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 settings often differ from the natural environment, which could ultimately be reflected in different stress responses. In this study, we investigated the impact of single versus simultaneous multiple stressor exposure on *Daphnia magna* life history traits and whether this tolerance was microbiome-mediated. *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 survival in *Daphnia* was microbiome-mediated as survival was only negatively impacted when *Daphnia* received a lab microbial community. *Daphnia* ~~which~~ received a natural microbial community have a broader environmental pool of microbiota to randomnly and selectively take up and showed no negative

impact on survival. Simultaneous exposure to both stressors also revealed an antagonistic interaction for survival. Fecundity and body size were negatively impacted by exposure to stress, however, responses were here not microbiome-mediated. 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.

Introduction

Organisms are constantly involved in biotic and abiotic interactions that can lead to the flourishment and diversity of life (Bøhn and Amundsen 2004, Ratzke et al. 2020). Interactions with environmental stressors have an important role in shaping co-evolutionary dynamics by altering the strength of and response to selection and/or population dynamics (Theodosiou et al. 2019, Thompson and Cunningham 2002). ~~We can fairly say that~~ all organisms, from plants to microorganisms to animals, in their natural settings are forced to cope with abiotic (caused by non-living factors) and/or biotic (caused by living organisms) stresses (Holmstrup et al. 2010), which ~~cause~~ a negative impact on the organisms. Abiotic stress, on the one hand, can be caused by a variety of factos such as soil salinity (e.g. Jha et al. 2019), water availability (shortage: e.g.: Joshi et al. 2016, excess: e.g.: Domisch et al. 2020), extreme temperatures (cold: e.g. Shahan 2020, heat: e.g. Rohde et al. 2013), oxidative stress (e.g. Gray and Jakob 2014) and toxicity (e.g. Azimi et al. 2021). Biotic stress, on the other hand, is mostly caused by organisms such as predators (e.g. Osvik et al. 2021), pathogens (e.g. Zhang et al. 2013), parasites (Decaestecker et al. 2005), and competitors (e.g. Dohn et al. 2013) ~~which~~ can consist of bacteria, fungi, viruses, animals and even

plants. Facing these stressful environmental conditions can trigger a number of responses in the stressed organism ~~imposed by their environment~~, ~~which~~ can vary greatly between host species (e.g. ~~sessil~~ versus mobile), but also between the type of stress experienced (e.g. toxicity versus pathogen). There is, however, increasing evidence that different single stressors generally co-occur and interact (Jackson et al. 2016) and generate complex effects on natural populations (Piggot et al. 2015). Organisms can simultaneously be affected by different biotic stresses (e.g. predator and pathogen: Adamo 2020), different abiotic stresses (e.g. drought and salinity: Sun et al. 2015), or even both combined (e.g. salinity and pathogen: Bai et al. 2018). ~~to the interaction between multiple stressors, can generate complex effects on natural host populations (Piggot et al. 2015).~~ A meta-analysis by Jackson et al. (2016) of the marine literature shows that 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) are less common in marine systems, but occur frequently in freshwater systems (Crain et al. 2008, Holmstrup et al. 2010, Jackson et al. 2016).

In this paper we focus on aquatic systems, and more specifically on the effect of multiple stressors on the zooplankter *Daphnia magna*. *Daphnia magna* is not only a keystone grazer in many ponds and lakes worldwide, but is also a well-known study system to study environmental stress via phenotypic plasticity (Stoks et al. 2015) or genetic adaptation (e.g. Hochmuth et al. 2015). One important aquatic stressor, cyanobacteria, is becoming increasingly dominant in aquatic ecosystems (Visser et al. 2016). The negative effect of cyanobacteria on zooplankton is well documented (Ferrão-Filho et al. 2000, Asselman et al. 2012, Lemaire et al. 2012). Cyanobacteria are

66 known to produce a wide range of toxic, secondary metabolites, classified as cyanotoxins among
67 which hepatotoxins, neurotoxins, dermatotoxins, and general cyanotoxins (De Figueiredo et al.
68 2004, Bittner et al. 2021). A commonly occurring and well-studied cyanobacterium is *Microcystis*
69 *sp.* (von Elert et al. 2003), which is known to be detrimental for *Daphnia* in many different ways.
70 First, *Microcystis* produce various toxins, such as the most-frequently occurring hepatotoxic
71 microcystin (Van appeldoorn et al. 2007). The toxic effects of microcystins are detrimental for the
72 survival and health of aquatic organisms such as zooplankton and fish (Penaloza et al. 1990).
73 Secondly, *Microcystis* is also of low food quality due to the absence of essential polyunsaturated
74 fatty acids and sterols (von Elert et al. 2003, Martin-Creuzburg et al. 2008). Thirdly, *Microcystis* is
75 known for its colony formation which interferes with the filtering process (DeMott et al. 2001),
76 which further negatively impacts zooplankton fitness. In response, zooplankton has developed
77 multiple anti-*Microcystis* tolerance mechanisms in e.g., production of proteases or increased ~~gene~~
78 expression of genes associated with secondary metabolite transport and catabolism
79 (Schwarzenberger et al. 2014).

80 ~~Not only cyanobacteria pose a severe threat to the zooplankton communities, also~~ parasites are
81 an increasing threat, especially upon global change. Parasites are a classic example of biotic stress
82 as they impose a negative impact on their host by exploiting the host to complete ~~the parasite's~~
83 life cycle. Fungal parasitism received increasing scientific interest in the last years (for
84 zooplankton: e.g. Decaestecker et al. 2005, Civitello et al. 2015, Banos et al. 2020; for cyanobacteria:
85 e.g. Gerphagnon et al. 2015, Gleason et al. 2015) and are omnipresent and diverse in morphology,
86 phylogeny and ecological functions. Fungal parasites, however, are still poorly understood in
87 their role in vital interactions and ecosystem functions in most aquatic ecosystems (Grossart et al.

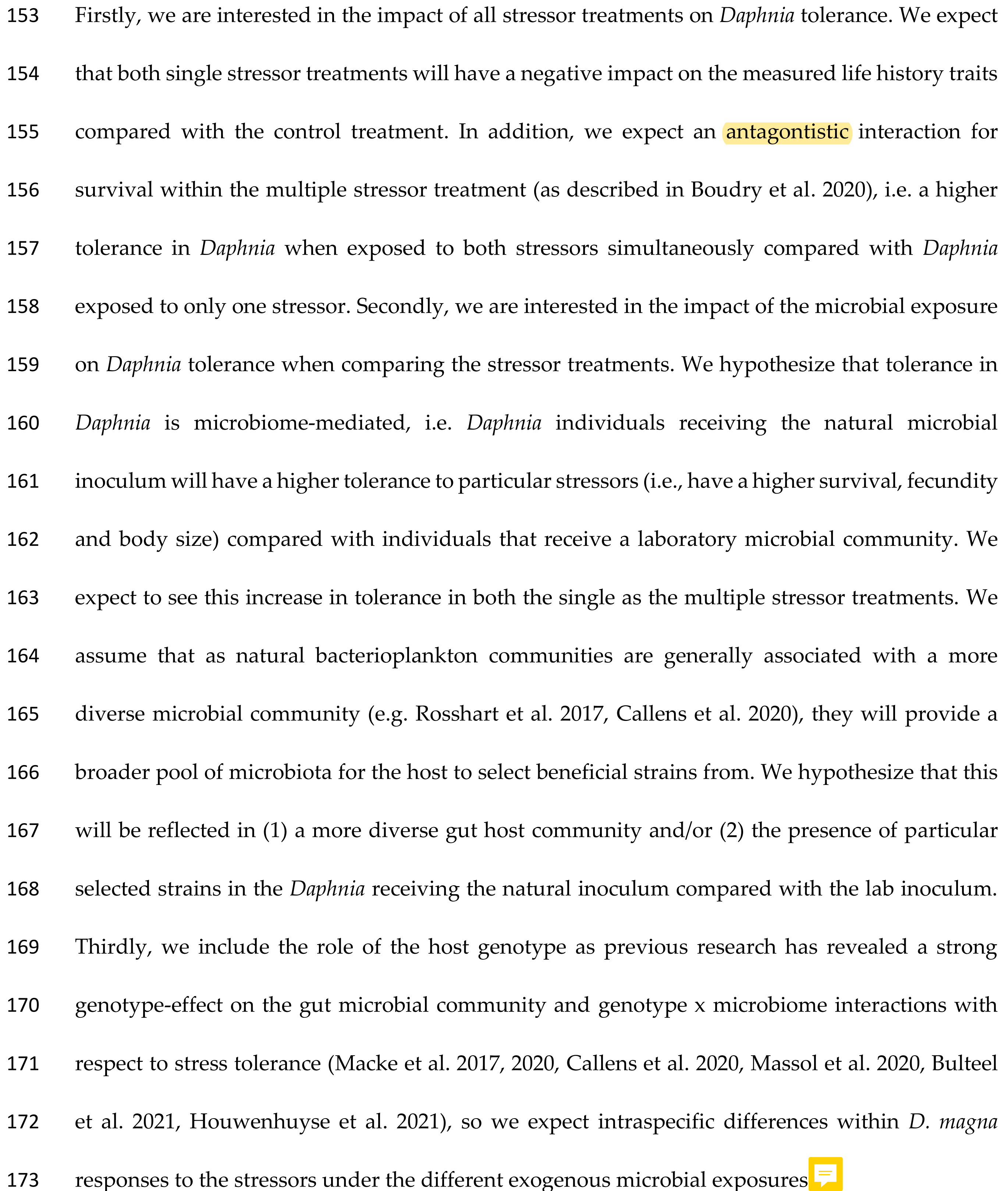
2019). Combined with the increasing abundance of cyanobacterial blooms, this sparked some studies to examine potential interactions between fungi and cyanobacteria impacting aquatic food webs. Some research focussed on altered predator-prey interactions by fungal infections of cyanobacterial blooms (e.g. Kagami et al. 2007, Tao et al. 2020). Agha et al. (2016) focussed on chytrid infection of cyanobacterial populations, revealing a positive impact on the freshwater zooplankter *Daphnia* by improving food quality. Other research focussed on altered host-parasite interactions by feeding infected *Daphnia* populations with cyanobacteria (Coopman et al. 2014, Boudry et al. 2020). Boudry et al. (2020) revealed an antagonistic interaction between a fungal parasite and *Microcystis* as a higher survival was obtained in infected *Daphnia* compared with non-infected *Daphnia* when fed on *M. aeruginosa*. Other studies have also revealed antagonistic interactions using other parasitic systems in *Daphnia* (e.g. predation x bacterium: Coors and De Meester 2008, pesticide x bacterium: De Coninck et al. 2013, salinity x bacterium: Hall et al. 2013, cyanobacteria x iridovirus: Coopman et al. 2014, microsporidium x bacterium: Lange et al. 2014).

The last decade, however, studies have shown that it is not just the host's genome that determines host fitness and reaction towards stressors, but rather a complex interplay of the host genome and microbiome (McFall-Ngai et al. 2013). Especially the gut microbiome, the genetic material of all microorganisms present in the host's gut, plays a key mediating role in host physiology (e.g. organ development: McFall-Ngai et al. 2013, immunoregulation: Renz et al. 2011, metabolism: Turnbaugh et al. 2006). Research has shown that the microbial community in *Daphnia* is structured by diet (Callens et al. 2016), host genetics (Macke et al. 2017, 2020, Bulteel et al. 2021), antibiotics (Callens et al. 2018, Motiei et al. 2020), temperature (Sullam et al. 2018, Frankel-Bricker et al. 2020) and cyanobacterial exposure (Macke et al. 2017). So far, little is known about the dynamics of

110 bacterial colonization within the *Daphnia* gut. Mushegian et al. (2018) suggested that *Daphnia*
111 functioning is largely determined by environmental bacteria, suggesting a strong role of
112 horizontally transmitted symbionts. Callens et al. (2020) showed that exogenous exposure to
113 different environmental pools of bacteria, resulted in different gut microbial communities,
114 reflected in both community composition and community structure. These results show an
115 important role of the bacterioplankton community in structuring the gut microbial community in
116 *Daphnia*. During the colonization process of these horizontally transmitted strains, attachment to
117 the gut epithelium seems crucial as *Daphnia* has a peritrophal matrix (PTM), which makes it ideal
118 for microbiota to establish in the gut epithelium. Throughout the colonization process, different
119 competitive processes, besides an initial priority effect, can influence the bacterial community.
120 Besides competition between bacterial strains, it is suggested that the host can select for certain
121 strains, such as studied in Macke et al. (2017) and in Houwenhuyse et al. (2021). As *D. magna* is a
122 well-established and key study system, many studies have been performed on this model
123 organism, but mostly under laboratory conditions. The bacterioplankton community under
124 laboratory conditions, however, differs from communities in natural conditions, among which a
125 reduced species richness in the laboratory communities (Callens et al. 2020). Similar studies on
126 fish and mice have shown that the gut microbiome from hosts in laboratory conditions are to some
127 extent the same, but also differ from its free-roaming counterpart under natural conditions, which
128 may modulate a different response to environmental stress (Roeselers et al. 2011, Adamovsky et
129 al. 2018, Rosshart et al. 2017, 2019).

130 In general, exposure to different bacterial environments could impact the strength and specifity
131 of stressor responses (e.g. host-parasite: Wolinska and King 2009). Host organisms under

132 laboratory conditions encounter fewer microbes compared with their free-roaming counterparts,
133 which could ultimately be reflected in a (1) less diverse or (2) less adapted laboratory host
134 microbiome. Previously, it has been shown that invasion of pathogens decreases when soil
135 bacterial diversity is high (van Elsas et al. 2012). Booth (2002) has also shown that bacterial
136 heterogeneity could aid in the survival of a bacterial host, whereby a small fraction of the bacterial
137 population would be able to survive the exposure to single or multiple stressors that kill the
138 majority of the population. These studies indicate that high bacterial diversity is a codetermining
139 factor in protecting the host against single or multiple stressors. In addition, when encountering
140 a smaller pool of available bacterioplankton, the host system could encounter fewer opportunities
141 to selection certain strains and as such obtain a less adapted host microbial community. As the
142 host microbiome plays a crucial role in immune responses, exogenous exposure to laboratory
143 microbiota could potentially not mirror expected tolerances (i.e. the ability to limit negative
144 impact of a given stressor) as occurring in natural populations (Greyson-Gaito et al. 2020). With
145 this experiment we aim to investigate the response of *D. magna* individuals to single or multiple
146 stressors when exposed to different exogenous microbial inocula. Individuals, inoculated with
147 either a natural or a laboratory microbial community, were exposed to one of the four stressor
148 treatments; the toxic cyanobacterium *M. aeruginosa* (further referred to as cyanobacterium or C),
149 infection with the fungus *Aspergillus aculeatus* (further referred to as fungus or F) , the combination
150 of both *M. aeruginosa* and the infection (further referred to as combination or F+C), and a control
151 treatment (fed with only *Chlorella vulgaris* instead of a mixture of *C. vulgaris* and *M. aeruginosa* and
152 no exposure to the infection, further referred to as control or CTL).



153 Firstly, we are interested in the impact of all stressor treatments on *Daphnia* tolerance. We expect
154 that both single stressor treatments will have a negative impact on the measured life history traits
155 compared with the control treatment. In addition, we expect an antagontistic interaction for
156 survival within the multiple stressor treatment (as described in Boudry et al. 2020), i.e. a higher
157 tolerance in *Daphnia* when exposed to both stressors simultaneously compared with *Daphnia*
158 exposed to only one stressor. Secondly, we are interested in the impact of the microbial exposure
159 on *Daphnia* tolerance when comparing the stressor treatments. We hypothesize that tolerance in
160 *Daphnia* is microbiome-mediated, i.e. *Daphnia* individuals receiving the natural microbial
161 inoculum will have a higher tolerance to particular stressors (i.e., have a higher survival, fecundity
162 and body size) compared with individuals that receive a laboratory microbial community. We
163 expect to see this increase in tolerance in both the single as the multiple stressor treatments. We
164 assume that as natural bacterioplankton communities are generally associated with a more
165 diverse microbial community (e.g. Rosshart et al. 2017, Callens et al. 2020), they will provide a
166 broader pool of microbiota for the host to select beneficial strains from. We hypothesize that this
167 will be reflected in (1) a more diverse gut host community and/or (2) the presence of particular
168 selected strains in the *Daphnia* receiving the natural inoculum compared with the lab inoculum.
169 Thirdly, we include the role of the host genotype as previous research has revealed a strong
170 genotype-effect on the gut microbial community and genotype x microbiome interactions with
171 respect to stress tolerance (Macke et al. 2017, 2020, Callens et al. 2020, Massol et al. 2020, Bulteel
172 et al. 2021, Houwenhuyse et al. 2021), so we expect intraspecific differences within *D. magna*
173 responses to the stressors under the different exogenous microbial exposures

174

175 **Materials and methods**

176 ***Daphnia* culturing**

177 To investigate the genotype effect, we used three different *D. magna* genotypes: KNO 15.04, OM2
178 11.3 and T8. The KNO 15.04 genotype (further referred to as KNO) was isolated from a small pond
179 (350m²) in Knokke, at the Belgian coast (51°20'05.62"N, 03°20'53.63"E) and is the same clone as
180 used in Macke et al. (2017, 2020). The OM2 11.3 genotype (further referred to as OM2) was isolated
181 from a 3.7 ha inland pond located in Heverlee, in Belgium (50°51'45.0"N, 04°42'58.8"E) and was
182 part of the clone set of Decaestecker et al. (2007). The T8 genotype was isolated from an 8.7 ha
183 shallow, manmade pond, located in Oud Heverlee, Belgium (50°50'24.0"N, 04°39'40.4"E) and was
184 part of the clone set of Cousyn et al. (2001). All clonal lineages were established from resting eggs,
185 isolated from the lake sediment. Two months before the start of the experiment, three independent
186 iso-female lines for each genotype were cultured in separate jars for at least two generations to
187 control for maternal effects. These iso-female lines were kept in a mixture of filtered tap and pond
188 water in a 9:1 ratio and fed every other day with a saturating amount of *C. vulgaris*. Medium
189 (filtered tap water) was refreshed once per week at a temperature of 19 ± 1°C and under a 16:8h
190 light:dark cycle in 2L glass jars (at a density of 20 individuals/L). They were fed three times per
191 week with saturating amounts of the green algae *C. vulgaris*. The first brood of the second
192 generation was discarded, whereas eggs from the second brood were collected to obtain axenic
193 (i.e. germ-free) juveniles following protocol from Bulteel et al. (2021) and Houwenhuyse et al.
194 (2021).

195 **Algae culturing**

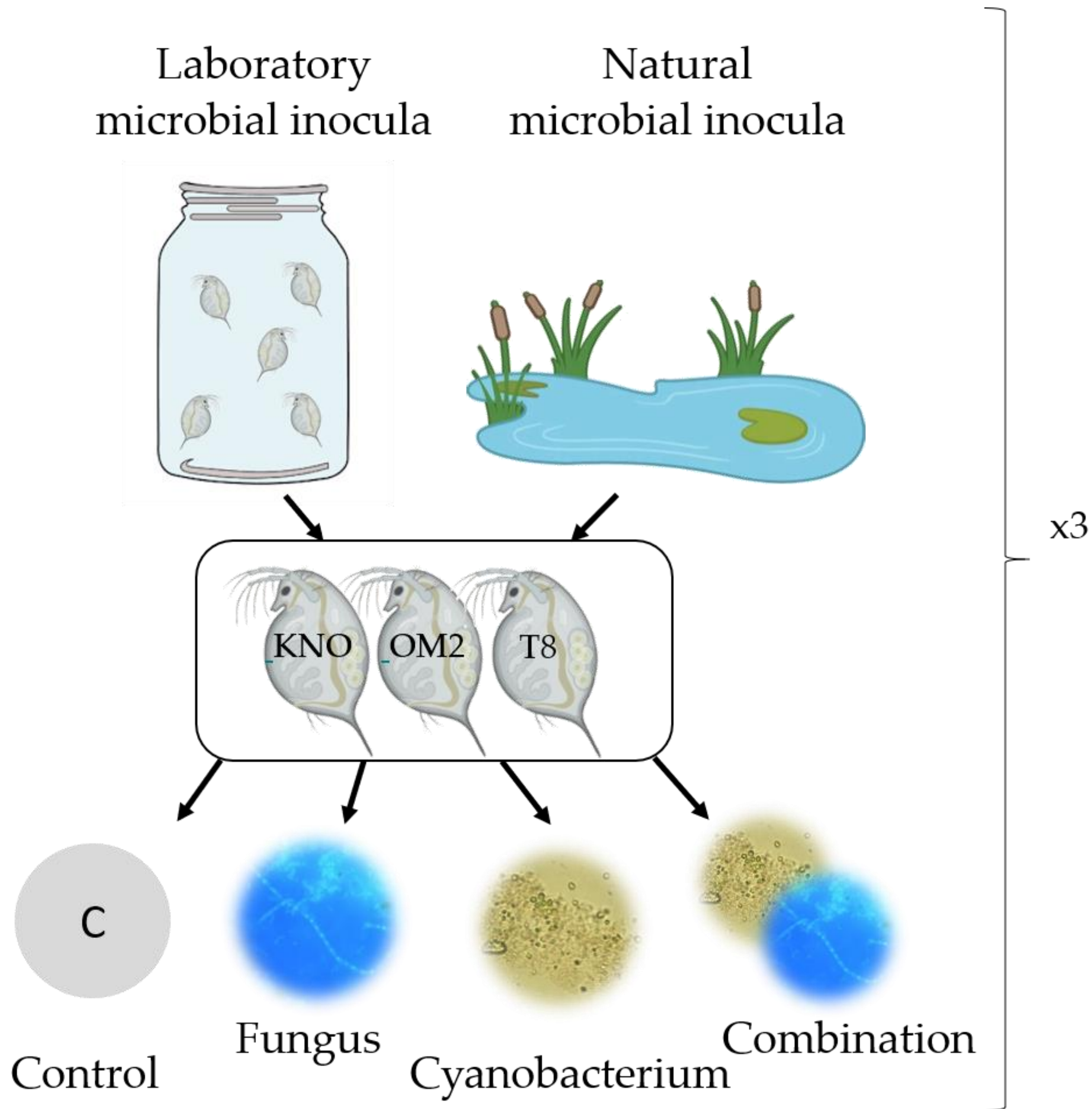
196 *Daphnia* were fed with *C. vulgaris* (strain SAG 211-11 B), which is considered as standard good-
197 quality food for *Daphnia* (Munirasu et al. 2016). One of the stressors used in this experiment is the
198 toxic cyanobacterial strain *M. aeruginosa* (strain PCC 7806), isolated from the Braakman reservoir
199 in the Netherlands (51°19′22″N, 3°44′16″E) and part of the Culture Collections at Institute Pasteur
200 (Paris, France). *Chlorella vulgaris* and *M. aeruginosa* were grown in WC medium (i.e. Wright's
201 Cryptophyte medium) and modified WC medium (without Tris) respectively. The algae were
202 cultured under sterile conditions in a climate chamber at 22 ± 1°C with a light:dark cycle of 16:8h
203 in 2L glass bottles, with constant stirring and aeration. Filters (0.22 μm) were placed at the input
204 and output of the aeration system to avoid any bacterial contamination. The algae were weekly
205 harvested in the stationary phase. The axenity of the algal cultures was checked by sequencing
206 and plating on LB- and R2A-plates.

207 **Experimental design**

208 With this experiment we aimed to investigate the impact of a natural versus a laboratory
209 microbiome on the tolerance of *D. magna* individuals when exposed to two different stressors in
210 single and combined exposures (Figure 1). Individuals, inoculated with either a natural or a
211 laboratory microbial community, were exposed to one of the four following stressor treatments:
212 an opportunistic fungus (characterized as *Aspergillus aculeatus*, further referred to as fungus or F,
213 Figure S1A), a toxic cyanobacterium *M. aeruginosa (*further referred to as cyanobacterium or C,
214 Figure S1B), the combination of both the cyanobacterium and the fungus (further referred to as
215 combination or F+C), and a control treatment (fed with *C. vulgaris* instead of the cyanobacterium
216 and no exposure to the fungus, further referred to as control or CTL). Each multifactorial

217 combination of stressor treatment, microbiome treatment and genotype was replicated

218 independently three times (independent iso-female lines).



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220 Figure 1: Experimental design. Axenic *Daphnia* individuals from three genotypes were exposed to

221 a natural or laboratory microbial community (microbiome treatment). *Daphnia* individuals

222 receiving a natural microbial community were exposed to 10 µm filtered pond water. *Daphnia*

223 individuals receiving a laboratory microbial community were exposed to 10 µm filtered tap water

224 originating from *Daphnia* stock cultures. All *Daphnia* individuals were then exposed to one of the

225 four different stressor treatments: control, fungus, cyanobacterium or combination. The
226 experiment was performed in triplicate for each treatment and factor combination.

227 **Microbial inocula**

228 All *Daphnia* individuals received either a natural or a lab microbial inoculum at the start of the
229 experiment. Each microbial inoculum consist of a water sample which encloses a microbial
230 community. All water samples were subsequently filtered over 100 μm and 10 μm to remove
231 debris. The natural microbial inocula were sampled from three local natural ponds from Kortrijk
232 (replicate 1 received bacterioplankton from the Kennedy pond (50°48'05.7"N 3°16'33.0"E),
233 replicate 2 received bacterioplankton from the Marionetten pond (50°47'43.5"N 3°15'00.2"E), and
234 replicate 3 received bacterioplankton from the Kulak pond (50°48'30.8"N 3°17'37.0"E)). The
235 laboratory microbial inocula, on the other hand, were sampled from the medium from three
236 different genotypes, which were cultured in the lab (replicate 1 received bacterioplankton from
237 the culture medium of M5 genotype, replicate 2 received tbacterioplankton from the culture
238 medium of theT7 genotype, and replicate 3 received bacterioplankton from the culture medium
239 of the ZWE 2B genotype). In this manner, we were able to mimic bacterioplankton communities
240 under natural (high bacterial diversity) and laboratory (low bacterial diversity) conditions. Each
241 experimental replicate received one of the three natural or laboratory microbial inocula.

242 **Stressor systems**

243 The opportunistic fungus used in this experiment was characterized as the fungus *Aspergillus*
244 *aculeatus* (see below for information on the characterization, Figure S1A). The *Aspergillus* genus is
245 generally associated with aspergillosis in humans, but also various wild and domestic animals
246 (Seyedmousavi et al. 2015), risk allergic responses or a fatal infection by inhaling *Aspergillus* spores

247 which generally infect the lungs. *Aspergillus aculeatus* is not generally associated as a causative
248 agent of aspergillosis, but some literature does suggest the species has pathogenic properties in
249 humans (e.g. Williams et al. 1984), but also in plants (e.g. Tanapichatsakul et al. 2020). No
250 description of a *Aspergillus* infection in *Daphnia* has been described in the literature to our
251 knowledge. However, as the *Aspergillus* genus is known as an opportunistic fungus and *Daphnia*
252 is prone to fungal infections (personal observations), it is possible that *Aspergillus* species could
253 also potentially affect *Daphnia*. Infection with the fungus, used and characterized in this
254 experiment, caused high mortality and reduced fecundity upon *Daphnia* individuals in the
255 laboratory before, especially in (germ-free) juveniles and during upscaling processes. Infection
256 with *A. aculeatus* was also visible by the presence of long, septated hyphae in biofilms on the wall
257 of the culture jar, on the medium surface, but also in dead individuals in cultures with high
258 infection rates. Infection with *A. aculeatus* in *Daphnia* also appears genotype specific (based on
259 visual inspections and experience by the authors) as exposure to the fungus resulted in a
260 differential respons for survival and fecundity between genotypes.

261 The cyanobacterium used in this experiment was the toxic cyanobacterial strain *M. aeruginosa*
262 (strain PCC 7806, Figure S1B), isolated from the Braakman reservoir in the Netherlands
263 (51°19′22″N, 3°44′16″E) and part of the Culture Collections at Institute Pasteur (Paris, France).
264 Cyanobacteria are generally accepted as poor food reducing zooplankton fitness. *Microcystis*
265 *aeruginosa* is a colonial cyanobacterium that produces toxic metabolites such as microcystins.
266 Many studies can be found that investigate the negative effects of *M. aeruginosa* on *Daphnia*. One
267 study showed that *M. aeruginosa* blooms could strongly inhibit the population growth of *D. magna*
268 through depression of survival, individual growth and gross fecundity (Liu et al. 2011). Another

269 study showed that *M. aeruginosa* negatively affected the survival, development and reproduction
270 of *Daphnia* (Huang et al. 2020). In addition, the effects of *M. aeruginosa* on *D. magna* are genotype
271 dependent (as the *A. aculeatus* infection), more specifically, the host genotype dependent gut
272 microbiome drives *D. magna* tolerance to *M. aeruginosa,* as shown by Macke et al. (2017).

273 **Stressor treatments**

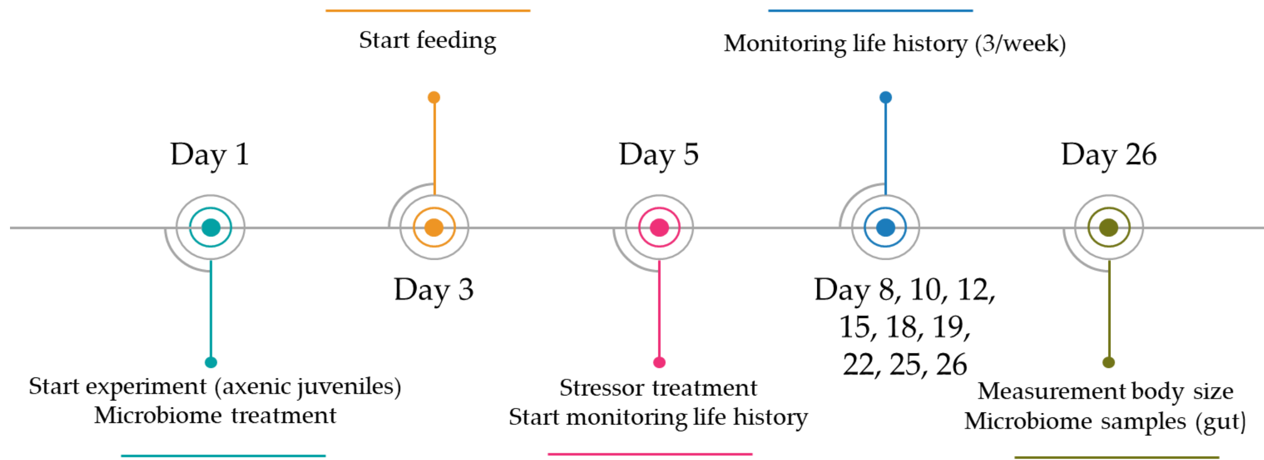
274 Individuals in the control treatment were not exposed to any stressor and were fed with *C. vulgaris*
275 from day 3 onwards. Individuals in the fungus treatment received a spore solution of *A. aculeatus*.
276 The spore solution was obtained by squashing infected *Daphnia* individuals and was administered
277 in a 1:3 ratio (1 infected individual per 3 to infect individuals). We assume little impact from the
278 small bacterial community associated with the spore solution as administered volume is low and
279 as administration occurred after the colonization of the microbial inocula (Vass and Langenheder
280 2017, Callens et al. 2020). Samples of the spore solution were sequenced to correct for
281 contamination if necessary. Individuals in the fungus treatment also received *C. vulgaris* as a food
282 source from day 3 onwards. Individuals in the cyanobacterium treatment received a mixture of
283 the toxic cyanobacterial strain *M. aeruginosa* and the non-toxic *C. vulgaris* in a 50:50 ratio on a daily
284 base from day 5 onwards. Before the start of the stressor treatment (day 3 and 4), cyanobacterium-
285 stressed individuals were fed with 100% *C. vulgaris*. Individuals in the combination treatment
286 received both the spore solution on day 5 and the combination of the toxic *M. aeruginosa* and the
287 non-toxic *C. vulgaris* in a 50:50 ratio from day 5 onwards. Similarly as in cyanobacterium-stressed
288 individuals, combination-stressed individuals were fed with 100% C. vulgaris on day 3 and 4
289 (before the stressor treatment).

290 **Execution of the experiment**

291 Axenic juveniles (0-1 day old) were individually placed in a closed vial filled with 18 mL sterile
292 filtered tap water and 2 mL of the corresponding microbiome treatment (natural or laboratory
293 microbial community). After receiving the corresponding microbial inoculum, the individuals
294 remained in these conditions for 48h, allowing for the microbiota to colonize the *Daphnia* guts. On
295 the third day, all individuals were fed with *C. vulgaris* ($100*10^3$ cells/mL). On the fifth day,
296 individuals were exposed to their corresponding stressor treatment (Figure 1 and 2). After being
297 exposed to their corresponding stressor treatment, the medium volume in the falcon tubes was
298 gradually increased to 50 mL by adding 10 mL of sterile filtered tap water per day, and this for
299 three consecutive days (day 6-8). Food concentration in the first 6 days was low ($100*10^3$ cells/mL)
300 to ensure a sufficient stress response. From day 7 onwards, food concentration was increased to
301 $200*10^3$ cells/mL. All individuals were monitored for survival and reproduction for 21 days. At
302 the end of the experiment (day 21), the body size was measured according to Telesh et al. (2009)
303 (from top of the head to the base of the tail) and guts were dissected and collected per treatment
304 in an Eppendorf tube filled with 10 µL of sterile MilliQ. Recipient guts were pooled per unique
305 combination (4 stressor treatments x 2 microbiome treatments x 3 genotypes x 3 replicates, number
306 of individuals per unique combination can be found in table S9). To characterize the gut microbial
307 communities from collected *Daphnia* guts, the guts of the surviving *Daphnia* per replicate were
308 dissected under a stereo-microscope with sterile dissection needles at the end of the experiment
309 and pooled per replicate (mean= 7.236 guts/sample; sd= 1.872 guts/sample; min= 2 guts; max= 10
310 guts; Table S6). Samples were stored under -20°C until further processing. In addition, samples of

311 the donor microbial inocula (n=6) were collected to compare bacterial communities. An overview

312 of the time line can be found in Figure 2.



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315 Figure 2: Timeline of the experiment with an overview of the essential steps, among which the

316 microbiome treatment (inoculation microbial inoculum), the stressor treatment (start exposure to

317 the corresponding stressor treatment; control, fungus, cyanobacterium or combination), life

318 history trait monitoring (three times per week) and microbiome sampling (dissection of the guts

319 of the surviving *Daphnia* individuals in order to analyze the gut microbial communities).

320 **Library preparation and sequencing**

321 DNA was extracted using a PowerSoil DNA isolation kit (MO BIO laboratories, Carlsbad, CA,

322 USA). DNA was dissolved in 20 µL milliQ water. Because of initially low bacterial DNA

323 concentrations in some samples, a nested PCR was applied to increase specificity and amplicon

324 yield. The full-length 16S rRNA gene was first amplified with EUB8F and 1492R primers on 10 ng

325 of template using a high-fidelity SuperFi polymerase (Thermofisher, Merelbeke, Belgium) for 30

326 cycles: 98°C – 10 s; 50°C – 45 s; 72°C – 30 s. PCR products were subsequently purified using the

327 CleanPCR kit (Qiagen, Antwerp, Belgium). To obtain dual-index amplicons of the V4 region, a

328 second amplification was performed on 5 μL (=20-50 ng) of PCR product using 515F and 806R
329 primers for 30 cycles: 98°C – 10 s; 50°C – 45 s; 72°C – 30 s. Both primers contained an Illumina
330 adapter and an 8-nucleotide (nt) barcode at the 5'-end. For each sample, PCRs were performed in
331 triplicate. Afterwards the PCR products were pooled and a small volume (5 μL) was loaded on a
332 gel to check if the PCR amplified the correct fragment. The remaining volume of the PCR products
333 were purified using the CleanPCR kit (Qiagen, Antwerp, Belgium). An equimolar library was
334 prepared by normalizing amplicon concentrations with a SequalPrep Normalization Plate
335 (Applied Biosystems, Geel, Belgium) and subsequent pooling. Amplicons were sequenced using
336 a v2 PE500 kit with custom primers on the Illumina Miseq platform (KU Leuven Genomics Core),
337 producing 2 x 250-nt paired-end reads. This way, 72 gut samples were generated representing 4
338 stressors x 2 microbiome inocula x 3 genotypes x 3 replicates. In addition, samples of the
339 microbiome inocula (n=6), the stressor treatments (n=2) and *C. vulgaris* (n=1) were sequenced.

340 **Life history traits data**

341 To explore tolerance of the *Daphnia* individuals to the different stressor treatments, we analyzed
342 survival, fecundity and body size. Survival was analyzed using a log-rank or Mantel-Haenszel
343 test. The survival times of individuals that were still alive at the end of the 21 day experiment
344 were coded as right-censored. Normality and skewness of body size and fecundity data were
345 examined with Shapiro–Wilk test and ggqqplot function (package ggpubr to make quantile-
346 quentile plots). For fecundity and body size, we used the Akaike information criterion (AIC) to
347 select the best subset of variables to represent the best model. We first evaluated to include
348 maternal line as a random factor (with a linear mixed-effect model) or not (with a general linear

349 model). Secondly, we tested the significance of the fixed factors in the model with the best random
350 effects factor according to the AIC. Type II ANOVA tables for fixed-effect terms with Satterhwaite
351 and Kenward-Roger methods for dominator degrees of freedom for F-tests and p-values were
352 created (Anova function of the car package). Following the AIC criterium, a linear mixed-effect
353 model was chosen to evaluate fecundity and body size. In the final model, we included
354 microbiome treatment, stressor treatment and genotype as fixed factors, and maternal line as
355 random effect. We also included all possible interactions. Post hoc analysis were performed using
356 the 'emmeans' function with a 'Tukey' adjustment from the emmeans R package. All statistical
357 tests were performed in R 4.0.2 (R Core Team 2020).

358 ***Daphnia* microbiome data**

359 To determine if the assumed microbiome-mediated tolerance could be reflected in the gut
360 microbial community of the *Daphnia* individuals, we analyzed the collected microbial DNA
361 sequences. DNA sequences were processed following Callahan et al. (2016a). Sequences were
362 trimmed (the first 10 nucleotides and from position 180 onwards) and filtered (maximum of 2
363 expected errors per read) on paired ends jointly. Sequence variants were inferred using the high-
364 resolution DADA2 method, which relies on a parameterized model of substitution errors to
365 distinguish sequencing errors from real biological variation (Callahan et al. 2016b). Chimeras were
366 subsequently removed from the data set. Taxonomy was assigned with a naïve Bayesian classifier
367 using the SILVA v132 training set. OTUs with no taxonomic assignment at the phylum level or
368 which were assigned as "chloroplast" or "cyanobacteria" were removed from the data set. After
369 filtering, a total of 3 552 490 reads were obtained with on average 39 038.35 reads per sample, with

370 most samples having more than 1000 reads. To visualize the bacterial families that differed
371 between the treatments, OTUs were grouped at the order level, and orders representing <1% of
372 the reads were discarded. Measures for α-diversity of the recipient gut microbial communities
373 within the different treatments (OTU richness) were calculated using the vegan package in R
374 following Borcard et al. (2011). All samples were rarified to a depth of 1000 reads, based on
375 rarefaction curves (Figure S2), before analyzing α-diversity. The effects of sample type (donor
376 bacterioplankton or recipient) stressor treatment (fugus, cyanobacterium, combination and
377 control), microbiome treatment (lab and natural), genotype (KNO, OM2 and T8), and all possible
378 interactions on OTU richness were assessed through a generalized linear model (GLM), assuming
379 a Poisson distribution of the data and corrected for overdispersion. Maternal line was not included
380 as a random factor as AIC criterium indicated that the model without inclusion of the maternal
381 line was a better predictive model of the data. After testing the full model, we used the AIC
382 criterium to select the best subset of variables to represent the best model. Pairwise comparisons
383 among significant variables and their interactions were performed by contrasting least-squares
384 means with Tukey adjustment. To examine differences in gut microbial community composition
385 (β-diversity) among samples, a Bray-Curtis dissimilarity matrix was calculated and plotted using
386 principal coordinates analysis with the phyloseq package in R. Multivariate community responses
387 to treatments and genotype were investigated by means of Principal Coordinates Analysis. The
388 effect of the stressor treatment, microbiome treatment, genotype, and all possible interactions on
389 β-diversity were assessed through a permutation MANOVA, using the Adonis2 function in the
390 vegan package in R. Obtained p-values were adjusted for multiple comparisons through the
391 control of the false discovery rate (FDR). Pearson correlations were executed between the number

392 of sequenced guts and the OTU richness to check for interdependence. Stressor treatment,
393 microbiome treatment, genotype, all two-way interactions, and the three-way interaction, all
394 showed no significant correlation, dismissing the issue of interdependence (Table S7).
395 Additionally, correlation tests were executed between the different life history traits and the OTU
396 richness of the gut microbial communities. Correlation coefficients and p-values were calculated
397 for all examined correlations. Obtained p-values were adjusted for multiple comparisons through
398 the control of the false discovery rate (FDR). To identify which bacterial classes significantly
399 differed between the main effects and the interaction effects, relative abundances per order were
400 calculated on the raw sequencing data, excluding the samples removed from the rarefaction.
401 Based on OTU presence, Union plots were created using the wilkox/unionplot function from
402 GitHub, to show the unique and shared OTUs within and between the stressor x microbiome
403 interaction. Unionplots are a visual representation of the present OTUs in a group of samples and
404 show which OTUs are unique or shared between three groups. The uptake of bacteria by the
405 recipient *Daphnia* from the donor bacterioplankton, was also analysed with Unionplots (Results
406 see supplementary information S1, Figure S3). Additionally, differential abundance analyses were
407 performed (edgeR function) on the raw sequencing data from which samples with less than 2
408 counts per million (CPM) in at least three samples were filtered out. All statistical tests were
409 performed in R 4.0.2 (R Core Team 2020).

410 ***Characterization of the fungus***

411 To characterize the fungal strain causing the infection in this experiment, samples of infected
412 *Daphnia* with visible signs of the fungal infection and *Daphnia* with no visible infections were

413 compared. Fifteen infected animals were transferred in whole per five individuals in a sterile
414 Eppendorf tube. Guts from 60 infected animals were dissected and transferred per 20 guts to 10
415 µl of sterile MilliQ water. Samples were stored under -20°C until further processing. DNA of all
416 samples was extracted using a PowerSoil DNA isolation kit (MO BIO laboratories). The total DNA
417 yield was determined using a Qubit dsDNA HS assay (Invitrogen) on 1 µL of sample. A PCR
418 reaction was run using a combination of primers for the large subunit (LSU) and small subunit
419 (SSU) region (see Table S9, White et al. 1990, Vilgalys and Sun 1994) on all of the template (98°C –
420 30s, 30 cycles of 98°C – 10s, 55°C – 45s, 72°C – 30s, and 72°C – 5s, 12°C hold) using the Platinum
421 SuperFi DNA polymerase (Thermofisher). PCR products were subsequently purified using the
422 QIAquick PCR purification kit (Qiagen) and were sent for Sanger sequencing to LGC Genomics
423 (Berlin, Germany). The sequences were first converted into consensus sequences using R (package
424 BioCManager). As little similarity was obtained to develop the consensus sequences, non-
425 consensus fasta files were used. The Basic Local Alignment Search Tool (BLAST), BLASTn was
426 performed on the non-consensus fasta files, using FungiDB (Basenko et al. 2018). All query
427 sequences were blasted with all the fungal species present in the database, including oomycetes.
428 The Expectation value (E-value, expected number of hits) was set as 50% of the length of the query
429 sequence. Maximum descriptions (number of descriptions/alignment to show) were set to 50 to
430 avoid compromising the e-value and possible sequence matches. Additionally, the low
431 complexity filter mode was set off to avoid omittance of results which contain repetitive and low
432 complexity sequences. Similar settings were performed for all blasted sequences. Obtained results
433 of fungiDB were verified using NCBI, emboss and wasabi. For NCBI the BLASTn protocol was
434 followed (Schoch et al. 2014). To improve the sequence matches with Fungi, BLAST search was

435 limited to RefSeq sequences only (using BioProject Number specific to Fungi, 177353, Schoch et
436 al. 2014). Furthermore, emboss, with the Emboss matcher algorithm, was used to create the
437 pairwise alignment between the sequences using the BLOSUM 62 matrix (Rice et al. 2000). Finally,
438 a reference based multiple sequence alignment was performed to create a multiple sequence
439 alignment table, using PRANK (probabilistic multiple alignment program for DNA) hosted by
440 wasabi using the HKY model (Veidenberg et al. 2015). The results were consistent across all
441 databases (FungiDB, NCBI, emboss and wasabi). After obtaining sequencing results (see Table
442 S8), *Daphnia* with visible or non-visible infection showed the highest match with *Aspergillus*
443 *aculeatus* and *Aspergillus niger*. Multiple sequence alignment further revealed a highly specific
444 match with nucleotides 1 to 1900 for *Aspergillus aculeatus* KV879170 (strain: ATCC 16872, Figure
445 S4). No specific match with *Aspergillus niger* was found in the multiple sequence alignment. Based
446 on these results, we conclude that the fungal infection is related to *Aspergillus aculeatus* ATCC
447 16872.

448

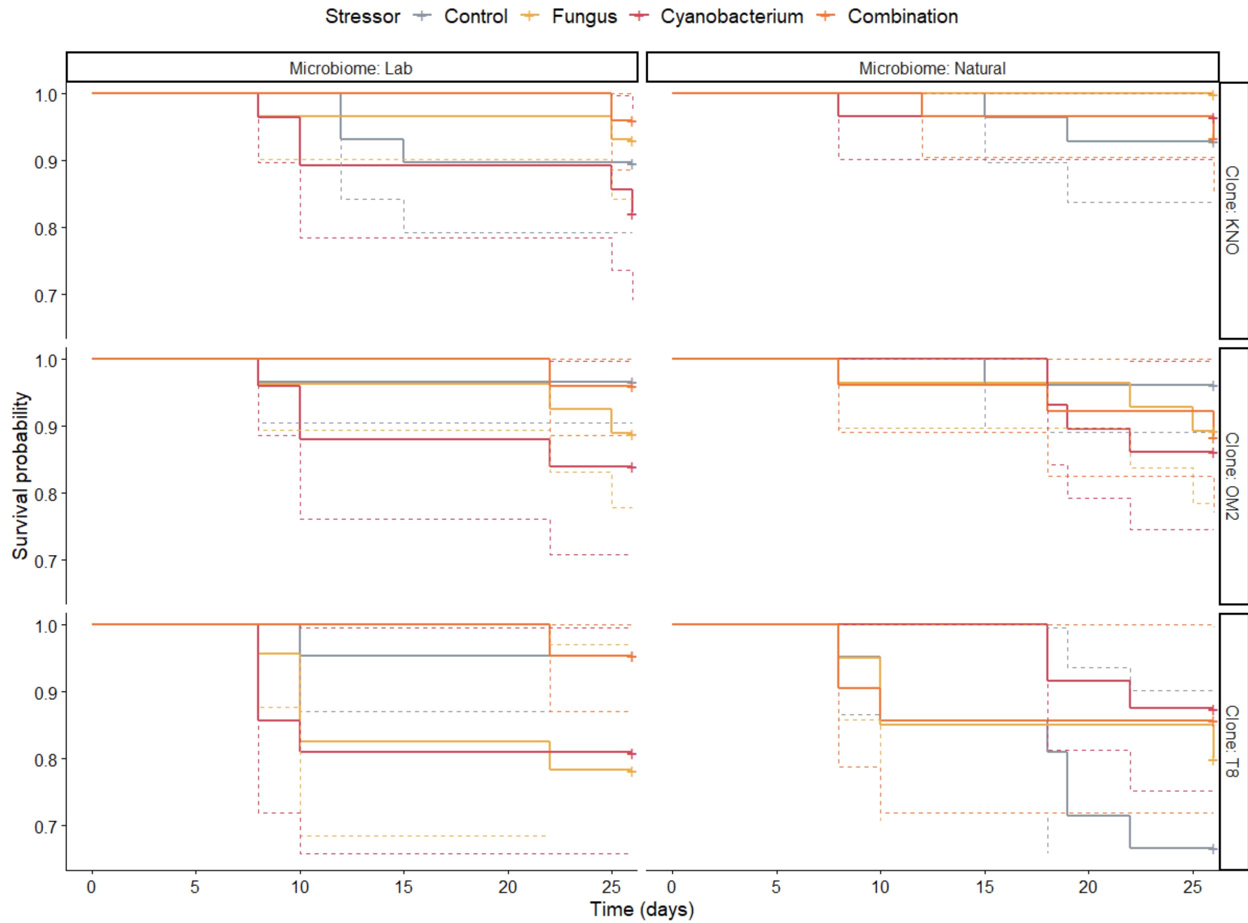
449 **Results**

450 ***Survival***

451 A survival analysis was performed to investigate (1) the impact of stress on *Daphnia* survival, (2)
452 whether this impact was influenced by the microbial environment, and (3) whether these
453 responses were genotype-specific. The survival analysis revealed a main genotype effect,
454 microbiome x genotype interaction, and stressor x microbiome x genotype interaction on *Daphnia*
455 survival. No stressor x microbiome interaction was present. Separate analyses per microbiome

456 treatment, however, revealed a significant main effect of the stressor treatment in *Daphnia*
457 individuals that received a laboratory microbial inoculum (X^2=9.5, df=3, p=0.02), but not in
458 *Daphnia* that received a natural microbial inoculum (X^2=0.8, df=3, p=0.9). In the lab microbial
459 treatment, *Daphnia* that received the cyanobacterium treatment had a significant lower survival
460 than *Daphnia* individuals that received the combination (X^2= 6.9, df=1, p=0.009; Figure S5) and
461 control treatment (X^2=4.9, df=1, p=0.03; Figure S5). Genotype, additionally, determined survival
462 as our analyses revealed a significant stressor x microbiome x genotype interaction (Table 1,
463 Figure 3). When tested separately per genotype, no significant stressor x microbiome interaction
464 was revealed for KNO, OM2 or T8 individuals (Table S1). We did, however, find a significant
465 stressor x genotype interaction in *Daphnia* receiving a natural microbial inoculum (X^2=22, df=11,
466 p=0.02), but not in *Daphnia* receiving a laboratory microbial inoculum (X^2=14, df=11, p=0.2). Within
467 the natural microbial treatment, the survival probability of the T8 individuals was significantly
468 lower compared with KNO individuals (X^2=5.6, df=1, p=0.02; Figure 3) and OM2 individuals
469 (X^2=7, df=1, p=0.008; Figure 3) for the control treatment. For all the other stressor treatments within
470 the natural microbial treatment, no significant differences between the genotypes were observed
471 (Table S1).

472



473

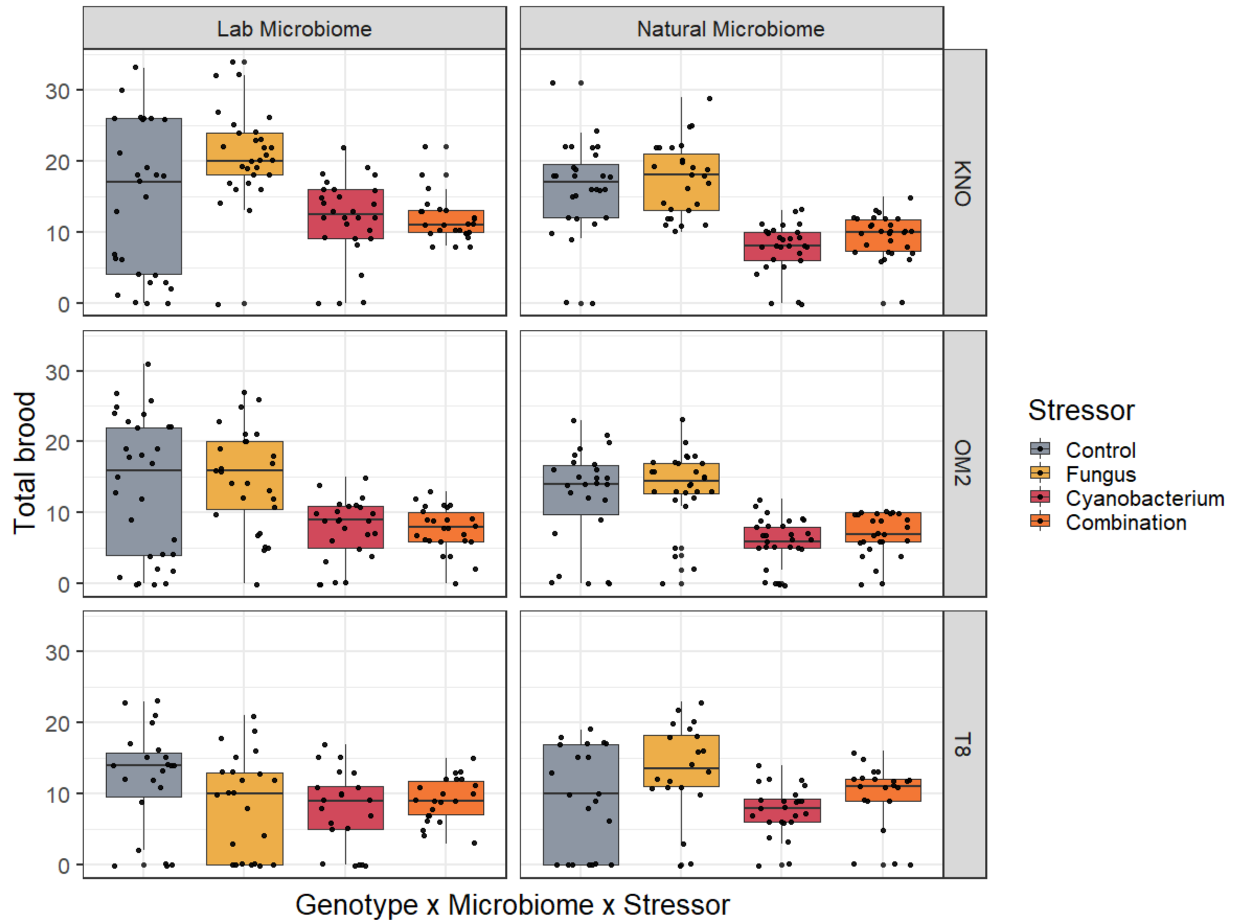
474 Figure 3: Survival plots of recipient *Daphnia* between the stressors treatments for the different
475 microbial inocula (columns) and genotypes (rows). Colors indicate the different stressor
476 treatments.

477

478 ***Total fecundity***

479 Analyses on the total fecundity (measured as the total number of hatched eggs per *Daphnia*
480 individual) were performed to investigate (1) the impact of stress on *Daphnia* reproduction, (2)
481 whether this impact was influenced by the microbial environment, and (3) whether these
482 responses were genotype-specific. The fecundity analysis revealed a significant main effect of the
483 stressor treatment and genotype, a significant stressor x genotype interaction, and stressor x

484 microbiome x genotype interaction on total fecundity (Table 1). Analyses revealed no significant
485 effect of stressor x microbiome on total fecundity (Table 1, Figure S6). Separate analysis per
486 microbiome treatment, revealed a significant main effect of the stressor treatment in *Daphnia*
487 individuals that received a natural ($F=47.36$, $df=3$, $p<0.0001$) and laboratory microbial inoculum
488 ($F=15.53$, $df=3$, $p<0.001$), with total fecundity significantly differing between the control treatment
489 and the cyanobacterium, and control and combination treatment. On average, *Daphnia* had a
490 lower reproduction when they received cyanobacterium (both as a single stressor and in the combi
491 treatment) compared with the control and fungus treatment (Figure S7). Genotype co-determined
492 total fecundity as our analyses revealed a significant stressor x microbiome x genotype interaction
493 (Table 1, Figure 4). The KNO genotype revealed significant differences between the fungus and
494 cyanobacterium treatment, and the fungus and combination treatment within both microbial
495 inocula (Table S1, Figure 4). The OM2 genotype revealed significant differences for fecundity
496 between the control and cyanobacterium, fungus and cyanobacterium, and fungus and
497 combination when exposed to the natural microbial inocula (Table S1, Figure 4). A similar pattern
498 was observed within the laboratory microbial inocula for OM2 with an additional significant
499 difference between the control and combination treatment (Table S1, Figure 4). The T8 genotype
500 revealed no significant differences between the stressor treatments within both microbial inocula
501 (Figure 4).



502

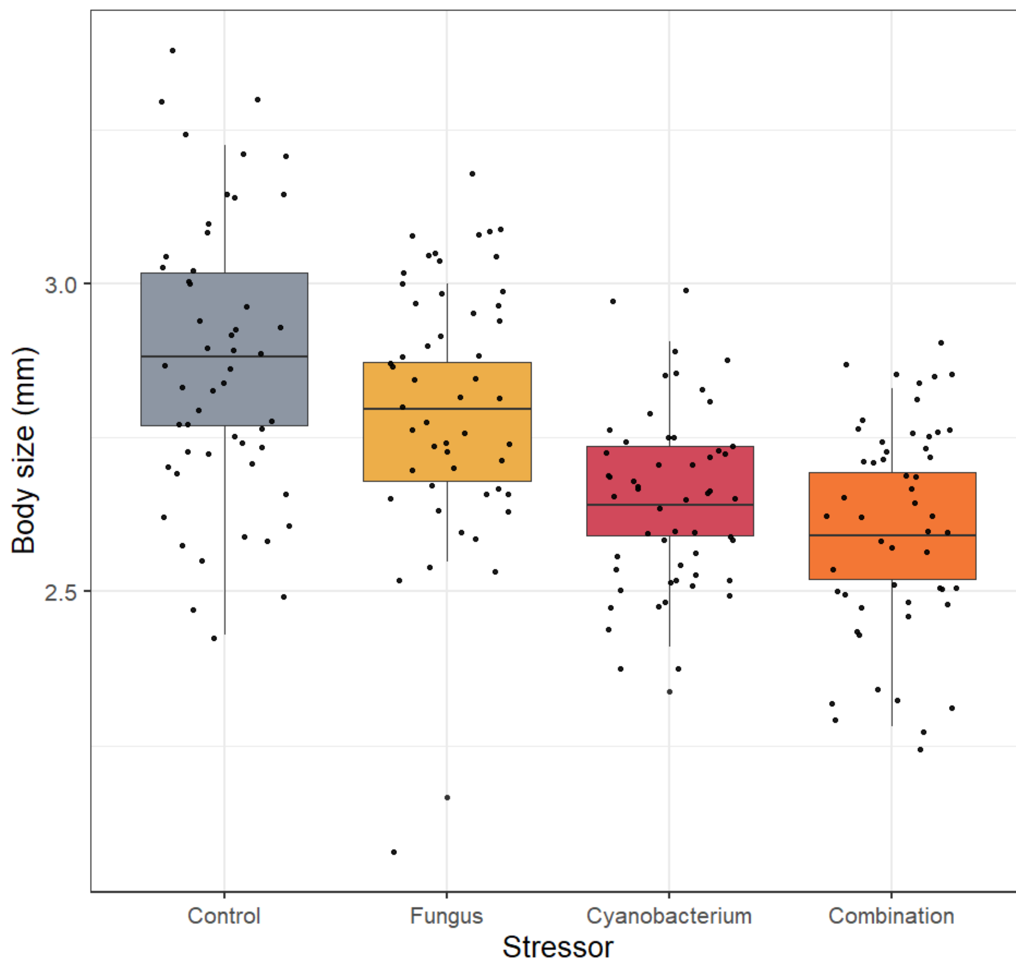
503 Figure 4: Box plots of the total brood for the different stressor treatments for the three-way
504 interaction (stressor x microbiome x genotype). Box plots are given for the two microbiome
505 treatments (columns) and the three genotypes (rows). Colors indicate the different stressor
506 treatments. Black dots represent the individual data points.

507

508 ***Body size***

509 Analyses on body size (measured at the end of the experiment) were performed to investigate (1)
510 the impact of stress on *Daphnia* body size, (2) whether this impact was influenced by the microbial
511 environment, and (3) whether these responses were genotype-specific. Analyses on *Daphnia* body

512 size revealed a significant main effect of the stressor treatment (Table 1, Figure 5). Post hoc
513 analyses showed a significant difference between all stressor treatments, except between the
514 single stressor cyanobacterium and the combination treatment (Table S1). Individuals in the
515 control treatment had the highest body size, followed by, in decreasing order of body size,
516 individuals exposed to the fungus, cyanobacterium and the combination treatment (Figure 5). No
517 impact of microbiome treatment or genotype were detected for *Daphnia* body size (Table 1).



518

519 Figure 5: Box plots of the recipient body size at the end of the experiment per stressor treatment.
520 Colors indicate the different stressor treatments. Black dots represent the individual data points.

521 Table 1: Overview results LMER for life history traits for the recipients and amplicon sequencing for the combination of donor
522 bacterioplankton and recipient *Daphnia*, the donor bacterioplankton separately and the recipient *Daphnia* separately. Sample type refers
523 to the origin of the sample, i.e. donor bacterioplankton or recipient gut *Daphnia*. Significant results (p<0.05) are indicated with *. Highly
524 significant results (p<0.001) are indicated with ***. df = degrees of freedom.

525

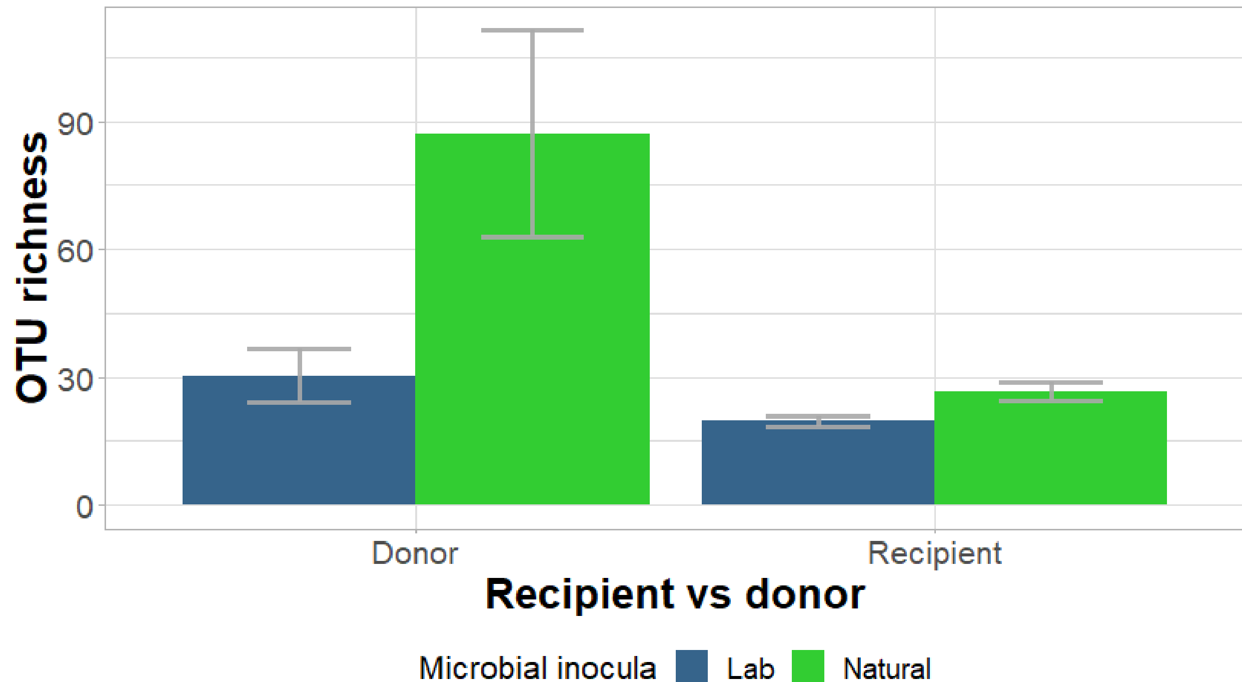
	df	Survival		Fecundity		Body Size		OTU richness		Beta diversity	
		p-value	**Chi²**	**p-value**	**F**	**p-value**	**F**	**p-value**	**res. dev.**	**p-value**	**R2**
Donor bacterioplankton + Recipient *Daphnia*											
Microbiome	1							1.434e-06***	179.85	0.003*	0.081
Sample type	1							1.113e-14***	255.77	0.006*	0.069
Microbiome x Sample type	1							0.001778*	147.94	0.022*	0.042
Donor bacterioplankton											
Microbiome	1							0.007906*	46.797	0.09	0.34698
Recipient *Daphnia*											
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

526 ***Microbial composition***

527 OTU richness

528 The OTU richness of the host gut communities was analyzed to examine whether a possible
529 microbiome-mediated tolerance in *Daphnia* was reflected in a higher OTU richness of the gut
530 microbial community. These analyses also can gives us an indication if this changes is mediated
531 by the stressor or genotype, which could reflect selection of the host. In addition, we included
532 samples on the donor bacterioplankton incoula to confirm whether the natural bacterioplankton
533 inocula and the resulting host gut microbial community exposed to these inocula indeed had a
534 higher OTU richness compared with the lab ones. Analysis of the data set containing both the
535 microbial donor inocula and the recipient gut microbiomes revealed a significant sample type
536 (donor bacterioplankton vs recipient *Daphnia*) x microbiome interaction (Table 1). Post hoc
537 analysis revealed significant differences between all combinations, except between the laboratory
538 donor bacterioplankton and the natural recipient guts(Table S4). In both donor inocula and
539 recipient microbiomes, OTU richness was significantly higher in the natural conditions (donor:
540 mean= 87.000, sd= 42.036, recipient: mean= 26.550, sd= 9.556) compared with the laboratory
541 conditions (donor: mean= 30.333, sd= 10.970, recipient: mean= 19.652, sd= 5.441, Table S4, Figure
542 6). OTU richness was also significantly higher in the donor bacterioplankton (mean= 58.667, sd=
543 41.452) compared with the recipient *Daphnia* (mean= 22.860, sd= 8.303; $p<0.001$, z-value=-12.13,
544 Figure 6). Analysis of the recipient *Daphnia* revealed a significant microbiome effect on OTU
545 richness (Table 1). No stressor, stressor x microbiome interaction or stressor x microbiome x
546 genotype interaction was observed (Table 1). A separate analysis per microbiome treatment did

547 not reveal a significant main effect of the stressor treatment in both *Daphnia* individuals that
548 received a laboratory bacterioplankton (Res. Dev.= 23.174, df=3, p= 0.129) or a natural
549 bacterioplankton (Res. Dev.= 57.756, df=3, p= 0.056).



550

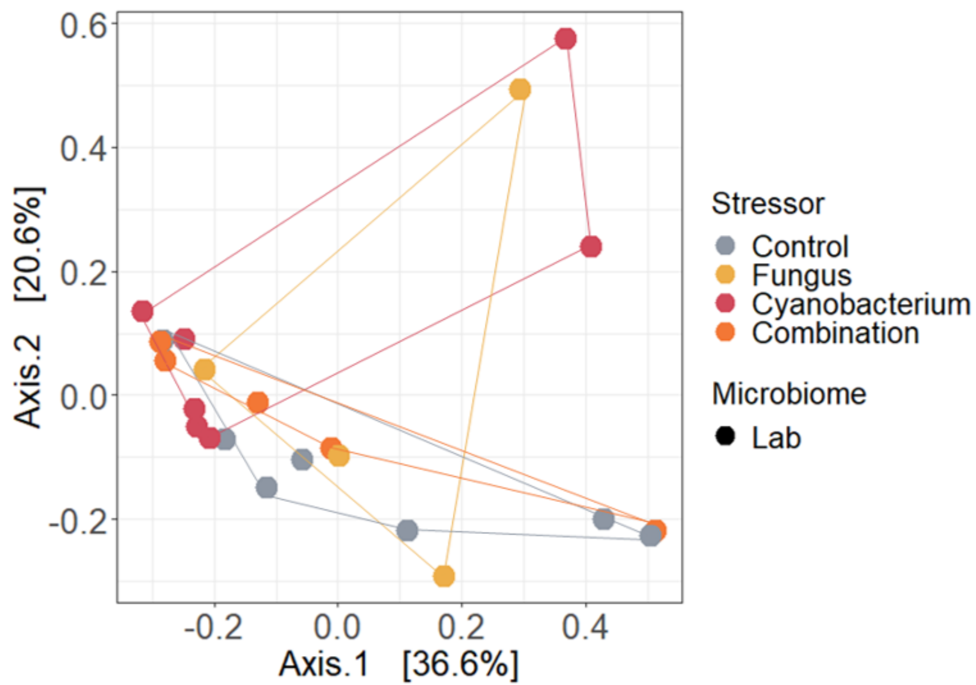
551 Figure 6: Bar plots of OTU richness of donor bacterioplankton and recipient *Daphnia* samples
552 which are grouped per sample type (donor bacterioplankton vs recipient *Daphnia*) and microbial
553 inocula. Colors indicate the different microbial inocula Error bars indicate standard error.

554 <u>Beta diversity</u>

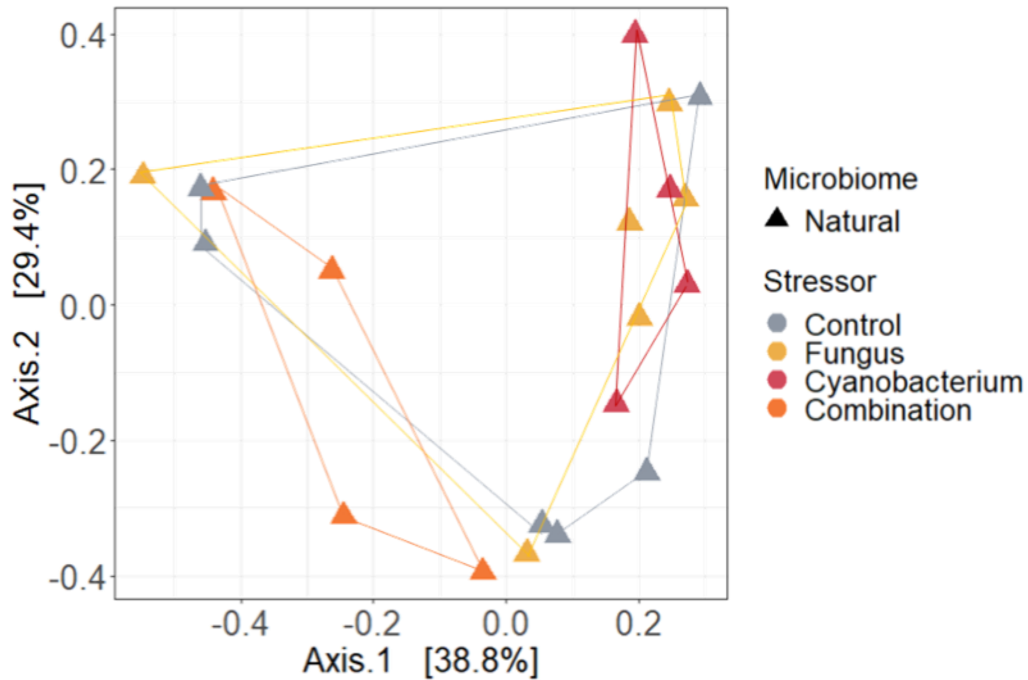
555 The beta diversity of the host gut communities was analyzed to examine whether a possible
556 microbiome-mediated tolerance in *Daphnia* was reflected in a differentially structured gut
557 microbial community. These analyses also can gives us an indication if this changes is mediated
558 by the stressor or genotype, which could reflect selection of the host In addition, we included
559 samples on the donor bacterioplankton incoula to confirm whether the natural bacterioplankton

560 inocula and the resulting host gut microbial community were differentially structured. Analysis
561 on beta diversity of the donor bacterioplankton and recipient *Daphnia* samples revealed a
562 significant sample type x microbiome treatment interaction (Table 1, Figure S8), and a significant
563 main effect of both the sample type and microbiome treatment (Table 1). All pairwise comparisons
564 for the main effects and the interaction effect on the combined data of recipients and donors were
565 significantly different, except for the difference between the laboratory and natural inoculum
566 treatment within the donor bacterioplankton (Table S4). The analyses on beta diversity on the
567 microbial donor inocula separately revealed no significant difference between the different
568 inocula or microbiome treatments (Table 1). Bray-Curtis ordinations, however, demonstrated a
569 complete separation between the natural and laboratory microbial donor inocula, indicating that
570 the bacterial community of the inocula were differently structured (Figure S9A). Analyses on beta
571 diversity on the recipient's gut microbial composition revealed that most of the variation was
572 explained by the microbiome (lab versus natural) treatment (Table 1). The bacterial composition
573 in recipients receiving the natural bacterioplankton differed significantly from those receiving
574 the lab bacterioplankton (Table 1). Stressor, stressor x microbiome interaction and stressor x
575 microbiome x genotype showed no significant contribution to the differences in beta diversity
576 (Table 1). Ordinations based on Bray-Curtis, however, demonstrated an overlap between
577 individuals exposed to natural and laboratory bacterioplankton, indicating that the bacterial
578 community of these communities were similarly structured (Figure S9B). Separate analyses per
579 microbiome treatment did not reveal a significant main effect of the stressor treatment in both
580 *Daphnia* individuals that received a laboratory (R2=0.11379, df=3, p=0.694) or a natural
581 bacterioplankton (R2=0.20147, df=3, p=0.18). Ordinations based on Bray-Curtis for *Daphnia*

582 individuals that received the laboratory bacterioplankton showed an overlap between all stressor
583 treatments (Figure 7A). Ordinations based on Bray-Curtis for *Daphnia* individuals that received
584 the natural bacterioplankton, however, demonstrated a complete separation between the
585 cyanobacterium and combination treatment, both showing small overlap with the control and
586 fungus treatment (Figure 7B) reflecting a specific cyanobacterium associated microbiome.



A



B

587 Figure 7: PCA of the gut microbial communities using weighted Bray-Curtis distance for
588 recipients exposed to (A) the lab microbial inoculation water, and (B) the natural microbial
589 inoculation water. Colors indicate stressor treatment. Symbols indicate microbial inoculum.

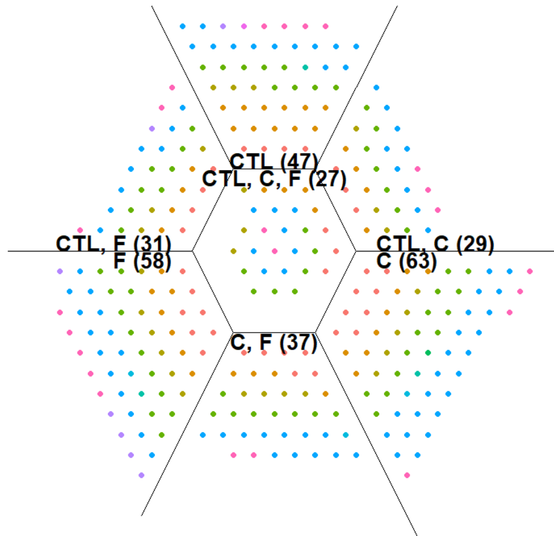
590 Microbial community

591 To examine whether different or specific strains were present between the different treatments,
592 we performed different analyses on the microbial community, such as a general overview of
593 present community, representation of number of unique and shared OTU via unionplots, and the
594 significantly different OTU's between treatment via an EdgeR analysis. In addition, we included
595 the donor bacterioplankton samples. Combined donor bacterioplankton and recipient *Daphnia*
596 microbial communities were dominated by Betaproteobacteriales (mean=46.484%, sd=26.554%),
597 Pseudomonadales (mean=20.005%, sd=23.323%) and Verrucomicrobiales (mean=5.388%,
598 sd=7.092%). Donor bacterioplankton microbial communities, analyzed separately, were

599 dominated by Betaproteobacteriales (mean=33.092%, sd=22.393%), Micrococcales
600 (mean=21.3728%, sd=31.5502%) and Chitinophagales (mean=11.525%, sd=17.676%), whereas
601 recipient *Daphnia* microbial communities were dominated by Betaproteobacteriales
602 (mean=48.397%, sd=26.780%), Pseudomonadales (mean=22.464%, sd=23.944%) and
603 Verrucomicrobiales (mean=5.811%, sd=7.317%, Figure S10). A similar top 3 was observed for all
604 recipient groups, whether they were exposed to the laboratory or natural microbial inoculum.
605 Additionally, the same top 3 was observed for recipient *Daphnia*, indifferently of the stressor
606 treatment, except for *Daphnia* exposed to the control, whereby the third most abundant order was
607 Rhizobiales instead of Verrucomicrobiales (Table S2).

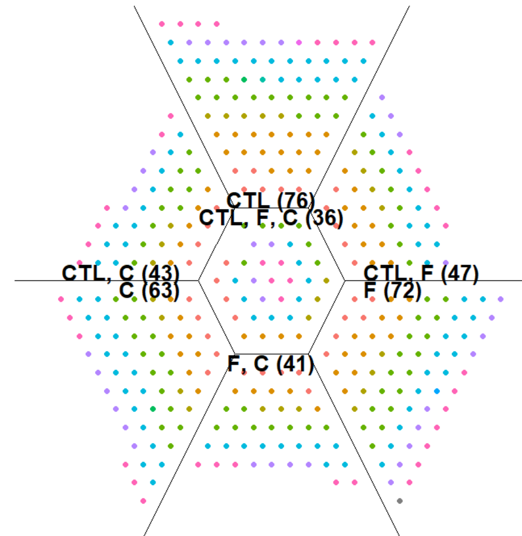
608 To examine whether selection of particular strains in specific stressor treatments was present, we
609 examined the number of unique and shared OTU's across the different microbial treatment and
610 stressor treatments. When comparing the total number of OTUs from *Daphnia* exposed to the
611 control treatment with the single stressor treatments (Figure 8A and 8B), a higher number of OTUs
612 in the single stressor treatments (fungus: 153, cyanobacterium: 156) was observed compared with
613 the control treatment (134) within the laboratory bacterioplankton. The opposite was observed
614 within the natural bacterioplankton, whereby *Daphnia* exposed to the single stressor treatments
615 (fungus: 196, cyanobacterium: 183) had a lower total number of OTUs compared with the control
616 treatment (202). Union plots comparing the single and multiple stressor treatments (Figure 8C
617 and 8D) showed that the total number of OTUs was lower in the combination treatment (lab: 138,
618 natural: 167) compared with the fungus treatment (lab: 154; natural: 186) and the cyanobacterium
619 treatment (lab: 161; natural: 170) for both microbial inocula.

Lab inocula	Natural inocula



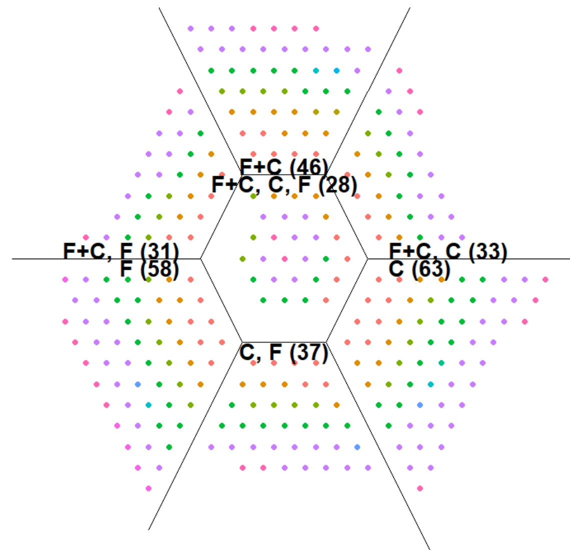
Class
- Actinobacteria
- Alphaproteobacteria
- Bacilli
- Bacteroidia
- Deinococci
- Deltaproteobacteria
- Fusobacteriia
- Gammaproteobacteria
- Planctomycetacia
- Rhodothermia
- Verrucomicrobiae

A



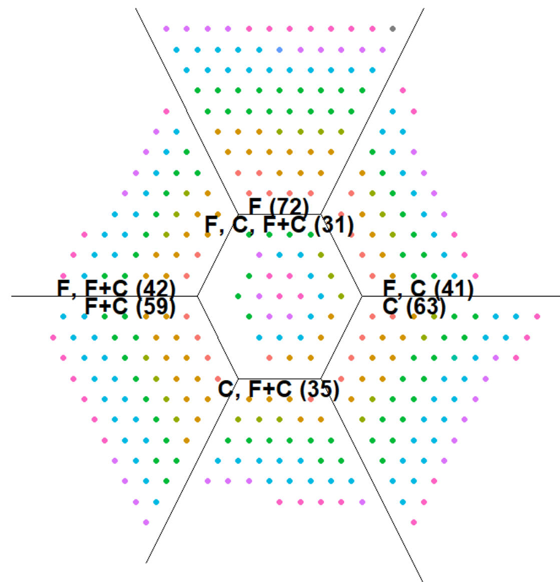
Class
- Actinobacteria
- Alphaproteobacteria
- Bacilli
- Bacteroidia
- Deltaproteobacteria
- Fimbriimonadia
- Gammaproteobacteria
- Negativicutes
- Planctomycetacia
- Thermoleophilia
- Verrucomicrobiae
- NA

B



Class
- Actinobacteria
- Alphaproteobacteria
- Babeliae
- Bacilli
- Bacteroidia
- Deinococci
- Deltaproteobacteria
- Fimbriimonadia
- Fusobacteriia
- Gammaproteobacteria
- Planctomycetacia
- Verrucomicrobiae

C



Class
- Actinobacteria
- Alphaproteobacteria
- Bacilli
- Bacteroidia
- Deltaproteobacteria
- Gammaproteobacteria
- Negativicutes
- Planctomycetacia
- Verrucomicrobiae
- NA

D

620 Figure 8: Union plots representing the OTUs that are unique within and shared between stressor
621 treatments when exposed to the lab (A and C) or natural microbiome bacterioplankton (B and D).
622 OTUs illustrated in A and B are: control (CTL), fungus (F) and cyanobacterium treatment (C).
623 OTUs illustrated in C and D are: fungus (F), cyanobacterium (C) and combination treatment (F+C).
624 Numbers between brackets indicate the total number of OTUs. Colors indicate OTUs grouped per
625 class.

626 Intrigued by the results on survival probablity for the KNO genotype within the natural microbial
627 for the control treatment, we explored the possibilty whether this could be reflected in the gut
628 microbiome community by performing union plots across microbial treatments and genotypes,
629 but within the control treatment (Figure 9). T8 had a higher number of unique OTUs (58) and total
630 number of OTUs (117), compared with KNO (unique: 30, total: 85) and OM2 (unique: 35, total: 95)
631 when receiving the natural bacterioplankton. When we examined the present OTUs after
632 receiving the laboratory bacterioplankton, T8 (unique: 32, total: 82) had the same number of
633 unique OTUs as KNO (unique: 32, total: 84) and a higher number of unique OTUs compared with
634 OM2 (unique: 21, total: 70).

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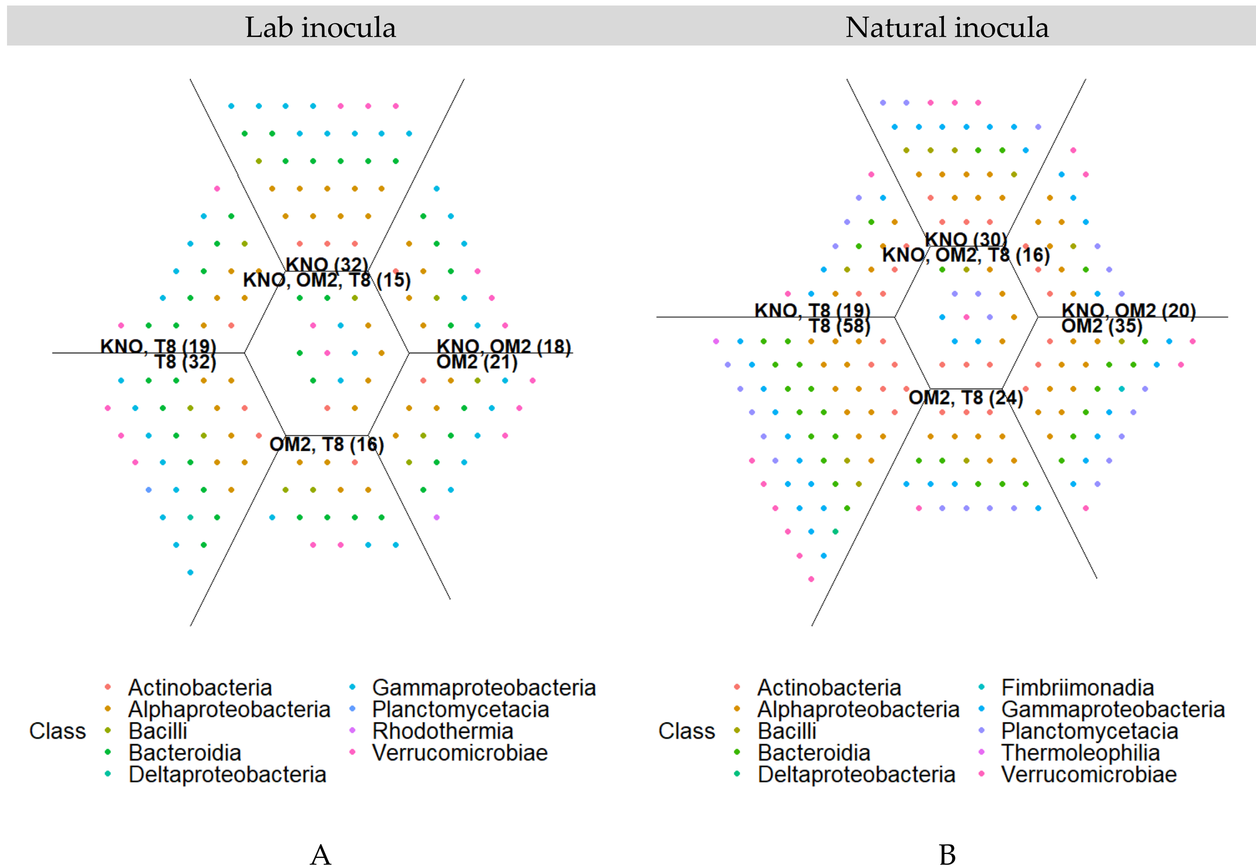


641 Figure 9: Union plots representing the OTUs that are unique within and shared between the
642 genotypes in the control treatment when exposed to the (A) lab and (B) natural bacterioplankton.
643 Numbers between brackets indicate the total number of OTUs present in that compartment.
644 Colors indicate the OTUs grouped per class.

645 Lastly, an EdgeR analysis was performed to examine which OTUs significantly differend in terms
646 of relative abundance between the different treatments. The Edge R analysis revealed highly
647 significant differences for 213 OTUs between the donor bacterioplankton and recipient *Daphnia*
648 (Table S3). Within the donor bacterioplankton, only the relative abundance of one OTU was highly
649 significantly different between the laboratory and natural microbial inocula (Table S3, the three
650 laboratory microbial inocula were pooled and the three natural microbial inocula were pooled).
651 Within the recipients the relative abundance of 141 OTUs were significantly different between the

652 four stressor treatments, the relative abundance of 285 OTUs between the microbiome treatments,

653 the relative abundance of 34 OTUs within the stressor x microbiome interaction and the relative

654 abundance of 5 OTUs were significantly different within the stressor x microbiome x genotype

655 interaction (Table S3; Figure S11). Analysis per microbiome treatment revealed that the relative

656 abundance significantly differed between the stressor treatments for 12 OTUs within the lab

657 bacterioplankton treatment and for 24 OTUs within the natural bacterioplankton treatment

658 (Figure S12, Table S3).

659 ***Correlations***

660 Correlation tests were performed to investigate possible links between the tested variables:

661 percentage of survived *D. magna*, total brood, body size and OTU richness. No correlation was

662 observed between the life history traits and OTU richness of the gut microbial community (Table

663 S7, Figure S13). We did observe a positive correlation between survival and fecundity (cor=0.32,

664 t=-2.84, df=70, p-adj=0.017; Table S5, Figure S14), and fecundity and body size (cor=0.33, t= 2.96,

665 df=70, p-adj=0.017; Table S5, Figure S14).

666

667 **Discussion**

668 We inoculated germ-free *Daphnia* to either a laboratory or natural bacterioplankton community

669 and compared host tolerance to a parasitic fungus, an *A. aculeatus*-like strain, and the toxic

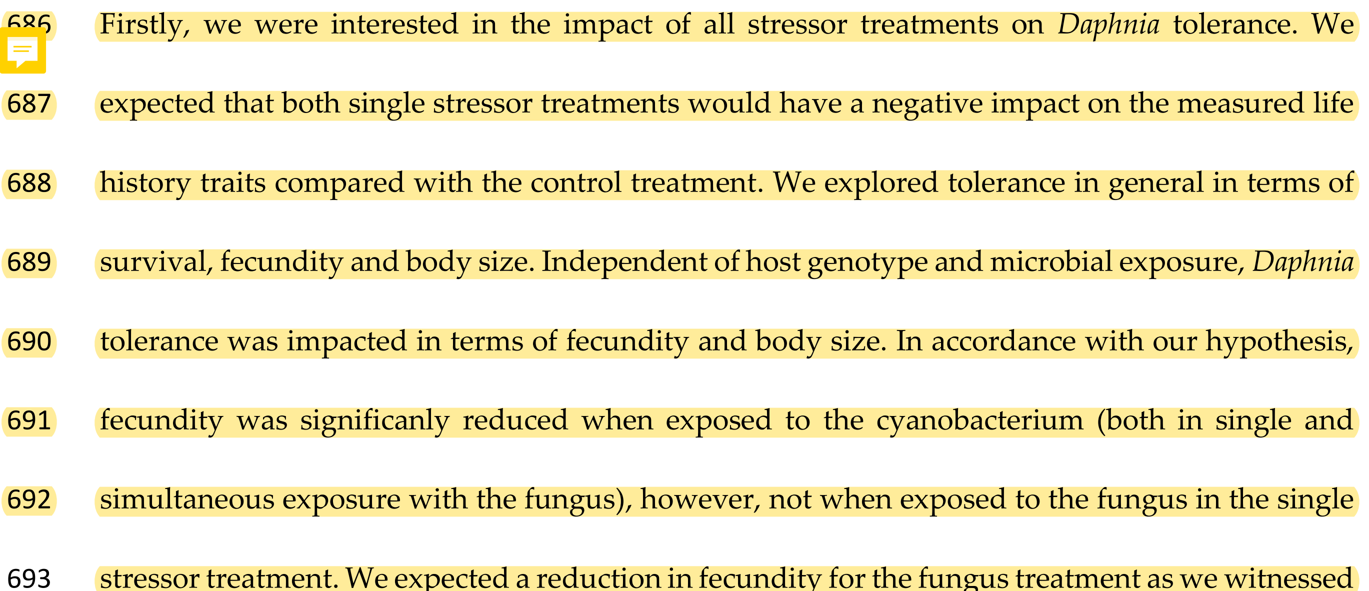
670 cyanobacterium *M. aeruginosa* in single and combined exposures. After exposure, we dissected

671 *Daphnia* gut's and determined the gut bacterial community. By performing this experiment we

672 aimed to tackle three questions: (1) how will these stressor treatments affect host life history traits,

(2) will exposure to a different microbial environment result in a different response to these stressor treatments, i.e. is *Daphnia* tolerance to these stressors microbiome-mediated, and (3) will these host responses differ between the different host genotypes.

Our results showed that (1) exposure to the cyanobacterium results in a decrease of fecundity and body size (both under single or multiple exposure). Exposure to the fungus did only result in a decrease of body size. In addition, the cyanobacterium and fungus seem to interact antagonistically as the reduction on *Daphnia* body size is less severe than expected under the multiple exposure, (2) tolerance in terms of survival, but not fecundity and body size, is microbiome-mediated as survival is only impact by stress under the lab bacterioplankton conditions an not under the natural conditions. Results on the gut microbial community reflect random take-up, but also stressor-dependent selection of bacteria from the environment. Our results also showed that (3) *Daphnia* responses are genotype dependent for survival and fecundity, but not for body size.



Firstly, we were interested in the impact of all stressor treatments on *Daphnia* tolerance. We expected that both single stressor treatments would have a negative impact on the measured life history traits compared with the control treatment. We explored tolerance in general in terms of survival, fecundity and body size. Independent of host genotype and microbial exposure, *Daphnia* tolerance was impacted in terms of fecundity and body size. In accordance with our hypothesis, fecundity was significanly reduced when exposed to the cyanobacterium (both in single and simultaneous exposure with the fungus), however, not when exposed to the fungus in the single stressor treatment. We expected a reduction in fecundity for the fungus treatment as we witnessed

694 damaged eggs in the brood poach during previous infections (personal observations). It is
695 possible that the genotypes used in this experiment are less susceptible towards this fungus and
696 as such have obtained a higher tolerance. Body size was significantly reduced under all stressor
697 treatment (both single as multiple stressor treatments). Exposure to both single stressors
698 separately resulted in a reduced body size, whereas body size after the simultaneous exposure
699 did not show an additive net effect of both stressors. It appears that body size is predominantly
700 driven by cyanobacterium-related stress. Interestingly, *Daphnia* survival was not significantly
701 impacted by single stressor or multiple stressor exposure when not taking the microbial treatment
702 into account. As the cyanobacterium impacts survival (e.g. Macke et al. 2017), we would expect a
703 decrease in survival when exposed to the single cynobacterium treatment and the combination
704 treatment.

705 Secondly, we were interested in the impact of the microbial exposure on *Daphnia* tolerance when
706 comparing the stressor treatments. We expected that tolerance in *Daphnia* was microbiome-
707 mediated, i.e. *Daphnia* individuals receiving the natural microbial inoculum would have a higher
708 tolerance to particular stressors (i.e., have a higher survival, fecundity and body size) compared
709 with individuals that received a laboratory microbial community. We expected to see this increase
710 in tolerance in both the single as the multiple stressor treatments. Our resuls suggest that tolerance
711 in terms of survival is microbiome-mediated as survival was only impacted when *Daphnia*
712 received a laboratory bacterioplankton inoculum, but not when they received a natural
713 bacterioplankton inoculum. This is in accordance with our expectations as we did expect and find
714 a positive effect on *Daphnia* tolerance when exposed to the natural bacterioplankton inoculum. In
715 addition, we hypothesized that this microbiome-mediated tolerance would be reflected in (a) a

more diverse gut host community and/or (b) the presence of particular selected strains in the *Daphnia* receiving the natural inoculum compared with the lab inoculum. We indeed observe that our natural bacterioplankton communities are generally associated with a more diverse microbial community (also seen in Callens et al. 2020) and as such possibly can provide a broader pool of microbiota. We observed that both the OTU richness was higher in the guts of *Daphnia* individuals which were exposed to the natural bacterioplankton inoculum as the community was differentially structured compared to the lab bacterioplankton inoculum. This more diverse community in *Daphnia* individuals inoculated with a natural bacterioplankton inoculum is in accordance with other study systems (e.g. Drosophila: Chandler et al. 2011, Limulus polyphemus: Friel et al. 2020, zebrafish: Roeselers et al. 2011, mice: Rosshart et al. 2017). In addition, not all strains of the environment were taken up, which could possibly imply (a) random colonization and competition of bacterial strains or (b) selection of certain bacterial strains by the *Daphnia* host as suggested in Macke et al. (2017) and Mushegian et al. (2018) and shown in Callens et al. (2020). We did not find ~~a possible~~ correlation between gut microbial diversity and the observed life history traits, which suggest that diversity of the gut community does not predominantly determine tolerance in *Daphnia*. 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 (as other

738 parasites often do, e.g., *Pasteuria ramosa*) instead of the gut cavity. Another possibility is that
739 selection processes were not triggered by the fungal infection as the impact was minimal
740 compared with the *Microcystis*-induced effects on life history. Combined with our results on
741 survival, it is possible that after initial colonization two processes are determining the *Daphnia* gut
742 community: (a) the general take-up of more strains which could possibly include strains which
743 have a positive effect on defence mechanisms linked with survival, and (b) selection of the *Daphnia*
744 host of particular microbial strains that have a phytoremediating effect against *Microcystis*. These
745 particular strains were possibly not present in the lab bacterioplankton environment. The high
746 amount of differing bacterial strains (as shown in EdgeR analysis and union plots) between
747 stressor treatments could also reflect a stressor-specific selection. Tolerance in terms of fecundity
748 and body size, however, was not microbiome-mediated. Trade-offs between survival and body
749 size under stress were previously found in *Daphnia* (Adamczuk 2010, Houwenhuyse et al. 2021)
750 and other organisms (Sterck et al. 2006, Mogensen and Post 2012) with Houwenhuyse et al. (2021)
751 suggesting a role of the microbiome for this trade-off under *Microcystis* stress. Here, we did,
752 however, not find support for such a trade-off.

753 In accordance with our initial expectations on antagonistic interactions, simultaneous exposure of
754 the fungus and cyanobacterium appears to interact antagonistically, but interestingly only in
755 *Daphnia* individuals with a lab gut microbial community. Similarly as in Boudry et al. (2020),
756 survival was not negatively impacted by simultaneous exposure of both stressors under lab
757 conditions. Boudry et al. (2020) described this antagonistic interaction as a potential protective
758 effect of the *Aspergillus* infection on *Daphnia* towards *Microcystis* exposure through a parasite-
759 mediated reduction in toxicity of *Microcystis.* Alternatively, *Daphnia* can be boosted through an

760 increase in general tolerance levels by ingestion of the produced zoospores. Cross-tolerance could
761 be initated as zoospores could function as an additional food source, which is in accordance with
762 Frenken et al. (2017), Kagami et al. (2007) and Agha et al. (2016), indicating that fungal parasites
763 can transfer energy and nutrients from otherwise inedible algae to *Daphnia*, and thereby increase
764 *Daphnia* growth and survival. These studies, however, used fungal parasites that are obligate
765 parasites from inedible diatoms and cyanobacteria. In this study, *Aspergillus* infects the *Daphnia*
766 host, resulting in reduced body size and a genotype dependent reduction in fecundity, as well a
767 high mortality in juveniles (L. Bulteel and S. Houwenhuyse, personal observations). It is, however,
768 not yet examined whether this specific *Aspergillus* can also infect cyanobacteria. The *Aspergillus*
769 genus is diverse and wide-spread containing up to 339 species (Samson et al. 2014), which consist
770 of several pathogenic species, significantly impacting food production (e.g. Alshannaq et al. 2018),
771 and animal and human health (e.g. Kousha et al. 2011, Seyedmousavi 2013). *Aspergillus aculeatus*
772 exposure, on the other hand, has been described to be associated with phytoremediation and
773 detoxification in plants (Xie et al. 2019).

864 Survival and fecundity analyses revealed a role of the genotype in the stress responses. These
865 results are in accordance with the literature as responses to cyanobacteria (e.g. Macke et al. 2017)
866 and parasites (e.g. Decaestecker et al. 2007) in *Daphnia* are generally genotype-dependent. In the
867 control treatment, T8 had a higher number of unique and total OTUs, compared with KNO and
868 OM2 when they received a natural microbial inoculum, but not when they received a laboratory
869 microbial inoculum. This increase in strains, whilst having a lower fitness appears
870 counterintuitive, but aquatic environments contain next to a plethora of beneficial and neutral
871 bacterial strains, also obligate and opportunistic bacterial pathogens (Schulze et al. 2006), so it

872 could be that with higher diversity more opportunistic microbiota are present (as also suggested
873 in Callens et al. 2016). As *Daphnia* genotypes differ in their selective capacities to take up bacteria
874 (Macke et al. 2017, Frankel-Bricker et al. 2020, Callens et al. 2020, Bulteel et al. 2021, Houwenhuyse
875 et al. 2021), our results would suggest that genotype T8 might be less selective and takes up
876 randomly also non-beneficial strains, at least in comparison with KNO and OM2.

877 In conclusion, *Daphnia* are negatively impacted by stress by exposure to cyanobacteria or fungal
878 infection. In addition, tolerance to these stressors in terms of survival appears to be microbiome-
879 mediated. When *Daphnia* were cultured in a rich microbial environment, the stress-induced
880 negative effects on survival are reduced to such an extent that no effect can be detected. In
881 contrast, *Daphnia* when cultured in an impoverished microbial environment do show this negative
882 impact of stress on survival. This microbiome-mediated tolerance could possibly be reflected by
883 a more diverse and differentially structured gut community which established by random take-
884 up from the environment, and stressor-dependent selection by the host This microbiome-
885 mediated tolerance, however, was not present in determining *Daphnia* fecundity and body size.
886 In accordance to Boudry et al. (2020) an antagonistic interaction for simultaneous stressor
887 exposure under lab conditions was observed on the survival of the *Daphnia*. In addition, stressor
888 responses were genotype specific for survival and fecundity, which could be linked with different
889 capabilities of the *Daphnia* genotypes to select beneficial or neutral microbial stains from the
890 environment.

891

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Data availability

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

Conflict of interest

The authors declare that there is no conflict of interest.

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1213

1214 **Supplementary information**

1215 Supplementary information S1: Uptake bacterial strains from environment by gut

1216 The uptake of bacteria by the recipient *Daphnia* from the environment (donor bacterioplankton),
1217 was analysed with Unionplots. Two Unionplots were made, one for each microbiome type. In
1218 each Unionplot, the donor bacterioplankton was compared with the gut microbiomes from the
1219 *Daphina* that received a control treatment or a stressor treatment (Figure S3). When *Daphnia*
1220 received a laboratory donor inoculum, they take up 35.8% of the donor inoculum. In the control
1221 treatment, 41.0% of the gut microbiomes consists of OTUs present in the donor laboratory
1222 bacterioplankton, while in the stressor treatments, only 34.5% of the gut microbiomes consists of
1223 OTUs present in the donor laboratory bacterioplankton. When *Daphnia* received a natural donor
1224 inoculum, they take up 31.4% of the donor inoculum. The difference in uptake between *Daphnia*
1225 that received a control or stressor treatment is smaller when they received a natural donor
1226 inoculum than when they received a laboratory donor inoculum. In the control treatment, 32.5%
1227 of the gut microbiomes consists of OTUs present in the donor natural bacterioplankton, andin the
1228 stressor treatments, 31.6% of the gut microbiomes consists of OTUs present in the donor natural
1229 bacterioplankton.

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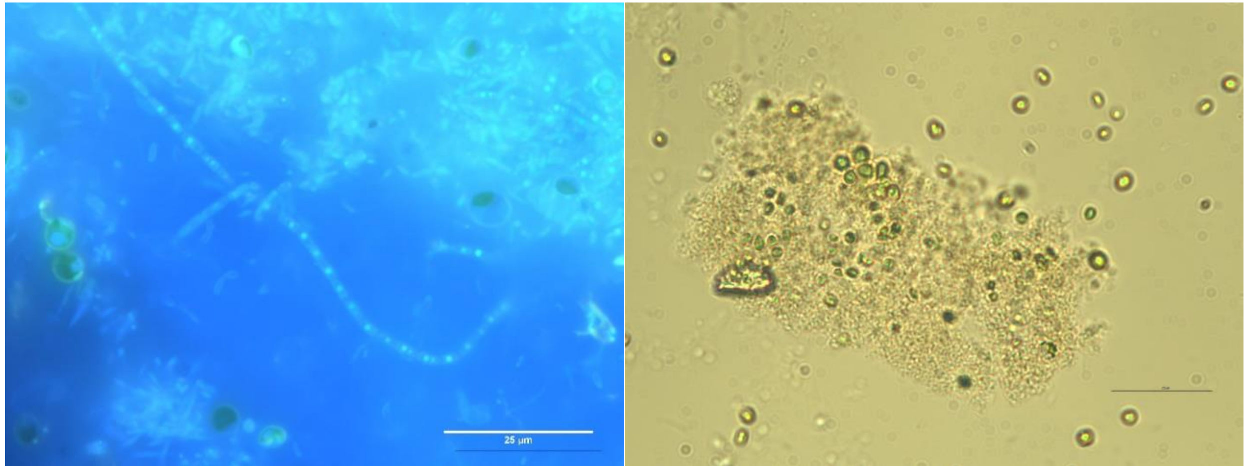
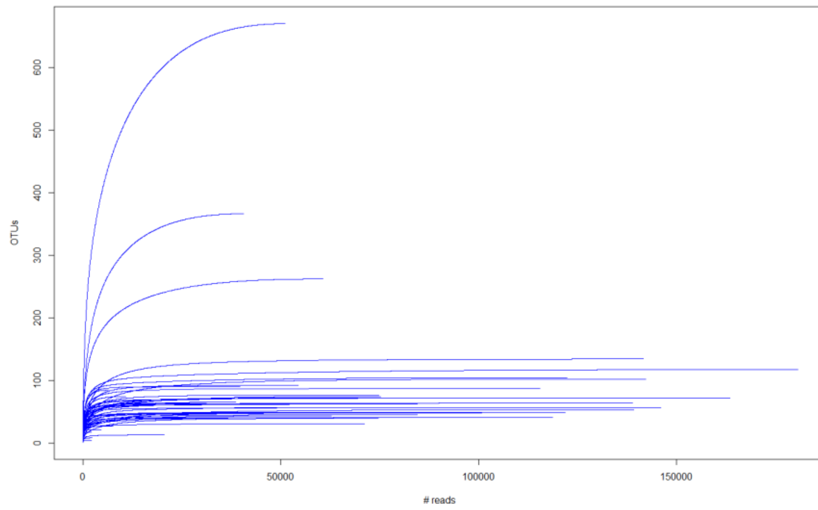
Supplementary figures

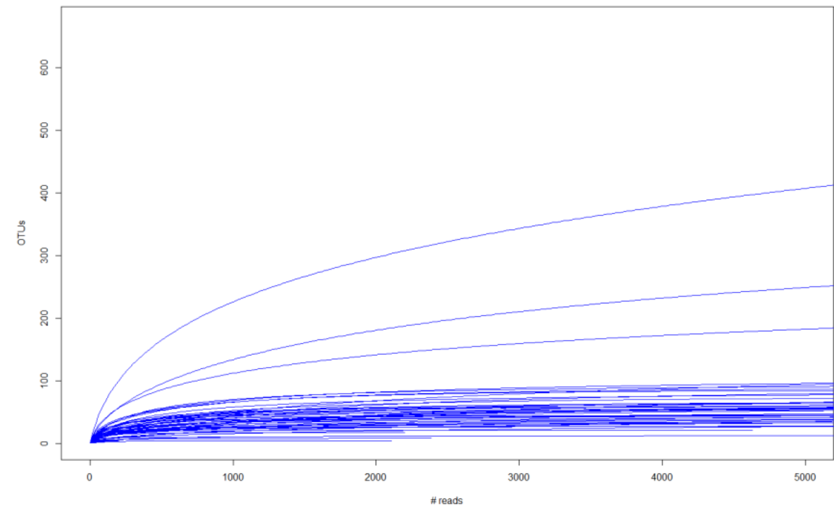


A B

Figure S1: Microscopic pictures of the stressor treatments; (A) *Aspergillus* infection treatment: hyphae and surrounding spores stained with dapi with 400 x magnification under UV fluorescence (for characterization process, see further) and (B) *Microcystis* treatment: Colony of *Microcystis* surrounded with individual cells with 160 x magnification.

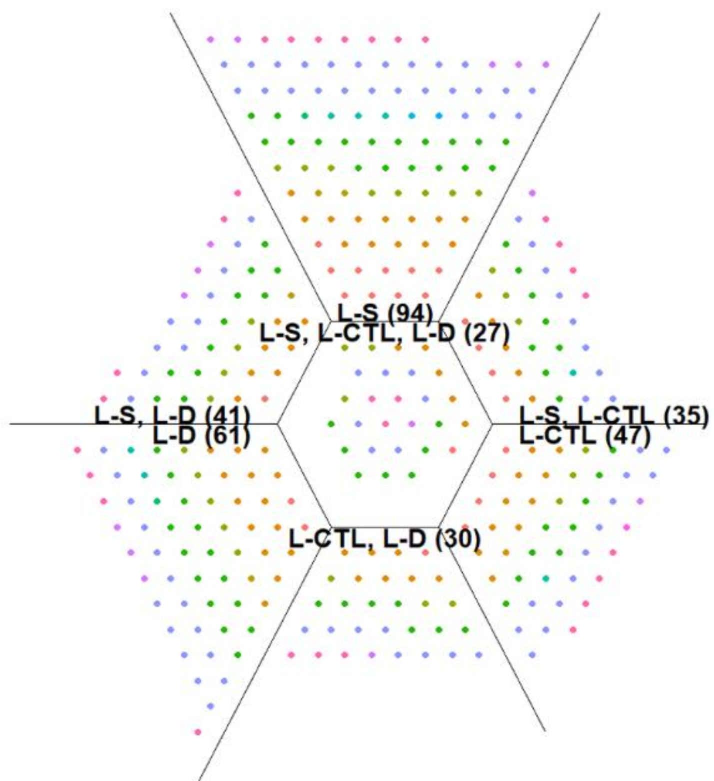


A

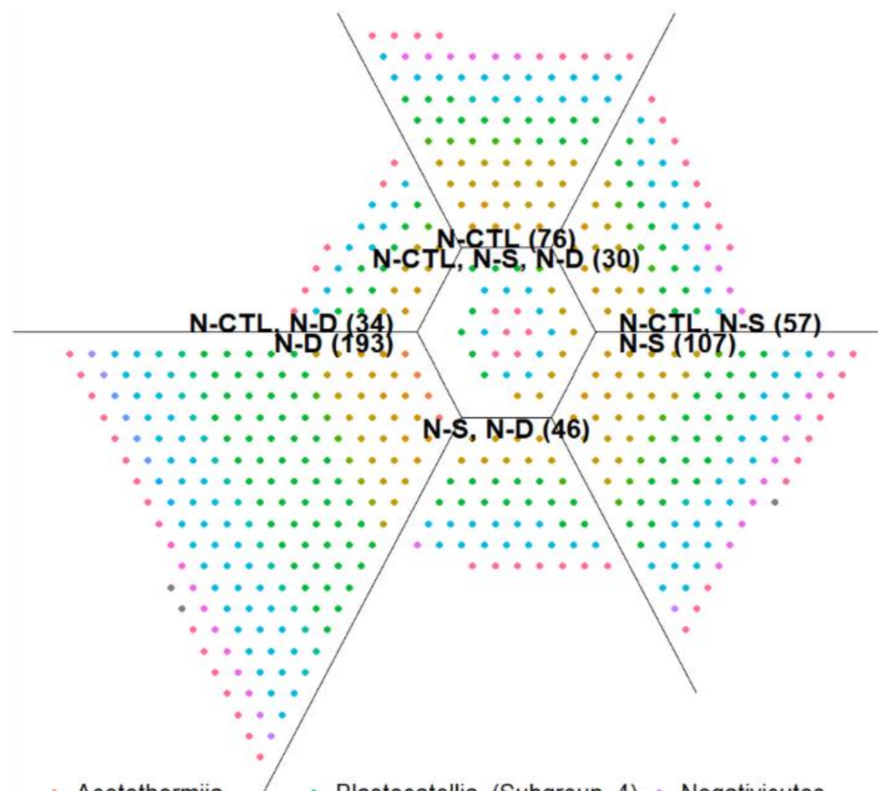


B

Figure S2: Rarefaction curve of the raw sequencing data. Number of reads are represented on the x-axis, number of OTUs are represented on the y-axis. (A) overview (B) zoomed in on the first 5000 reads.



Class
- Actinobacteria
- Alphaproteobacteria
- Babeliae
- Bacilli
- Bacteroidia
- Deinococci
- Deltaproteobacteria
- Fimbriimonadia
- Fusobacteriia
- Gammaproteobacteria
- Planctomycetacia
- Rhodothermia
- Verrucomicrobiae



Class
- Acetothermiia
- Acidimicrobiia
- Acidobacteriia
- Actinobacteria
- Alphaproteobacteria
- Aminicenantia
- Anaerolineae
- Armatimonadia
- Bacilli
- Bacteroidia
- Blastocatellia_(Subgroup_4)
- Campylobacteria
- Clostridia
- Deltaproteobacteria
- Fimbriimonadia
- Gammaproteobacteria
- Gemmatimonadetes
- Gracilibacteria
- Ignavibacteria
- Mollicutes
- Negativicutes
- Phycisphaerae
- Planctomycetacia
- Rhodothermia
- Thermoanaerobaculia
- Thermoleophilia
- Verrucomicrobiae
- NA

1243

Figure S3: Unionplos representing the unique and shared OTUs between donor bacterioplankton and recipient *Daphnia* in the (A) laboratory treatment and (B) natural treatment. L=laboratory treatment, N=natural treatment, S=stressor treatment (fungus, cyanobacterium and combination), CTL=control treatment, D=donor bacterioplankton.

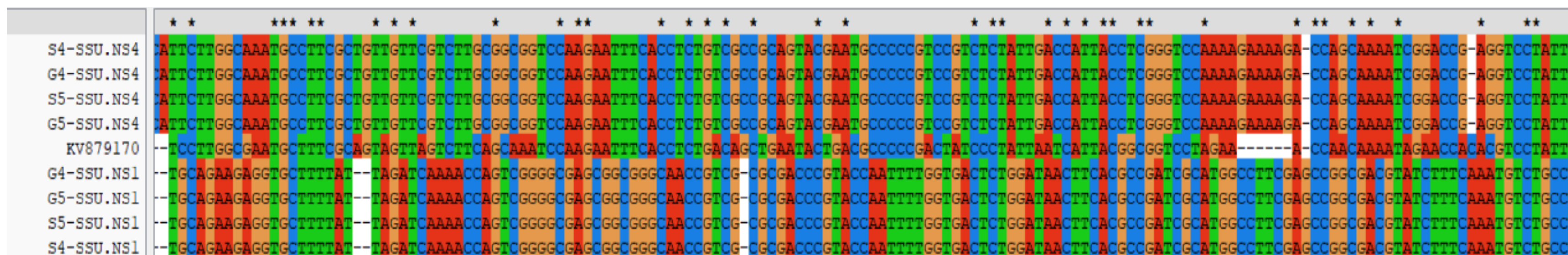
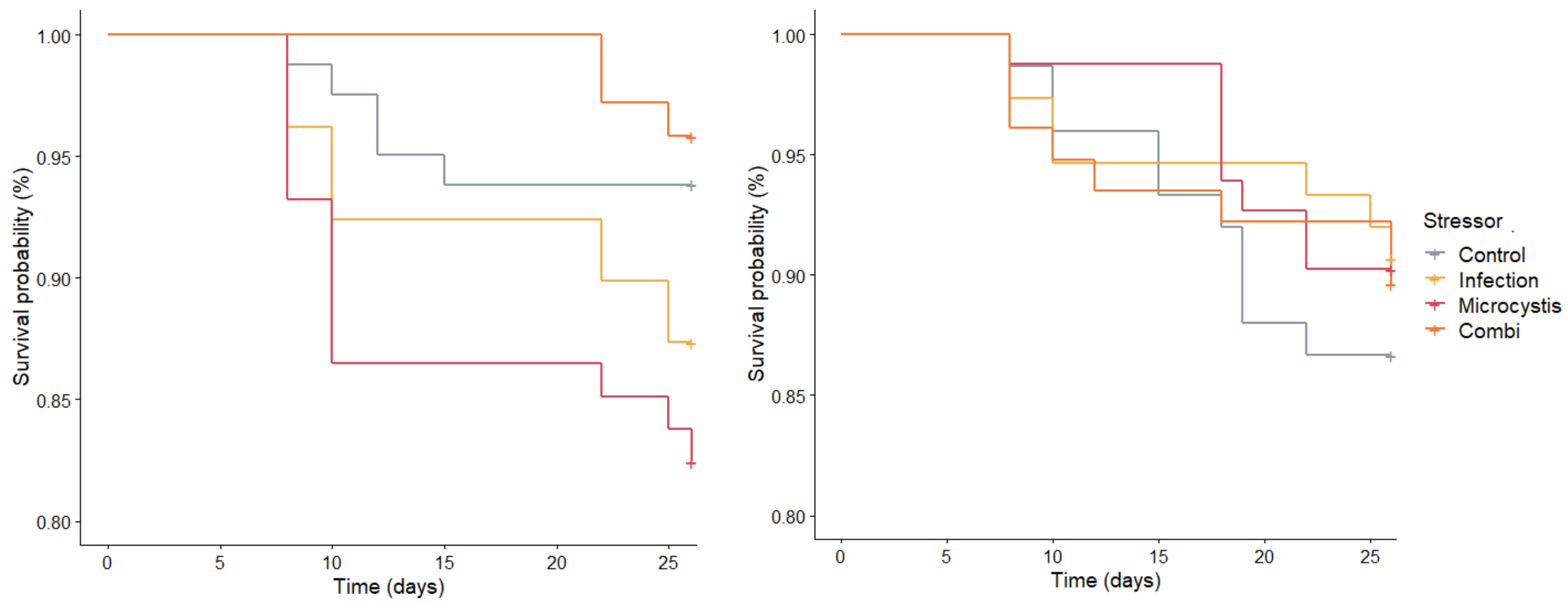


Figure S4: Part (155 to 310 nucleotides) of the multiple sequence alignment pattern of the sample sequences with *Aspergillus aculeatus* ATCC 16872. Sequences of *Daphnia* with a visible and no visible infection, together with the *Aspergillus aculeatus* ATCC 16872 strain are aligned (sample names are shown in the left column). Color represents a specific type of nucleotide that matches with the *Aspergillus aculeatus* strain. Hyphen (-) represents a gap where no match between the nucleotides of the *Aspergillus aculeatus* strain and the aligned sequence of the sample is found. Asterisk on the top represents the nucleotides that are common in all the aligned sequences.



1254 A B

1255 Figure S5: Survival plots recipient *Daphnia* between the stressors treatments for (A) the lab microbial inocula and (B) the natural
1256 microbial inocula. Colors indicate the different stressor treatments.
1257

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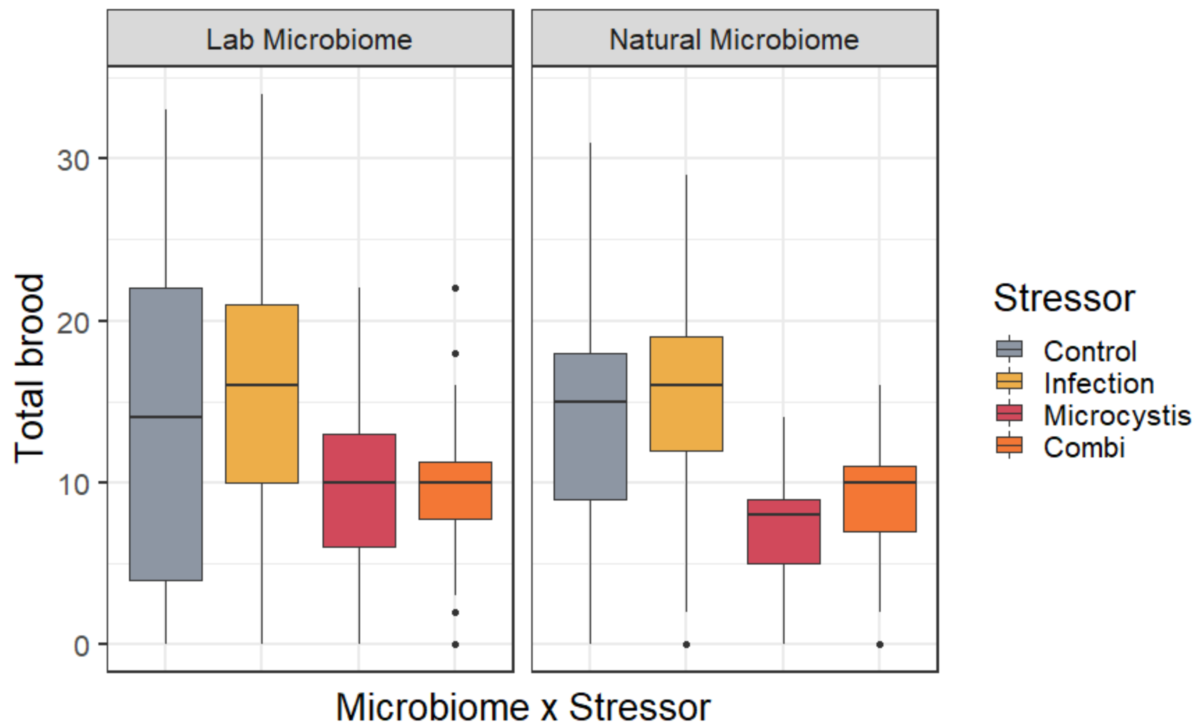
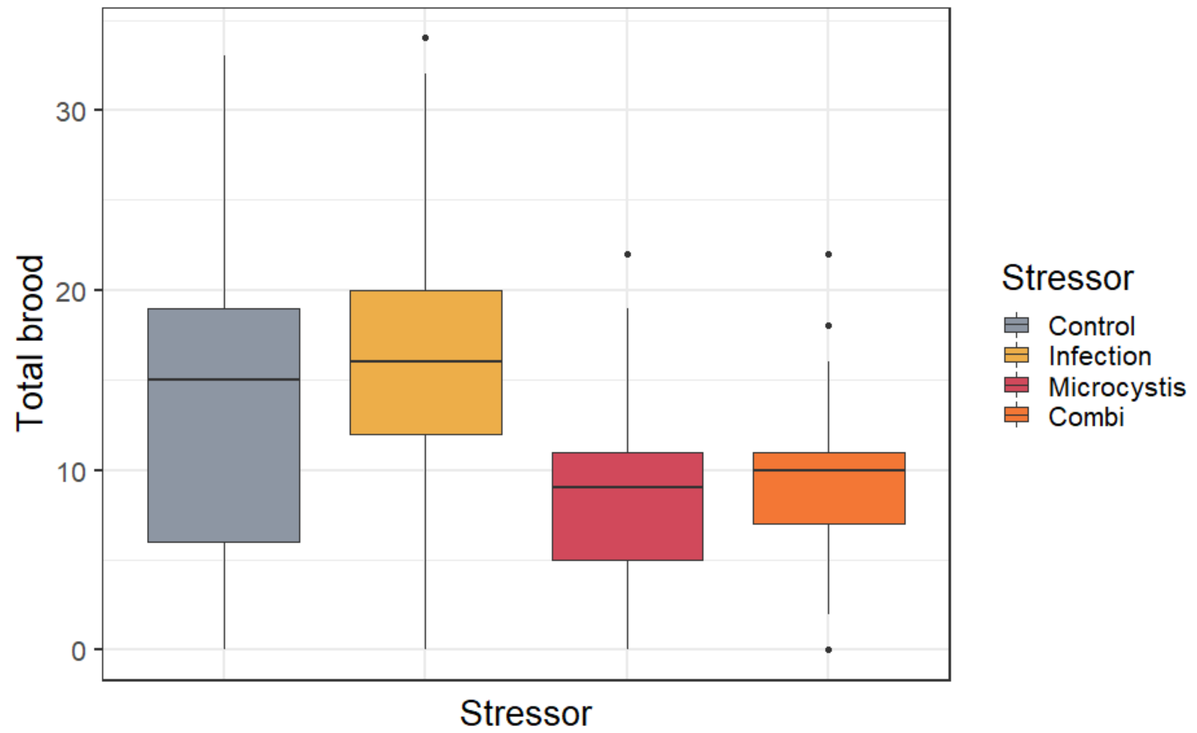


1259

1260 Figure S6: Boxplots of the total brood for the different stressor treatments under the different

1261 microbial inocula treatment. Colors indicate the different stressor treatments.

1262



1263

1264 Figure S7: Boxplots of the total brood for the different stressor treatments. Colors indicate the

1265 different stressor treatments.

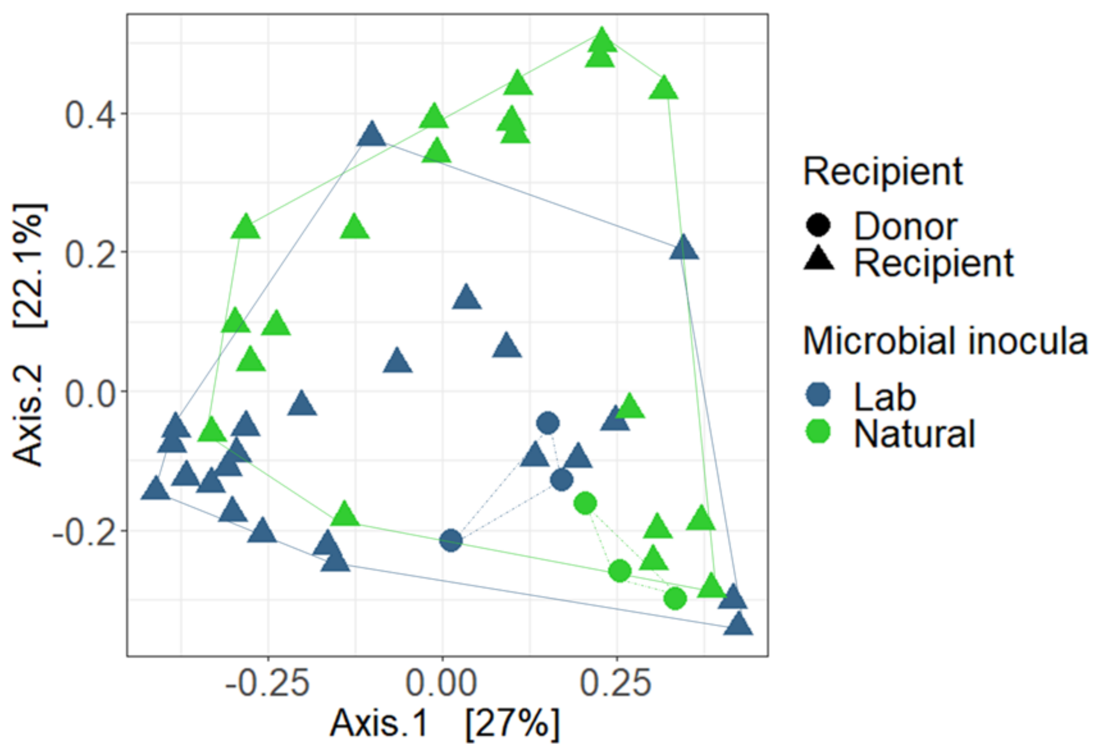
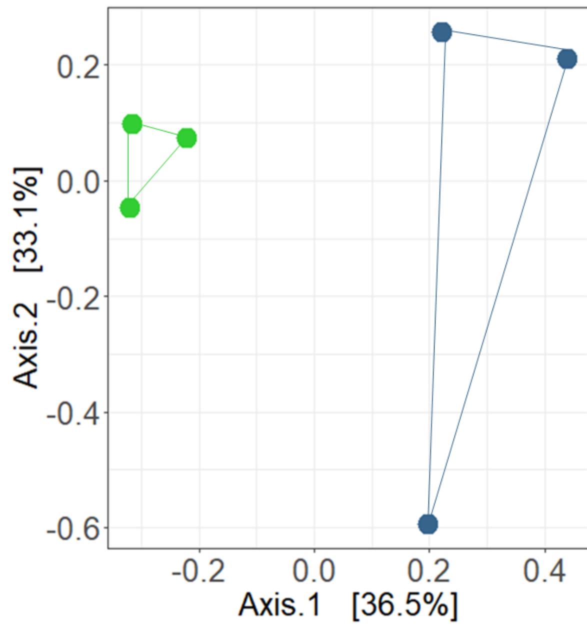
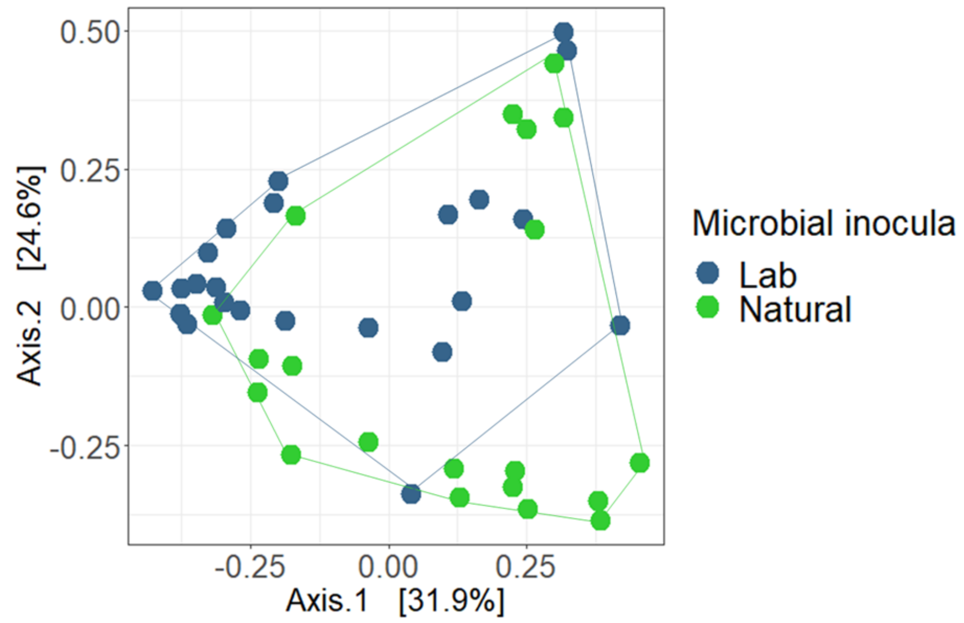


Figure S8: PCA of the gut microbial communities using weighted Bray-Curtis distance for donor and recipient data. Colors indicate microbiome treatment. Symbols and line type indicate sample type (donor bacterioplankton vs recipient *Daphnia*).

1266



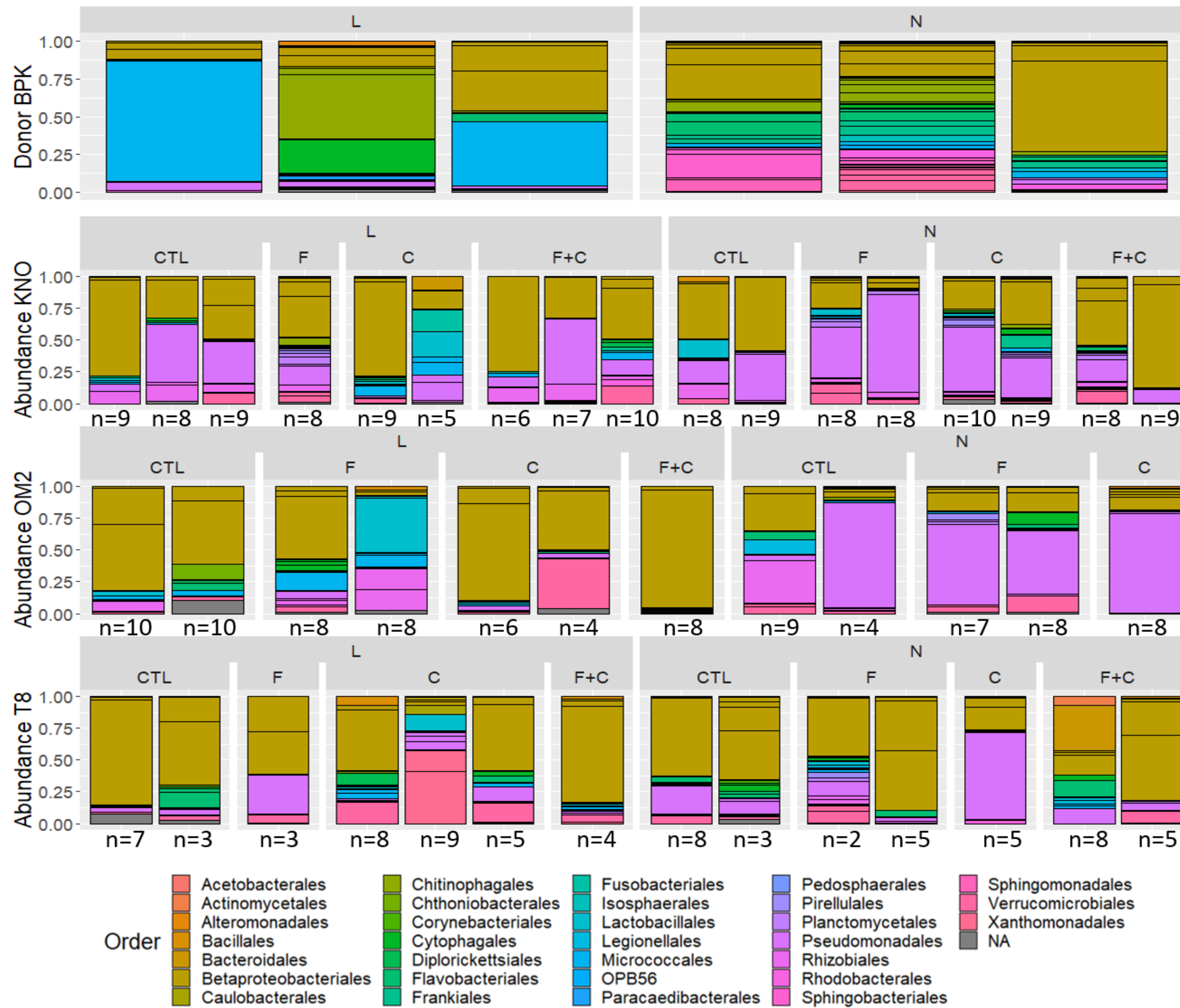


1268 A B

1269 Figure S9: PCA of the (A) donor and (B) recipient microbial communities using weighted Bray-Curtis distance. Colors indicate the

1270 different microbiome treatments.

1271



1272 Figure S10: Relative abundance of the donor bacterioplankton (BPK) and gut microbial composition of the recipient population
1273 grouped per genotype x microbiome x stressor interaction. Number of guts per sample are represented at the bottom of each bar.
1274 Colors indicate the bacterial order. OTUs with a relative abundance lower than 1% are not included. Analyses are performed on
1275 rarefied data. Explanation of the abbreviations: L=laboratory inoculum, N=natural inoculum, CTL= control treatment, F=fungal
1276 treatment, C=cyanobacterium treatment and F+C=combination treatment.

1277

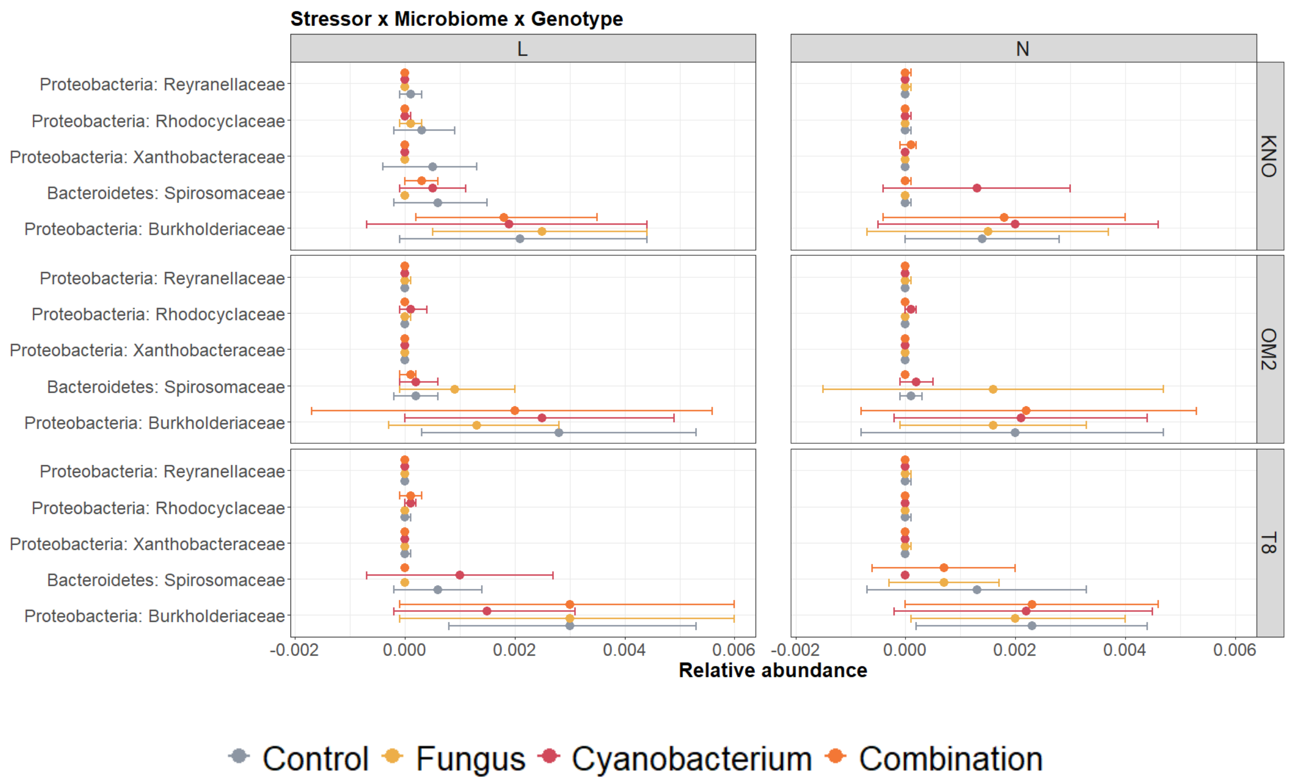
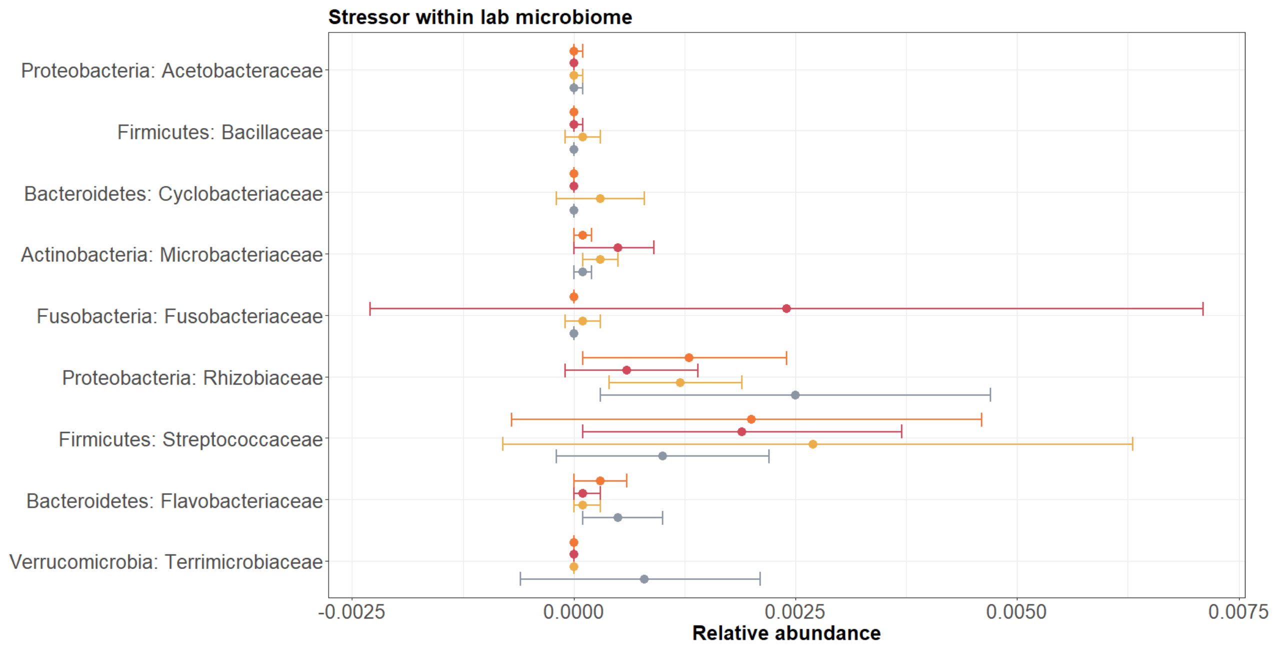
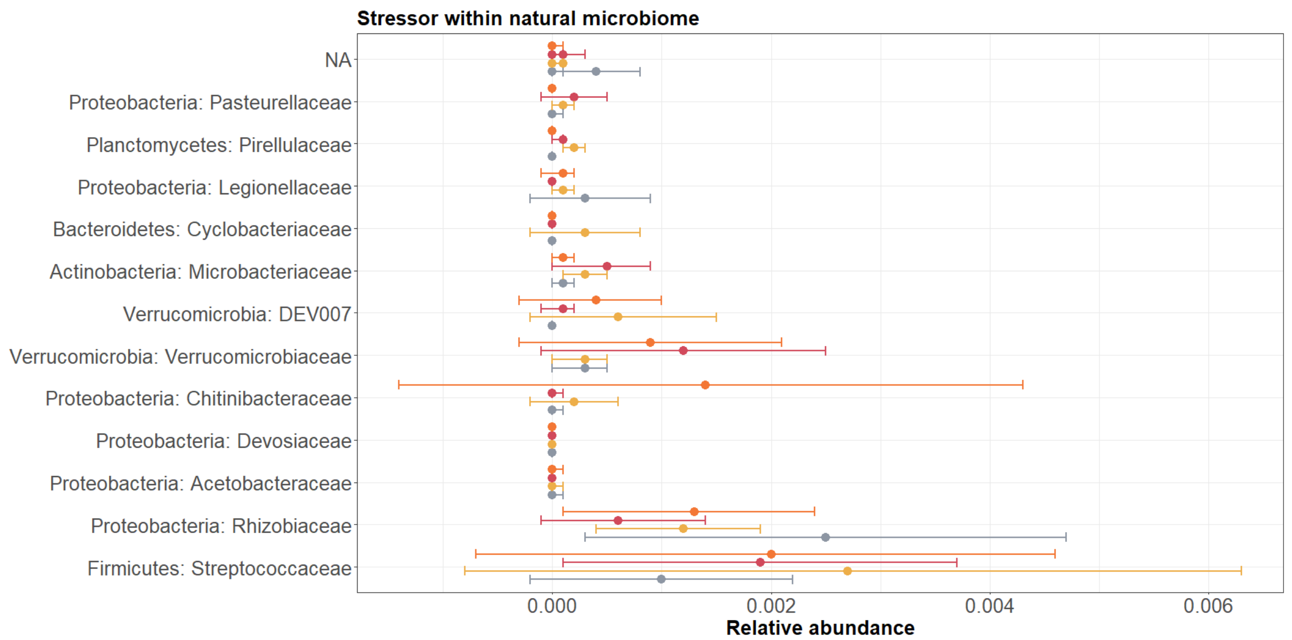


Figure S11: ggplot representing the OTUs at family level that were significantly different between the stressor x microbiome x genotype interaction. Colors indicate the stressor treatments.



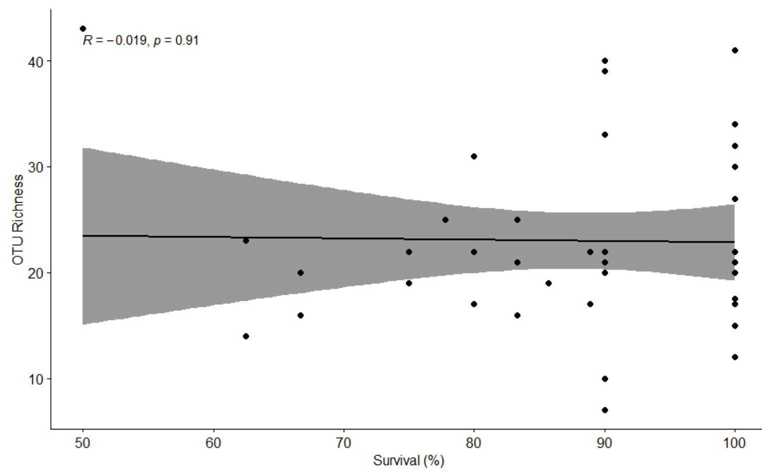
A



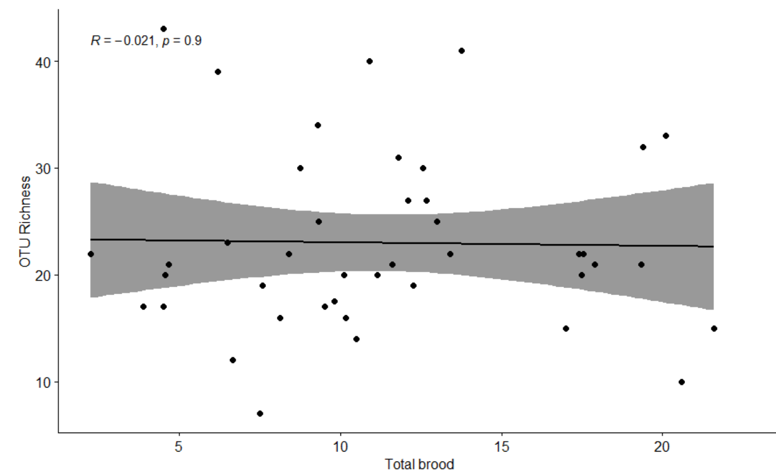
Control Fungus Cyanobacterium Combination

B

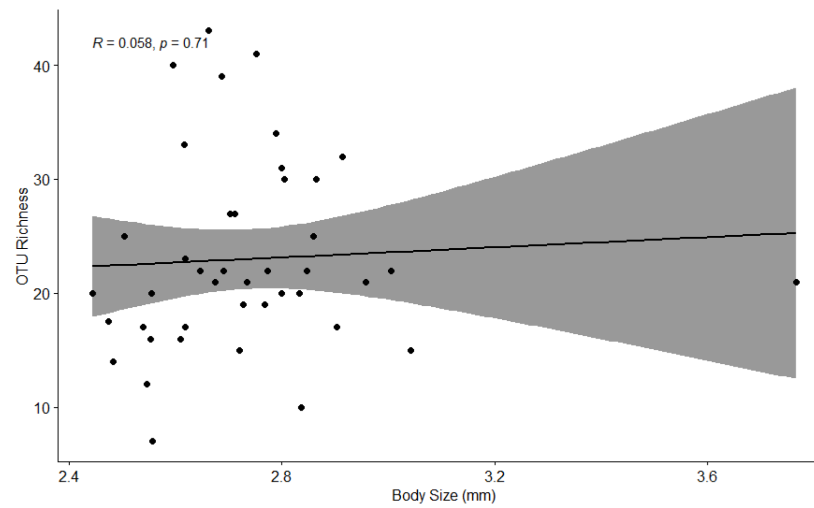
1278 Figure S12: ggplot representing the OTUs at family level that were significantly different between
1279 the different stressor treatments within the (A) lab and (B) natural microbial water treatment.
1280 Colors indicate the stressor treatments.



A

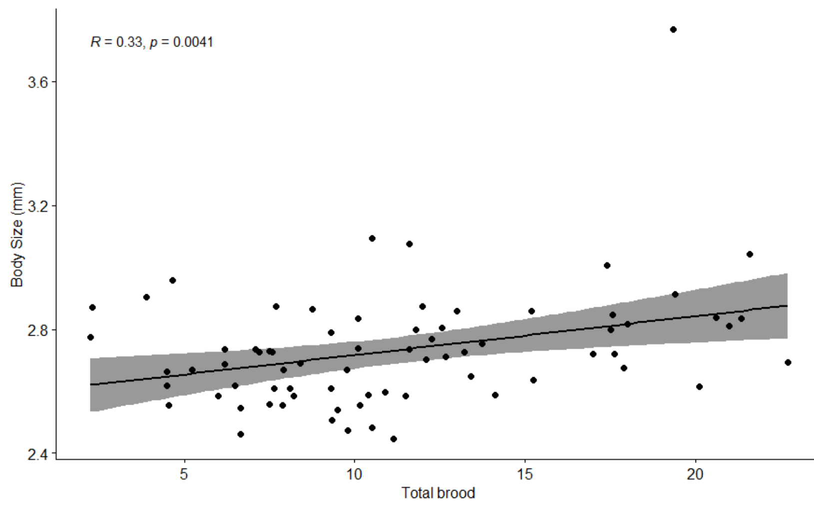


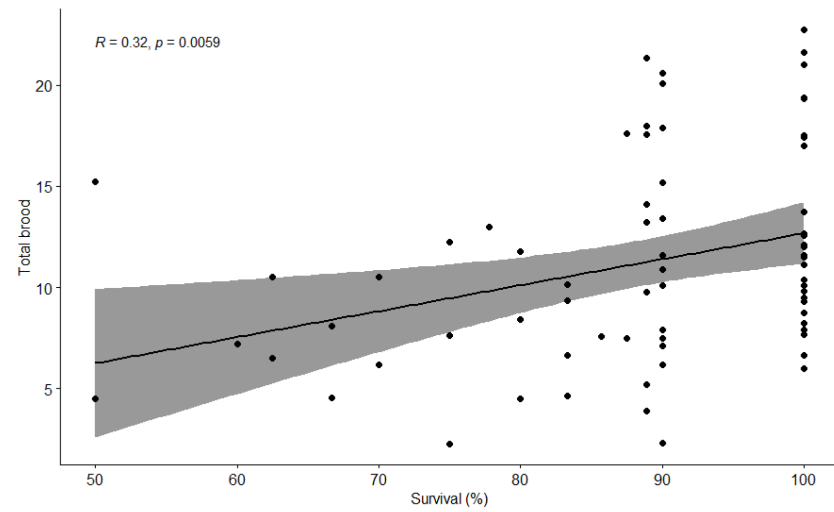
B



C

Figure S13: Pearson regression between (A) Survival, (B) Fecundity, (C) Body size and OTU richness of the gut microbial community of recipient *Daphnia*. Non-adjusted p-values and correlation coefficient (R) are noted per figure.





A B

Figure S14: Pearson regression between (A) Fecundity and body size, and (B) Survival and Fecundity. Non-adjusted p-values and correlation coefficient (R) are noted per figure.

A B

1281

1282