500 to the multiple stressors showed a partial overlap with the communities exposed to all other
501 stressor treatments (control, *Aspergillus* and *Microcystis*), which already hints at a possible
502 microbiome-mitigated defense mechanism.

503 Exposure to a laboratory or a natural microbial community, however, had no impact on *Daphnia*
504 stress-responses for fecundity and body size. The antagonistic interaction between the stressors

505 observed in the survival analysis was not present for fecundity and body size. Both fecundity and
506 body size did reveal a main stressor effect, independent of microbial exposure, showing a lower
507 reproduction and body size for *Daphnia* that were exposed to the *Microcystis* and combi treatment
508 compared with *Daphnia* that were exposed to the infection and control treatment. Body size, in
509 addition, was lower for individuals exposed to *Aspergillus* compared with the control, whereas
510 the opposite was observed for the total number of brood. Trade-offs between survival and body
511 size under stress were previously found in *Daphnia* (Adamczuk 2010, Houwenhuyse et al. 2021)
512 and other organisms (Sterck et al. 2006, Mogensen and Post 2012) with Houwenhuyse et al. (2021)
513 suggesting a role of the microbiome for this trade-off under *Microcystis* stress. Here, we did,
514 however, not find support for such a trade-off. Interestingly, both *Microcystis* and combi-exposed
515 *Daphnia* show a stressor-specific microbiome, which could be a co-determinant of this lower body
516 size and fecundity.

517 Survival and fecundity analyses revealed a role of the genotype in the stress responses, with for
518 survival the genotype x stressor response being microbiome dependent. Body size, however, was
519 not determined by genotype. The survival probability of genotype T8 was lower compared to
520 KNO and OM2 when they were exposed to a control treatment and received a natural microbiome
521 inoculum. No significant differences, however, were observed between the different genotypes
522 under the control treatment when receiving a laboratory microbial inoculum. For fecundity, KNO
523 and OM2 individuals reproduced a lower number of juveniles when they received a *Microcystis*
524 or combi treatment compared with when they received the infection or control treatment.
525 Fecundity of T8 individuals, however, was not differentially impacted by the different stressor
526 treatments as no significant differences on the number of offspring were observed. When

527 examining the Union plots, we observed that for the control treatment, T8 had a higher number
528 of unique and total OTUs, compared with KNO and OM2 when they received a natural microbial
529 inoculum, but not when they received a laboratory microbial inoculum. This appears
530 counterintuitive, but aquatic environments contain next to a plethora of beneficial and neutral
531 bacterial strains, also obligate and opportunistic bacterial pathogens (Schulze et al. 2006), so it
532 could be that with higher diversity more opportunistic microbiota are present (as also suggested
533 in Callens et al. 2016). As *Daphnia* genotypes differ in their selective capacities to take up bacteria
534 (Macke et al. 2017, Frankel-Bricker et al. 2020, Callens et al. 2020, Bulteel et al. 2021, Houwenhuyse
535 et al. 2021), our results would suggest that genotype T8 might be less selective and takes up
536 randomly also non-beneficial strains, at least in comparison with KNO and OM2.

537 In conclusion, laboratory conditions only contain a subset of the bacterial community from natural
538 environments. We found that these different microbial conditions can affect stressor responses
539 when looking at *Daphnia* survival. Antagonistic interactions between multiple stressors were
540 present when *Daphnia* were exposed to a laboratory bacterial community, but not when exposed
541 to a natural bacterial community. The microbial condition, however, did not play a role in
542 determining *Daphnia* fecundity and body size. We did detect a main stressor effect for both
543 fecundity and body size, with a lower reproduction and body size when exposed to the *Microcystis*
544 and the combined stressor treatment compared with *Daphnia* that were exposed to the infection
545 and control treatment. These effects could possibly be linked to the stressor specific microbiomes
546 observed in the *Microcystis* and combi treatment. In addition, stressor responses were genotype
547 specific for survival and fecundity, which could be linked with different capabilities of the *Daphnia*
548 genotypes to select beneficial or neutral microbial stains from the environment.

549

550 **<u>Materials and methods</u>**

551 ***Daphnia* and algae culturing**

552 To investigate the genotype effect, we used three different *D. magna* genotypes: KNO 15.04, OM2
553 11.3 and T8. The KNO 15.04 genotype (further referred to as KNO) was isolated from a small pond
554 (350m²) in Knokke, at the Belgian coast (51°20′05.62″N, 03°20′53.63″E) and is the same clone as
555 used in Macke et al. (2017, 2020). The OM2 11.3 genotype (further referred to as OM2) was isolated
556 from a 3.7 ha inland pond located in Heverlee, in Belgium (50°51′45.0″N, 04°42′58.8″E) and was
557 part of the clone set of Decaestecker et al. (2007). The T8 genotype was isolated from an 8.7 ha
558 shallow, manmade pond, located in Oud Heverlee, Belgium (50°50′24.0″N, 04°39′40.4″E) and was
559 part of the clone set of Cousyn et al. (2001). All clonal lineages were established from resting eggs,
560 isolated from the lake sediment. All genotypes were maintained in the laboratory under
561 standardized conditions for several years prior to the experiment. Stock *Daphnia* clonal lineages
562 were cultured in filtered tap water at a temperature of 19 ± 1°C and under a 16:8h light:dark cycle
563 in 2L glass jars (at a density of 20 individuals/L). They were fed three times per week with
564 saturating amounts of the green algae *C. vulgaris*. The medium of the stock cultures was refreshed
565 once per three weeks.

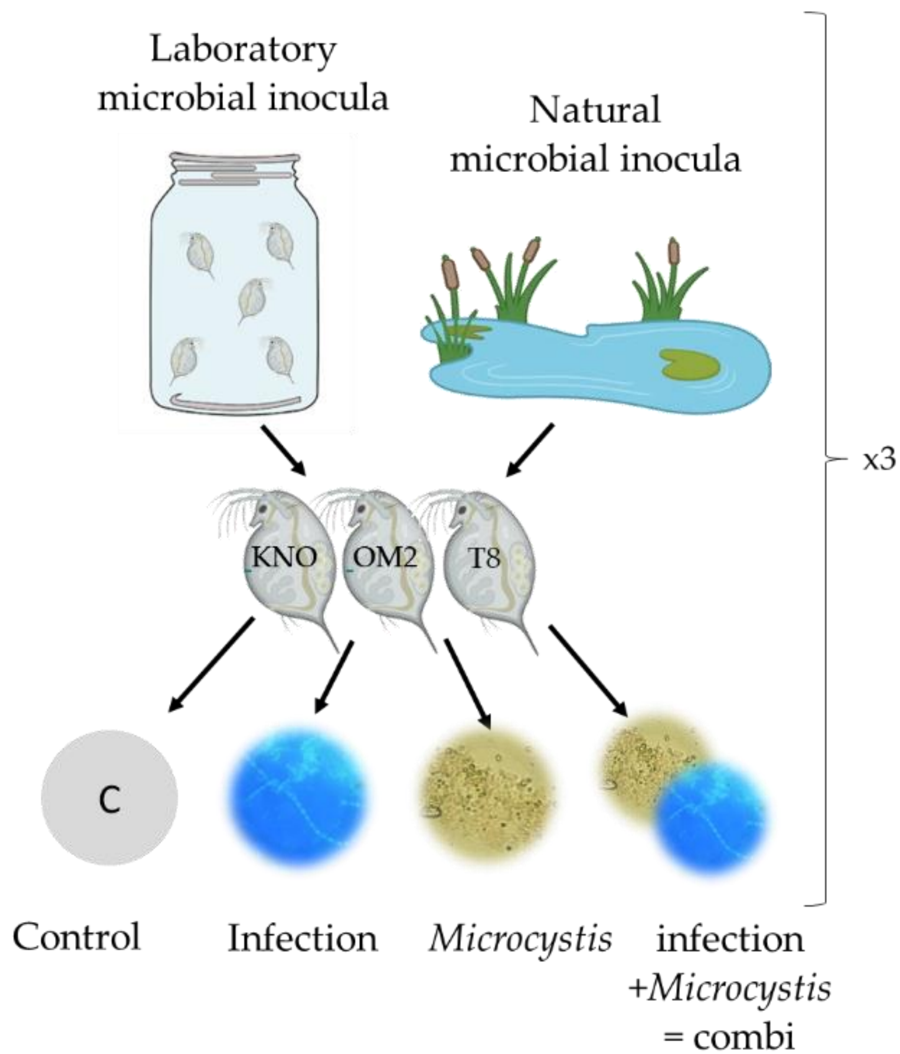
566 *Daphnia* were fed with *C. vulgaris* (strain SAG 211-11 B), which is considered as standard good-
567 quality food for *Daphnia* (Munirasu et al. 2016). One of the stressors used in this experiment is the
568 toxic cyanobacterial strain *M. aeruginosa* (strain PCC 7806), isolated from the Braakman reservoir
569 in the Netherlands (51°19′22″N, 3°44′16″E) and part of the Culture Collections at Institute Pasteur
570 (Paris, France). *Chlorella vulgaris* and *M. aeruginosa* were grown in WC medium (i.e. Wright's

571 Cryptophyte medium) and modified WC medium (without Tris) respectively. The algae were
572 cultured under sterile conditions in a climate chamber at 22 ± 1°C with a light:dark cycle of 16:8h
573 in 2L glass bottles, with constant stirring and aeration. Filters (0.22 µm) were placed at the input
574 and output of the aeration system to avoid any bacterial contamination. The algae were weekly
575 harvested in the stationary phase. The axenity of the algal cultures was checked by sequencing
576 and plating on LB- and R2A-plates.

577 **Experimental design**

578 With this experiment we aimed to investigate the impact of a natural versus a laboratory
579 microbiome on the tolerance of *D. magna* individuals when exposed to two different stressors in
580 single and combined exposures (Figure 12). Individuals, inoculated with either a natural or a
581 laboratory microbial community, were exposed to one of the four following stressor treatments:
582 an opportunistic infection (characterized as *A. aculeatus*, Figure 13A), a toxic cyanobacterium *M.*
583 *aeruginosa* (Figure 13B), the combination of both *Microcystis* and the infection, and a control
584 treatment (fed with *C. vulgaris* instead of *Microcystis* and no exposure to the infection). The
585 opportunistic infection used in this experiment was characterized as a fungal *Aspergillus aculeatus*
586 infection, which causes high mortality and reduced fecundity upon *Daphnia* individuals in the
587 laboratory before, especially in (germ-free) juveniles. The infection is genotype specific (based on
588 visual inspections and experience by the authors).

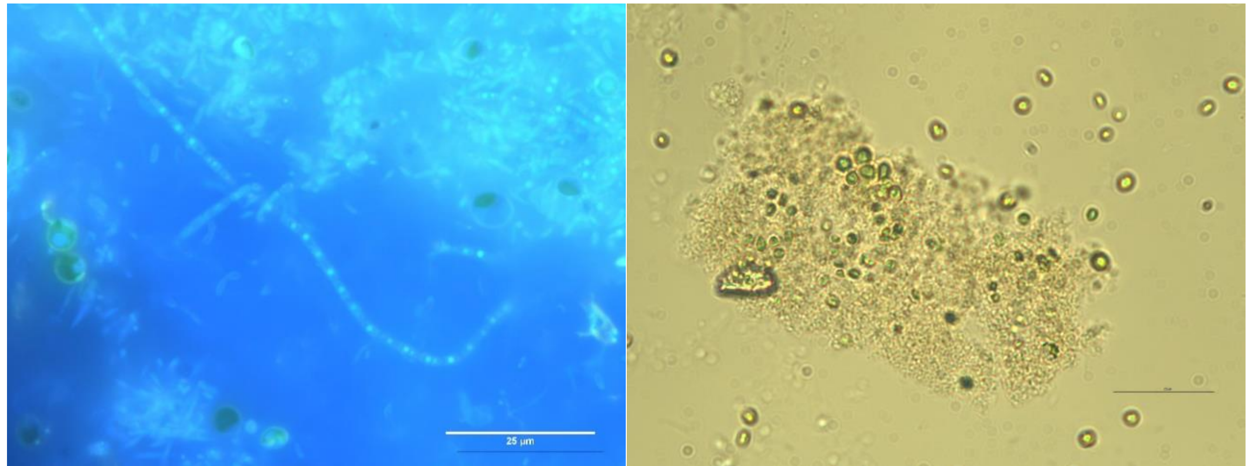
589



590

591 Figure 12: Experimental design. Axenic *Daphnia* individuals from three genotypes were exposed
592 to a natural or laboratory microbial community (microbiome treatment). *Daphnia* individuals
593 receiving a natural microbial community were exposed to filtered pond water. *Daphnia*
594 individuals receiving a laboratory microbial community were exposed to filtered tap water
595 originating from *Daphnia* stock cultures. All *Daphnia* individuals were then exposed to one of the
596 four different stressor treatments: infection, *Microcystis*, combination of both *Microcystis* and
597 infection or a control treatment. The experiment was performed in triplicate for each treatment
598 and factor combination.

599



A B

600 Figure 13: Microscopic pictures of the stressor treatments; (A) *Aspergillus* infection treatment:
601 hyphae and surrounding spores stained with dapi with 400 x magnification under UV
602 fluorescence (for characterization process, see further) and (B) *Microcystis* treatment: Colony of
603 *Microcystis* surrounded with individual cells with 160 x magnification.

604 Each multifactorial combination of stressor treatment, microbiome treatment and genotype was
605 replicated independently three times (independent maternal lines). To establish the independent
606 maternal lines, three iso-female lines for each genotype were cultured in separate jars for at least
607 two generations to control for maternal effects. These iso-female lines were kept in a mixture of
608 filtered tap and pond water in a 9:1 ratio and fed every other day with a saturating amount of *C.*
609 *vulgaris*. Medium was refreshed once per week. The first brood of the second generation was
610 discarded, whereas eggs from the second brood were collected to obtain axenic (i.e. germ-free)
611 juveniles following protocol from Bulteel et al. (2021) and Houwenhuyse et al. (2021).

612 The axenic individuals were then placed individually in a closed vial filled with 18 mL sterile
613 filtered tap water and 2 mL of the corresponding microbiome treatment (natural or laboratory
614 microbial community). The natural microbial communities were sampled from three local natural
615 ponds. The laboratory microbial communities, on the other hand, were sampled from the medium

616 from three different genotypes, which were cultured in the lab. In this manner, we were able to
617 mimic bacterioplankton communities under natural (high bacterial diversity) and laboratory (low
618 bacterial diversity) conditions. Each microbial community was subsequently filtered over 100 µm
619 and 10 µm to remove debris. Each maternal line received one of the three natural or laboratory
620 microbial communities (Table 2).

621 After receiving the corresponding microbial inoculum, the individuals remained in these
622 conditions for 48h, allowing for the microbiota to colonize the *Daphnia* guts. On the third day, all
623 individuals were fed with *C. vulgaris* ($100*10^3$ cells/mL). On the fifth day, individuals were
624 exposed to their corresponding stressor treatment (Figure 12). Individuals in the *Microcystis*
625 treatment received a mixture of the toxic cyanobacterial strain *M. aeruginosa* and the non-toxic *C.*
626 *vulgaris* in a 50:50 ratio on a daily base from day 5 onwards. Individuals in the infection treatment
627 received a spore solution. The spore solution was obtained by squashing infected *Daphnia*
628 individuals and was administered in a 1:3 ratio (1 infected individual per 3 to infect individuals).
629 We assume little impact from the small bacterial community associated with the spore solution as
630 administered volume is low and as administration occurred after the colonization of the microbial
631 inocula (Vass and Langenheder 2017, Callens et al. 2020). Samples of the spore solution were
632 sequenced to correct for contamination if necessary. Individuals in the combi treatment received
633 both the spore solution on day 5 and the combination of the toxic *M. aeruginosa* and the non-toxic
634 *C. vulgaris* in a 50:50 ratio from day 5 onwards. Individuals in the control treatment were not
635 exposed to any stressor and were fed with *C. vulgaris* from day 5 onwards. After being exposed
636 to their corresponding stressor treatment, the volume in the falcon tubes was gradually increased
637 to 50 mL by adding 10 mL of sterile filtered tap water per day, and this for three consecutive days

638 (day 6-8). Food concentration in the first 6 days was low (100*10³ cells/mL) to ensure a sufficient
639 stress response. From day 7 onwards, food concentration was increased to 200*10³ cells/mL. All
640 individuals were monitored for survival and reproduction for 21 days. At the end of the
641 experiment (day 21), the body size was measured (from top of the head to the base of the tail) and
642 guts were dissected and collected per treatment in an Eppendorf tube filled with 10 µL of sterile
643 MilliQ. Recipient guts were pooled per unique combination (4 stressor treatments x 2 microbiome
644 treatments x 3 genotypes x 3 replicates). In addition, samples of the donor microbial inocula (n=6)
645 were collected to compare bacterial communities.

646 Table 2: Overview of the origin of the microbial communities divided over the replicates.

Replicate	Laboratory microbial inoculum	Natural microbial inoculum
1	M5 lab medium	Kennedy pond (50°48'05.7"N 3°16'33.0"E)
2	T7 lab medium	Marionetten pond (50°47'43.5"N 3°15'00.2"E)
3	ZWE 2B lab medium	Kulak pond (50°48'30.8"N 3°17'37.0"E)

647

648 **Library preparation and sequencing**

649 To characterize the gut microbial communities from collected *Daphnia* guts, the guts of the
650 surviving *Daphnia* per replicate were dissected under a stereo-microscope with sterile dissection
651 needles at the end of the experiment and pooled per replicate (mean= 7.236 guts/sample; sd= 1.872
652 guts/sample; min= 2 guts; max= 10 guts; Table S6). Samples were stored under -20°C until further
653 processing. DNA was extracted using a PowerSoil DNA isolation kit (MO BIO laboratories,
654 Carlsbad, CA, USA). DNA was dissolved in 20 µL milliQ water. Because of initially low bacterial

655 DNA concentrations in some samples, a nested PCR was applied to increase specificity and
656 amplicon yield. The full-length 16S rRNA gene was first amplified with EUB8F and 1492R primers
657 on 10 ng of template using a high-fidelity SuperFi polymerase (Thermofisher, Merelbeke,
658 Belgium) for 30 cycles: 98°C – 10 s; 50°C – 45 s; 72°C – 30 s. PCR products were subsequently
659 purified using the CleanPCR kit (Qiagen, Antwerp, Belgium). To obtain dual-index amplicons of
660 the V4 region, a second amplification was performed on 5 μL (=20-50 ng) of PCR product using
661 515F and 806R primers for 30 cycles: 98°C – 10 s; 50°C – 45 s; 72°C – 30 s. Both primers contained
662 an Illumina adapter and an 8-nucleotide (nt) barcode at the 5'-end. For each sample, PCRs were
663 performed in triplicate. Afterwards the PCR products were pooled and a small volume (5 μL) was
664 loaded on a gel to check if the PCR amplified the correct fragment. The remaining volume of the
665 PCR products were purified using the CleanPCR kit (Qiagen, Antwerp, Belgium). An equimolar
666 library was prepared by normalizing amplicon concentrations with a SequalPrep Normalization
667 Plate (Applied Biosystems, Geel, Belgium) and subsequent pooling. Amplicons were sequenced
668 using a v2 PE500 kit with custom primers on the Illumina Miseq platform (KU Leuven Genomics
669 Core), producing 2 x 250-nt paired-end reads. This way, 72 gut samples were generated
670 representing 4 stressors x 2 microbiome inocula x 3 genotypes x 3 replicates. In addition, samples
671 of the microbiome inocula (n=6), the stressor treatments (n=2) and *C. vulgaris* (n=1) were
672 sequenced.

673 **Life history traits data**

674 Survival was analyzed using a log-rank or Mantel-Haenszel test. The survival times of individuals
675 that were still alive at the end of the 21 day experiment were coded as right-censored. Normality

676 and skewness of body size and fecundity data were examined with Shapiro–Wilk test and
677 ggqqplot function (package ggpubr). For fecundity and body size, we used the Akaike
678 information criterion (AIC) to select the best subset of variables to represent the best model. We
679 first evaluated to include maternal line as a random factor (with a linear mixed-effect model) or
680 not (with a general linear model). Secondly, we tested the significance of the fixed factors in the
681 model with the best random effects factor. Type II ANOVA tables for fixed-effect terms with
682 Satterhwaite and Kenward-Roger methods for dominator degrees of freedom for F-tests and p-
683 values were created (Anova function of the car package). Following the AIC criterium, a linear
684 mixed-effect model was chosen to evaluate fecundity and body size. In the final model, we
685 included microbiome treatment, stressor treatment and genotype as fixed factors, and maternal
686 line as random effect. We also included all possible interactions. Post hoc analysis were performed
687 using the 'emmeans' function with a 'Tukey' adjustment from the emmeans R package. All
688 statistical tests were performed in R 4.0.2 (R Core Team 2020).

689 ***Daphnia* microbiome data**

690 DNA sequences were processed following Callahan et al. (2016a). Sequences were trimmed (the
691 first 10 nucleotides and from position 180 onwards) and filtered (maximum of 2 expected errors
692 per read) on paired ends jointly. Sequence variants were inferred using the high-resolution
693 DADA2 method, which relies on a parameterized model of substitution errors to distinguish
694 sequencing errors from real biological variation (Callahan et al. 2016b). Chimeras were
695 subsequently removed from the data set. Taxonomy was assigned with a naïve Bayesian classifier
696 using the SILVA v132 training set. OTUs with no taxonomic assignment at the phylum level or

697 which were assigned as "chloroplast" or "cyanobacteria" were removed from the data set. After
698 filtering, a total of 3 552 490 reads were obtained with on average 39 038.35 reads per sample, with
699 most samples having more than 1000 reads. To visualize the bacterial families that differed
700 between the treatments, OTUs were grouped at the order level, and orders representing <1% of
701 the reads were discarded. Measures for α-diversity of the recipient gut microbial communities
702 within the different treatments (OTU richness) were calculated using the vegan package in R
703 following Borcard et al. (2011). All samples were rarified to a depth of 1000 reads before analyzing
704 α-diversity. The effects of stressor treatment, microbiome treatment, genotype, and all possible
705 interactions on OTU richness were assessed through a generalized linear model (GLM), assuming
706 a Poisson distribution of the data and corrected for overdispersion. Maternal line was not included
707 as a random factor as AIC criterium indicated that the model without inclusion of the maternal
708 line was a better predictive model of the data. After testing the full model, we used the AIC
709 criterium to select the best subset of variables to represent the best model. Pairwise comparisons
710 among significant variables and their interactions were performed by contrasting least-squares
711 means with Tukey adjustment. To examine differences in gut microbial community composition
712 (β-diversity) among samples, a Bray-Curtis dissimilarity matrix was calculated and plotted using
713 principal coordinates analysis with the phyloseq package in R. Multivariate community responses
714 to treatments and genotype were investigated by means of Principal Coordinates Analysis. The
715 effect of the stressor treatment, microbiome treatment, genotype, and all possible interactions on
716 β-diversity were assessed through a permutation MANOVA, using the Adonis2 function in the
717 vegan package in R. Obtained p-values were adjusted for multiple comparisons through the
718 control of the false discovery rate (FDR). Pearson correlations were executed between the number

719 of sequenced guts and the OTU richness to check for interdependence. Stressor treatment,
720 microbiome treatment, genotype, all two-way interactions, and the three-way interaction, all
721 showed no significant correlation, dismissing the issue of interdependence (Table S7).
722 Additionally, correlation tests were executed between the different life history traits and the OTU
723 richness of the gut microbial communities. Correlation coefficients and p-values were calculated
724 for all examined correlations. Obtained p-values were adjusted for multiple comparisons through
725 the control of the false discovery rate (FDR). To identify which bacterial classes significantly
726 differed between the main effects and the interaction effects, relative abundances per order were
727 calculated on the raw sequencing data, excluding the samples removed from the rarefaction.
728 Based on OTU presence, Union plots were created using the wilkox/unionplot function from
729 GitHub, to show the unique and shared OTUs within and between the stressor x microbiome
730 interaction. Additionally, differential abundance analyses were performed (edgeR function) on
731 the raw sequencing data from which samples with less than 2 counts per million (CPM) in at least
732 three samples were filtered out. All statistical tests were performed in R 4.0.2 (R Core Team 2020).

733 ***Characterization infection***

734 To characterize the strain causing the infection in this experiment, samples of infected *Daphnia*
735 with visible signs of the fungal infection and *Daphnia* with no visible infections were compared.
736 Fifteen infected animals were transferred in whole per five individuals in a sterile Eppendorf tube.
737 Guts from 60 infected animals were dissected and transferred per 20 guts to 10 µl of sterile MilliQ
738 water. Samples were stored under -20°C until further processing. DNA of all samples was
739 extracted using a PowerSoil DNA isolation kit (MO BIO laboratories). The total DNA yield was

740 determined using a Qubit dsDNA HS assay (Invitrogen) on 1 µL of sample. A PCR reaction was
741 run using a combination of primers for the LSU and SSU region (see Table S9, White et al. 1990,
742 Vilgalys and Sun 1994) on all of the template (98°C – 30s, 30 cycles of 98°C – 10s, 55°C – 45s, 72°C
743 – 30s, and 72°C – 5s, 12°C hold) using the Platinum SuperFi DNA polymerase (Thermofisher).
744 PCR products were subsequently purified using the QIAquick PCR purification kit (Qiagen) and
745 were sent for Sanger sequencing to LGC Genomics (Berlin, Germany). The sequences were first
746 converted into consensus sequences using R (package BioCManager). As little similarity was
747 obtained to develop the consensus sequences, non-consensus fasta files were used. The Basic Local
748 Alignment Search Tool (BLAST), BLASTn was performed on the non-consensus fasta files, using
749 FungiDB (Basenko et al. 2018). All query sequences were blasted with all the fungal species
750 present in the database, including oomycetes. The Expectation value (E-value, expected number
751 of hits) was set as 50% of the length of the query sequence. Maximum descriptions (number of
752 descriptions/alignment to show) were set to 50 to avoid compromising the e-value and possible
753 sequence matches. Additionally, the low complexity filter mode was set off to avoid omittance of
754 results which contain repetitive and low complexity sequences. Similar settings were performed
755 for all blasted sequences. Obtained results of fungiDB were verified using NCBI, emboss and
756 wasabi. For NCBI the BLASTn protocol was followed (Schoch et al. 2014). To improve the
757 sequence matches with Fungi, BLAST search was limited to RefSeq sequences only (using
758 BioProject Number specific to Fungi, 177353, Schoch et al. 2014). Furthermore, emboss, with the
759 Emboss matcher algorithm, was used to create the pairwise alignment between the sequences
760 using the BLOSUM 62 matrix (Rice et al. 2000). Finally, a reference based multiple sequence
761 alignment was performed to create a multiple sequence alignment table, using PRANK

762 (probabilistic multiple alignment program for DNA) hosted by wasabi using the HKY model
763 (Veidenberg et al. 2015). The results were consistent across all databases (FungiDB, NCBI, emboss
764 and wasabi).

765

766 **Acknowledgments**

767 The authors thank Noé Cuvelier, Bart Soens and Jesse Vandenbussche for their assistance with
768 the experimental work.

769

770 **Funding**

771 Funding was provided by the KU Leuven research project C16/17/002 and the FWO projects
772 G092619N and G06216N.

773

774 **Data availability**

775 The datasets and scripts generated for this study can be found in the NCBI, under accession
776 number PRJNA731313 and on Zenoda with DOI: 10.5281/zenodo.4778716.

777

778 **Conflict of interest**

779 The authors declare that there is no conflict of interest.

780

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1023 and photosynthetic efficiency of rice. *Ecotoxicology and Environmental Safety,* 171: 373-381. DOI:
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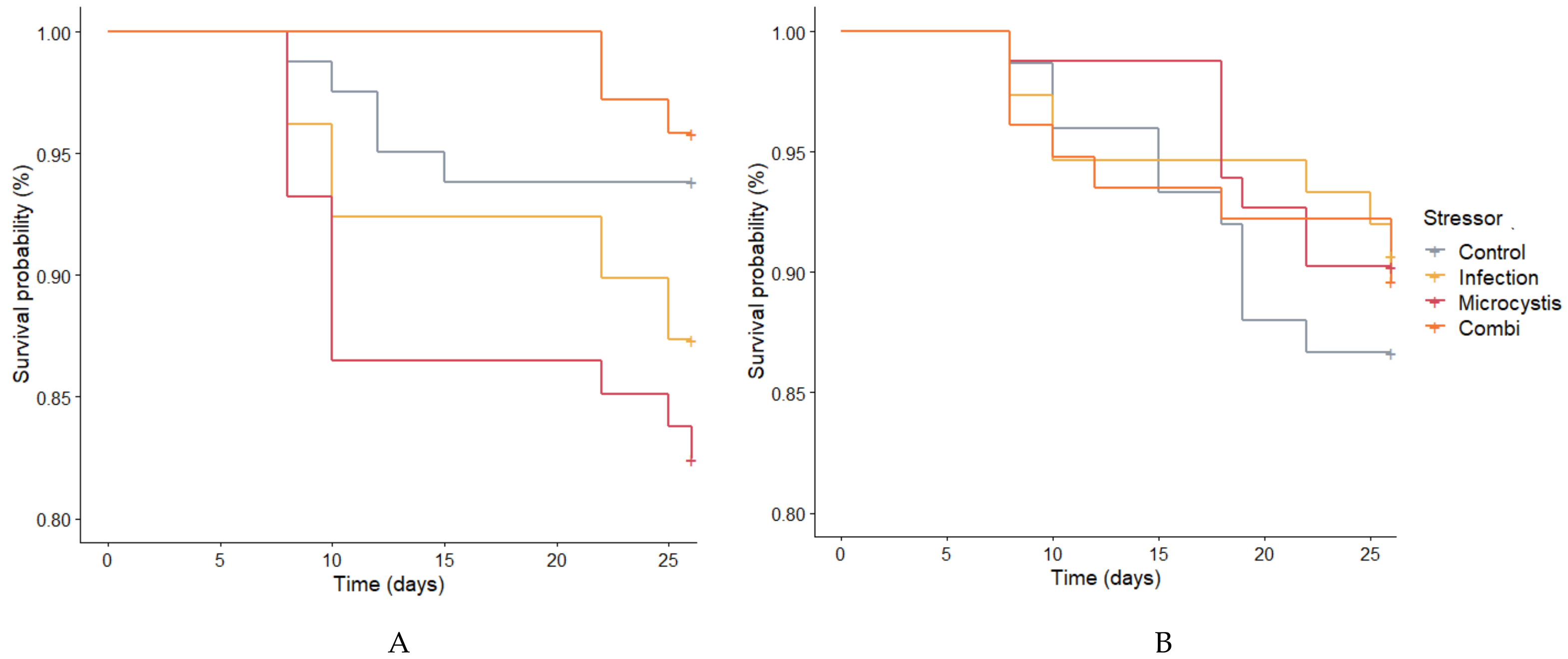
1025

1026 **Supplementary information**



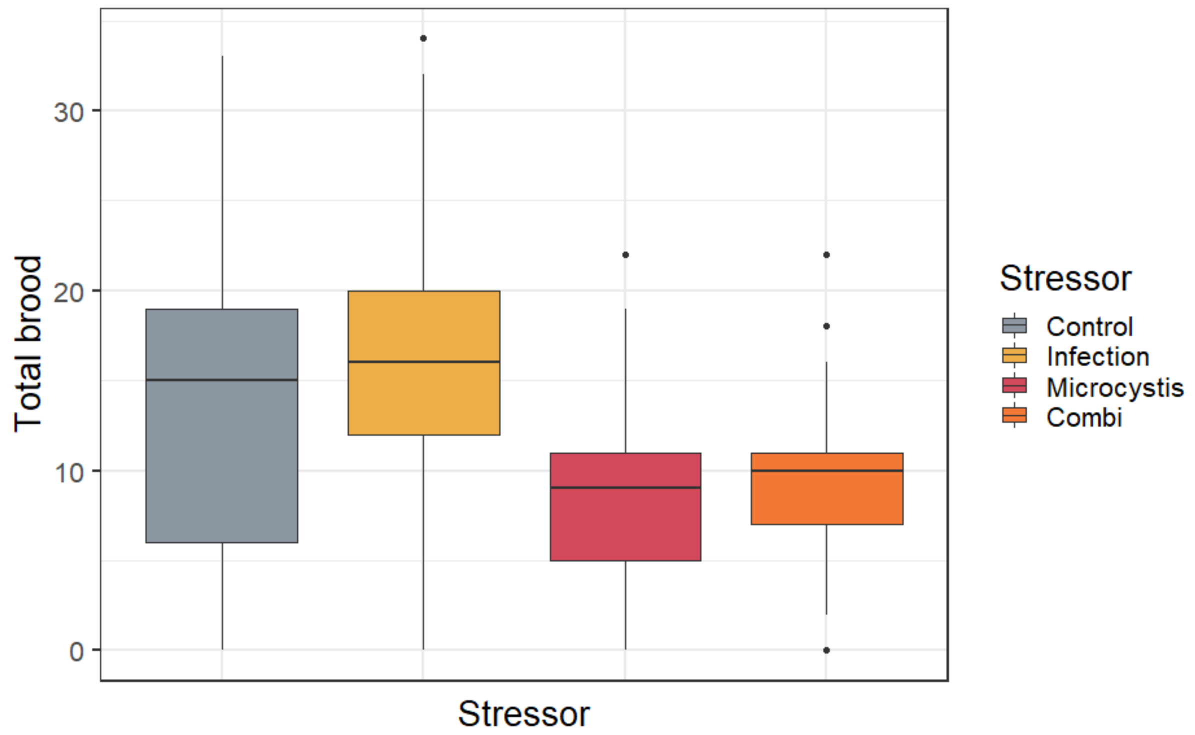
1028 A B

1029 Figure S1: Survival plots recipient *Daphnia* between the stressors treatments for (A) the lab microbial inocula and (B) the natural

1030 microbial inocula. Colors indicate the different stressor treatments.

1031

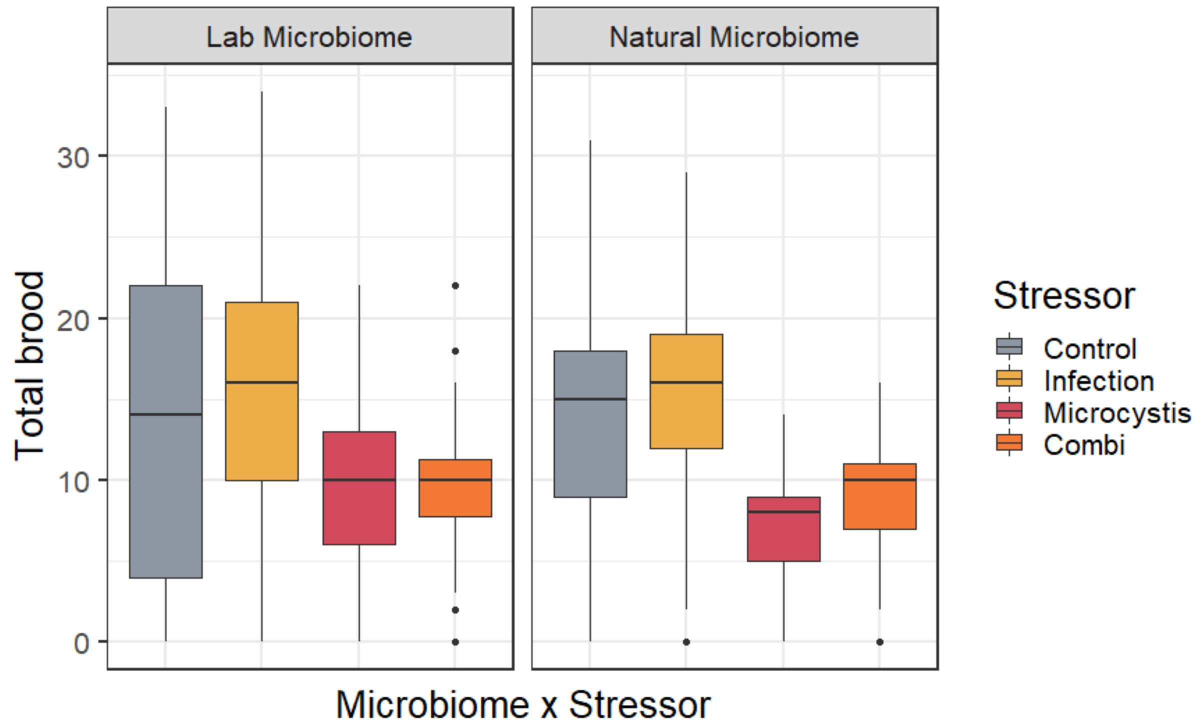
1032



1033

1034 Figure S2: Boxplots of the total brood for the different stressor treatments. Colors indicate the

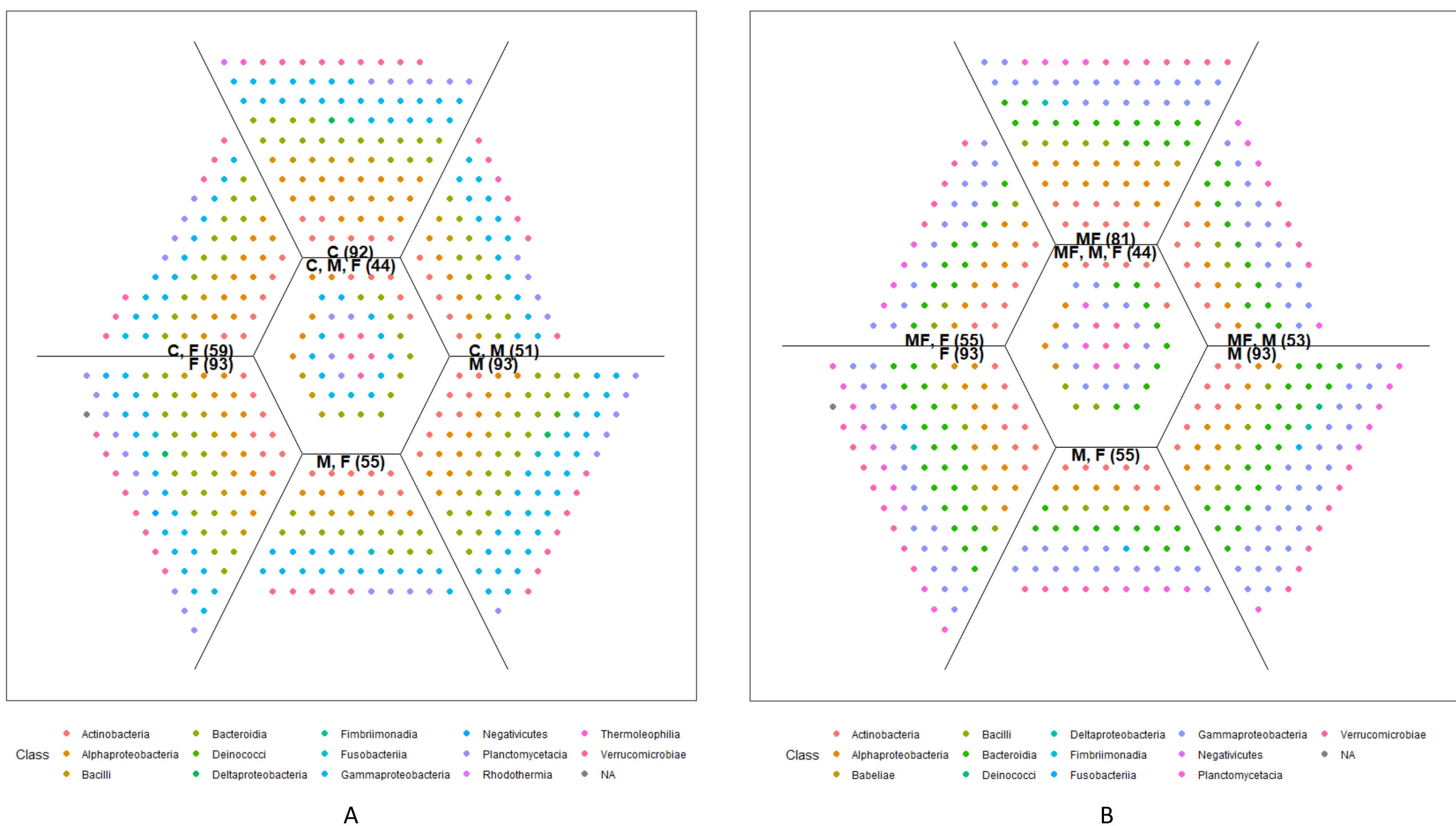
1035 different stressor treatments.



1036

1037 Figure S3: Boxplots of the total brood for the different stressor treatments under the different

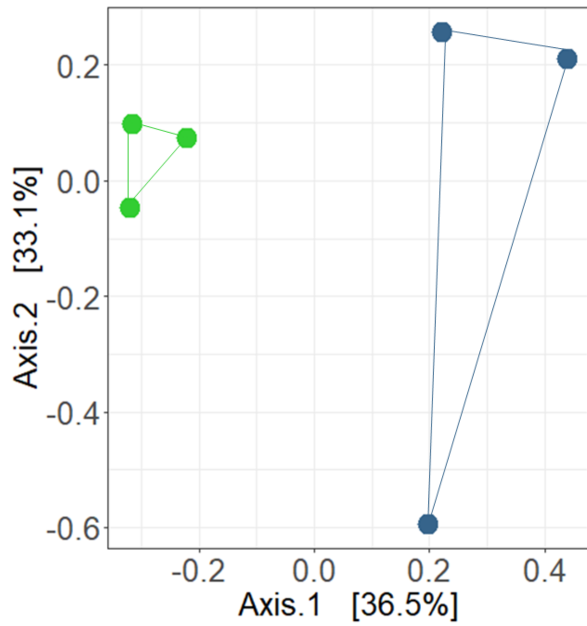
1038 microbial inocula treatment. Colors indicate the different stressor treatments.

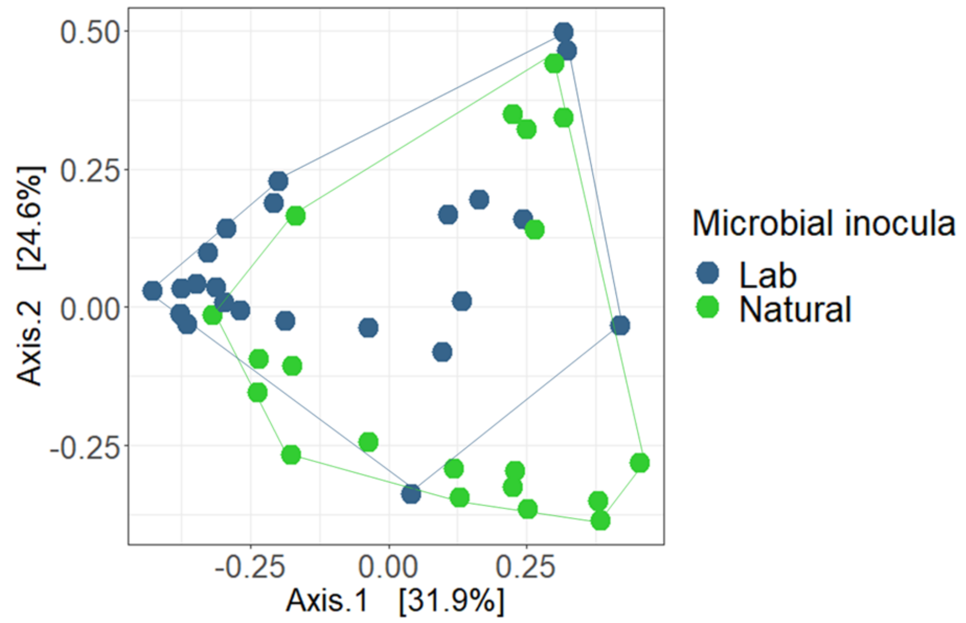


A B

1039 Figure S4: Unionplots representing the OTUs that are unique within and shared between (A) the control treatment and single stressor
1040 treatments and (B) the single treatments and the multiple stressor treatment. OTUs are illustrated for A and B: the control treatment
1041 (C), infection treatment (F) and Microcystis treatment (F) and C and D: infection treatment (F), Microcystis treatment (M) and combi
1042 treatment (MF). Numbers between brackets indicate OTUs present in that compartment. Colors indicate the class level per OTU.

1043

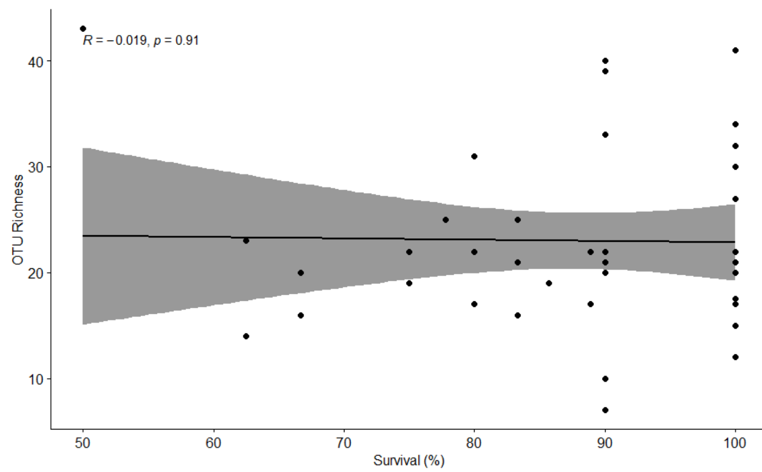




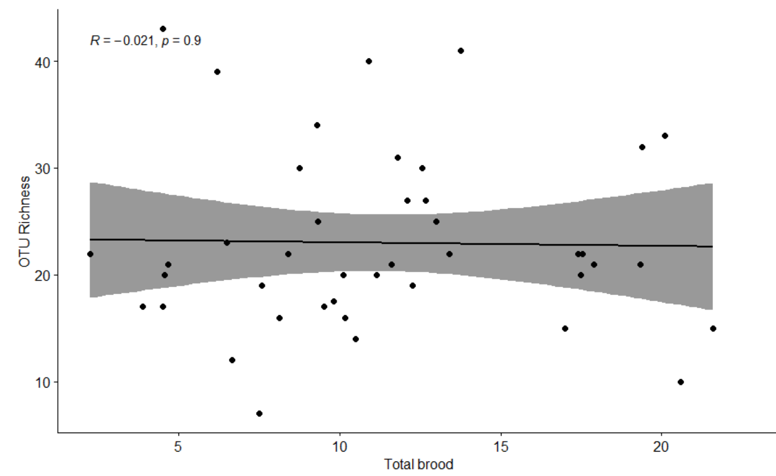
1045 A B

1046 Figure S5: PCA of the (A) donor and (B) recipient microbial communities using weighted Bray-Curtis distance. Colors indicate the

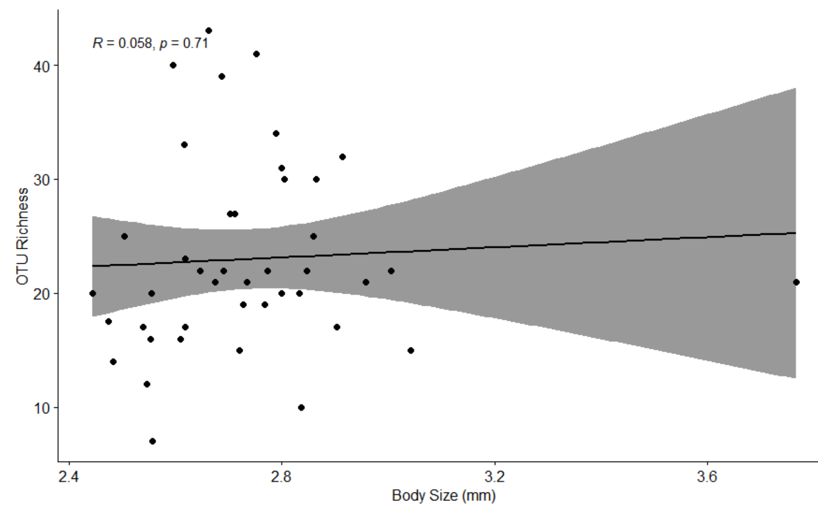
1047 different microbiome treatments.



A

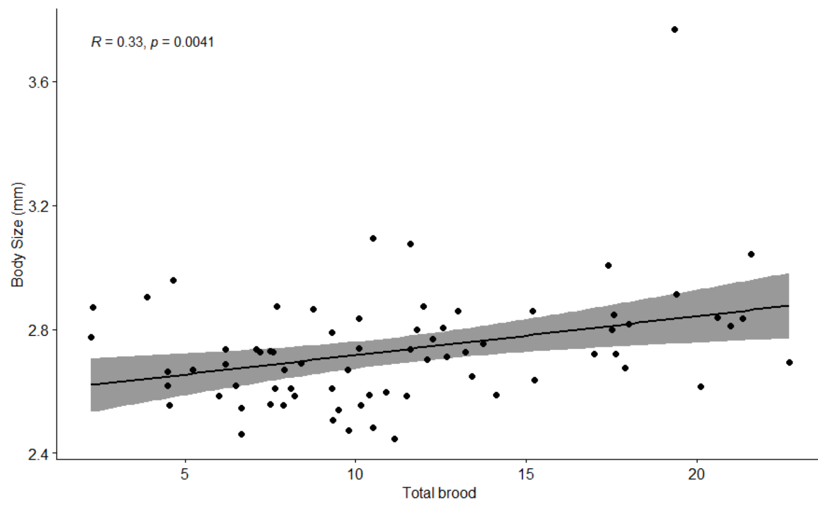


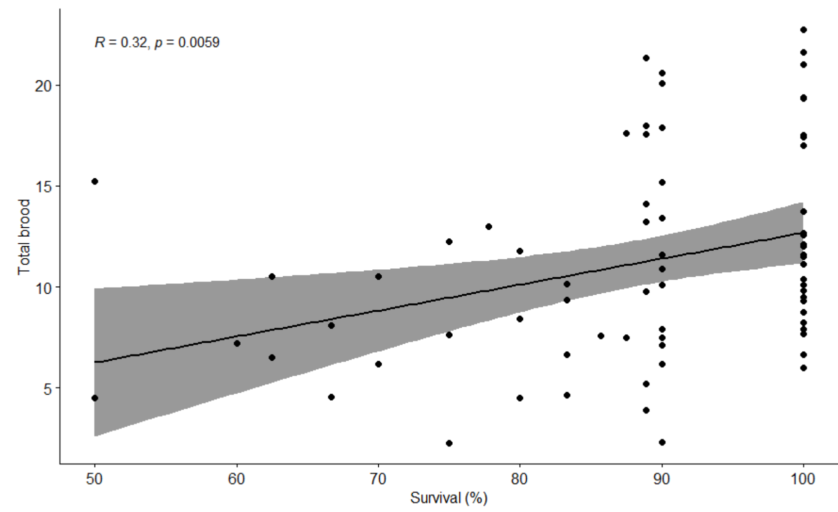
B



C

1048 Figure S6: Pearson regression between (A) Survival, (B) Fecundity, (C) Body size and OTU richness of the gut microbial community of
1049 recipient *Daphnia*. Non-adjusted p-values and correlation coefficient (R) are noted per figure.





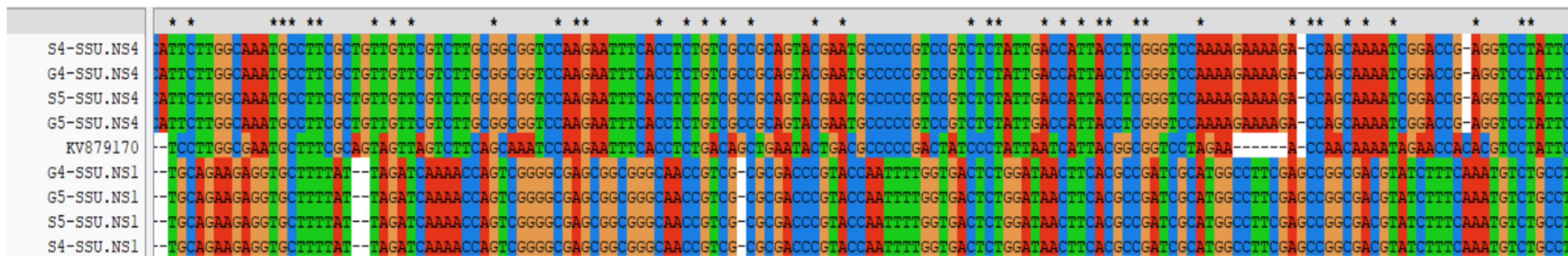
A B

1050 Figure S7: Pearson regression between (A) Fecundity and body size, and (B) Survival and Fecundity. Non-adjusted p-values and
1051 correlation coefficient (R) are noted per figure.

1052

1053

1054



1055 Figure S8: Part (155 to 310 nucleotides) of the multiple sequence alignment pattern of the sample sequences with *Aspergillus aculeatus*
1056 ATCC 16872. Sequences of *Daphnia* with a visible and no visible infection, together with the *Aspergillus aculeatus* ATCC 16872 strain are
1057 aligned (sample names are shown in the left column). Color represents a specific type of nucleotide that matches with the *Aspergillus*
1058 *aculeatus* strain. Hyphen (-) represents a gap where no match between the nucleotides of the *Aspergillus aculeatus* strain and the aligned
1059 sequence of the sample is found. Asterisk on the top represents the nucleotides that are common in all the aligned sequences.

1060