Environmental sources of bacteria and genetic variation in behavior influence host-associated microbiota

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Summary statement: Genetic differences in Daphnia behavior contribute to the amount of environmental bacteria present in their microbiome

Abstract

In many organisms, host-associated microbial communities are acquired horizontally after birth. This process is believed to be shaped by a combination of environmental and host genetic factors. We examined whether genetic variation in animal behavior could affect the composition of the animal's microbiota in different environments. The freshwater crustacean *Daphnia magna* is primarily planktonic, but exhibits variation in the degree to which it browses in benthic sediments. We performed an experiment with clonal lines of *D. magna* showing different levels of sediment-browsing intensity exposed to either bacteria-rich or bacteria-poor sediment or whose access to sediments was prevented. We find that the bacterial composition of the environment and genotype-specific browsing intensity together influence the diversity and composition of the *Daphnia*-associated bacterial community. Exposure to more diverse bacteria did not lead to a more diverse microbiome, but greater abundances of environment-specific bacteria were found associated with host genotypes that exhibited greater browsing behavior. Our results indicate that individual behavior can mediate genotype-by-environment interaction effects on microbiome composition.

Introduction

Every multicellular organism is colonized by a community of microorganisms: its microbiota (McFall-Ngai et al. 2013). The host provides a habitat for a complex and dynamic consortium of microorganisms, many of which have fundamental influences on the host's well-being (Zilber-Rosenberg & Rosenberg 2008). A central concern in both infectious disease epidemiology and in studies of host-associated microbial community ecology is the transmission of microbes between host individuals and between hosts and the environment. Many bacterial assemblages are transmitted from host mother to offspring (Funkhouser & Bordenstein 2013; Salem et al. 2015), but the diversity of microbiota typically changes over time depending on the microbes available in the environment (Rajilic-Stojanovic et al. 2009; Shin et al. 2015). In some cases, environmentally acquired microbes are even essential for the completion of postembryonic development (e.g.

49 Cheesman et al. 2011; Diaz Heijtz et al. 2011). Thus microbes from the environment
50 can be co-opted as part of the microbiota, or can affect host health during a transient
51 occupation (Voss et al. 2015).
52 Environmental effects on microbiota community structure have been
53 extensively documented (Fan et al. 2013; Seedorf et al. 2014) and studies on model
54 organisms have started to shed light on the relative importance of environmental
55 and host genetic factors in determining microbiota composition (Campbell et al.
56 2012; Spor et al. 2011). Recently, the focus has been moving towards a better
57 understanding of the mechanisms of bacterial aqusition from the environment. Host
58 genetics have been shown to play a role in the establishment of microbial
59 associations through microbial recognition, immune selection, and determination of
60 the biochemical niche (Spor et al. 2011). Importantly, these processes select
61 microbes after the host has come in contact with bacterial communities in the
62 environment. The initial encounter may be a key phase of the host's colonization by
63 microbes. If host genetics influence interaction with the environment, for example
64 through the expression of behavioral variation, it may influence the initial
65 encounters with environmental bacteria and thus affect the composition of the host
66 microbiota.
67 Many animals utilize different habitats according to behavioral strategies
68 collectively termed habitat selection. If habitats differ in their microbial
69 communities, host behavior influencing habitat choice and the microbiome may
70 influence each other. Hosts may have evolved strategies to ensure or avoid
71 encounter with beneficial and pathogenic microorganisms. Avoidance behaviors of
72 harmful bacteria are well documented, and behavior is considered one of the first
73 lines of defense against infectious disease. For example, the nematode
74 *Caenorhabditis elegans* actively avoids pathogenic bacteria and the genetic
75 determinants of this behavior have been worked out (Meisel & Kim 2014). The
76 opposite case, where a host's behavior is involved in the acquisition of beneficial
77 bacteria from the environment, has received less attention, despite speculation
78 about the role of human behaviors such as outdoor play in preventing autoimmune
79 diseases (Rook 2013). The overall effects of host habitat choice behavior on

80 microbiome composition have not, to our knowledge, been explored in any system.
81 An analysis of natural genetic variation in behavioral traits potentially influencing
82 microbiota acquisition is therefore timely (Ezenwa et al. 2012). If variation in
83 behavior affects the composition of the host's microbial community, then behavior
84 could underlie some genotype-environment interaction effects on microbiota. The
85 goal of this study was to examine the effect of genetic variation in host behavior on
86 microbiota composition in different environments using the freshwater planktonic
87 crustacean *Daphnia magna*.
88 Recently, it has been shown that *D. magna* microbiota play a major role in
89 host fitness (Sison-Mangus et al. 2015), that both host clonal line and environmental
90 factors are determinants of microbiota community structure (Sullam et al. 2017)
91 and that genotype-specific microbiomes can mediate daphnids' adaptive traits
92 (Macke et al. 2017). However, little is known about the mechanisms by which the
93 host acquires microbiota from the environment. A specific behavior, termed
94 sediment browsing, mediates the interaction between *D. magna* and bottom
95 sediments of ponds and lakes (Horton et al. 1979; Decaestecker et al. 2002). During
96 browsing, the animals swim along the sediment surface, stirring up particles, and
97 then ingest the particles by filter feeding. Besides representing valuable food
98 reservoirs, sediments are likely important environmental sources of bacteria.
99 Therefore, the physical contact with the sediments resulting from browsing might
100 present both disease risks and benefits from increased contact with bacteria.
101 Previous work found evidence of genetic variation for the levels of browsing activity
102 in *D. magna* (Arbore et. al. 2016).
103 We performed a laboratory experiment in which we analyzed the browsing
104 behavior and microbiota of 12 genetically distinct *D. magna* clones allowed to
105 browse in sediment. The animals were exposed to three different treatment
106 conditions, where they had access to either previously autoclaved (i) or untreated
107 ("natural" and therefore microbe-rich) sediments (ii), or where their access to
108 natural sediment was prevented (iii) (Figure 1A). We hypothesized that *D. magna*
109 clones exhibiting more intense browsing behavior would have more diverse
110 microbiota in conditions where they had access to bacteria-rich sediment. In this

experiment, we made no assumptions about whether bacteria found in the sediments were beneficial, harmful, or neutral for the host, nor whether they colonized *Daphnia* stably or transiently; therefore, the patterns observed here could be applicable to studies of disease, microbiota, or general environmental microbial community dynamics. Our analysis illustrates how a behavioral trait can mediate the interplay between genetic and environmental variation in the establishment of host-microbe associations.

Methods

Overview of the experiment

In this study, we combined the analysis of animals with constitutive (genetic) differences in browsing behavior with manipulations of the environment that affected animals' access to the sediments. Animals were either exposed to natural sediment, to autoclaved sediment, or to natural sediment blocked by a permeable net barrier (Figure 1A). In order to analyze both the browsing behavior of the animals and their microbiota, we placed two animals in each jar; of these pairs of animals, after 6 days of exposure to the different treatments, one animal was used to assay browsing behavior while the other was used for microbiota analyses.

Experimental animals

D. magna reproduces by cyclical parthenogenesis. Clonal populations can be generated and propagated in the laboratory through asexual reproduction. Here, we refer to such genetically identical individuals as "replicates" or "animals" while we refer to different genetic lines as "clones." In this study we used 12 *D. magna* clones from our stock collection, originating from different populations (Table 1). The animals were propagated from stock cultures maintained in the laboratory in standardized conditions and without any effort to modify their microbiota. The browsing behavior of these clones has been assessed before (Arbore et al. 2016) and was shown to differ among genotypes.

141 All animals used in this study were females. Prior to the experiment, every
142 clone was propagated in individual replicates for three generations in order to
143 minimize variation due to maternal effects. These animals were kept individually in
144 100-ml glass jars filled with 80 ml of ADaM (*Daphnia* medium (Kluttgen et al. 1994))
145 randomly distributed within trays in incubators with a 16:8 light/dark cycle and
146 constant temperature of 20 °C. To establish every generation, the animals were
147 isolated at 4 days old and fed daily with chemostat-grown green algae *Scenedesmus*
148 sp: 1 x 10^6 algae cells/animal until day 5, 2 x 10^6 until day 8, 2.5 x 10^6 until day 10, 3
149 x 10^6 until day 12, and 5 x 10^6 onwards. The animals were transferred to fresh
150 medium when they were 12 days old and thereafter every day.
151 For the experiment, we used animals from the 4th generation of each of the
152 12 clones. These animals were kept in groups of 8 siblings belonging to one clutch of
153 one mother. At 4 days old (± 1 day), 6 animals from every clutch were randomly
154 assigned in pairs to individual jars divided into the three different treatments (split
155 brood design); each such jar containing a pair of animals was an experimental
156 replicate. In total, we included in the experiment 540 animals (270 pairs)
157 corresponding to 15 pairs of clone BE-OHZ-T10, 18 pairs of clones DE-K35-Mu10
158 and NO-V-7, 21 pairs of clone F2-918, and 24 pairs of each of the remaining clones.
159 Variation in replicate numbers resulted from differences in availability of female
160 offspring at the time that the treatments were established.
161
162 *Experimental design*
163 The experiment was conducted in cylindrical glass jars (height = 20 cm;
164 diameter = 6.5 cm) (Fig. 1A) kept in cardboard boxes on shelves in a climate room
165 (16:8 light/dark cycle at 20 °C), loosely covered with transparent plastic film and
166 top-illuminated with neon lights. In this way, light only entered the jars from the
167 top. All the experimental jars were first filled with 400 ml of medium. 15 ml of a
168 suspension of loess (fine silt) was then carefully deposited on the bottom using a
169 serological pipette. The loess was previously collected from a soil stock, suspended
170 in water, passed through a 200 μm filter and washed to remove very fine particles.
171 After two days of sedimentation, the loess formed a 1 cm layer at the bottom of the

172 jar. Then, an acrylic tube (height = 21 cm; diameter = 5 cm) was inserted into the
173 jars and kept in position with a plastic ring fixed to the opening of the jar, so that its
174 lower end was positioned close to the sediment surface. In one treatment (NET), the
175 acrylic tube was closed with a 500 µm net at the lower end (suspended 5 mm above
176 the sediment surface) preventing animals from direct contact with the sediment
177 (Fig.1A left). In the other two treatments (AUT and SED), the acrylic tubes had no
178 net so that the animals had free access to the sediment (Fig.1A right). In the AUT
179 treatment, the loess was previously autoclaved while in the SED and the NET
180 treatment the loess was left untreated ("natural"). (After autoclaving, AUT sediment
181 was handled in the same way as natural sediment, i.e. exposed to nonsterile media
182 and laboratory environment.) After inserting the tubes, the jars were left
183 undisturbed for two days before the animals were introduced in order to allow the
184 sediment to settle. Immediately before the experiment, the sediments of three jars
185 of each of the SED and the AUT treatment were sampled and frozen at -20 °C; these
186 sampled jars were not used further.
187 Two animals from the same clutch were carefully introduced into the inner
188 tube of each jar. The 264 jars, each containing one pair of animals, were evenly
189 distributed among the treatments and their positions in the incubator room were
190 randomized. The animals remained in the experimental jars for 6 days. During this
191 time, the animals were carefully fed twice daily with 2.5 x 10^6 algal cells. At day 6, all
192 animals were collected and one member of every pair was assigned to the
193 behavioral assay (see below) and the other was frozen for later DNA extraction. 32
194 pairs of animals were lost or damaged during the experiment and were excluded
195 from further analyses. At the end of the experiment, 3 sediment samples from the
196 NET treatment and 3 sediment samples from the AUT treatment were collected and
197 frozen at -20°C.
198
199 *Behavioral analysis*
200 The animals for the behavioral assay were transferred individually from the
201 sediment jars to 100-ml glass jars filled with medium and kept in an incubator and
202 fed daily with 5 x 10^6 algal cells. The behavior assay was conducted over two days

203 when the animals were 12 to 14 days old with all replicates for the different clone
204 by treatment combinations evenly distributed across time. The behavior assay was
205 performed as described in Arbore et al. (2016). Briefly, we measured the traces left
206 by individual replicate animals on a smooth surface layer of sediment (loess) at the
207 bottom of tall cylindrical glass jars (20 cm tall, 6.5 cm diameter; Fig. 1B) during 30
208 minutes. The sediment surface was photographed before animals were released
209 (time 0), using a ring light to ensure uniform illumination. The jar was then
210 transferred into a cardboard tube and illuminated from the top with a neon light
211 and one animal was introduced in each jar. After exactly 30 minutes, the animal was
212 removed and the sediment surface was again photographed (time 1), in the same
213 position and under the same light conditions. Using the software ImageJ
214 (http://rsb.info.nih. gov/ij/), the pictures were converted to grey scale and a central
215 circular area was cropped to exclude shadows from the edge of the jar (Fig. 1B).
216 Pictures were processed such that the browsing traces of the animals on the
217 sediment surface resulted black areas against a white background. Then the number
218 of black pixels was quantified. Pictures taken at time 0 were used to correct the
219 values calculated for the browsing traces when irregularities on the sediment
220 surface were detected (i.e. in cases the picture of time 0 was not entirely white). The
221 pixel values were then log-transformed ([$\log_{10}(X+1000)$]; 1000 corresponds
222 approximately to the number of pixels of one individual browsing trace). During the
223 assay, four animals were accidentally damaged while handling and were excluded
224 from the analyses. The body lengths of the animals used for behavior analysis were
225 measured after the behavioral assay.
226 The adjusted intra-class correlation coefficient for the browsing behavior
227 (equivalent to broad sense heritability) was calculated with a linear mixed effect
228 (LMM) model, with treatment as a fixed effect and clone as a random effect (R
229 software package rptR developmental version; (Nakagawa & Schielzeth 2010)).
230 Confidence intervals and statistical significance were calculated using parametric
231 bootstrapping with 5000 iterations and a randomization procedure with 5000
232 permutations.
233

234 *DNA extraction, library preparation and sequencing*

235 The animals assigned to the microbiota analysis were transferred
236 individually from the sediment treatment jars to 40 mL of autoclaved ADaM for
237 about 2 hours to dilute carryover of unattached bacteria. Then, the animals were
238 transferred into 1.5 ml Eppendorf tubes, the ADaM was removed and the tubes were
239 stored at -20 °C until DNA extraction.

240 DNA was extracted from single animals using a cetyltrimethylammonium
241 bromide (CTAB)-based protocol. The animals were ground with a sterile pestle in
242 1.5 ml Eppendorf tubes in a 10 mg/ml lysozyme solution and mixed at 850 rpm and
243 37 °C for 45 minutes. Then, a 20 mg/ml solution of proteinase K was added and the
244 tubes mixed at 850 rpm and 55 °C for 1 hour. After an RNase treatment (20 mg/ml)
245 at room temperature for 10 minutes, a preheated 2X solution of CTAB was added
246 and the tubes mixed at 300 rpm and 65 °C for 1 hour. After two rounds of
247 chloroform isoamyl alcohol (CIA) purification (1 volume CIA; 8 minutes
248 centrifugation at 12,0000 rpm and 15 °C), a solution of sodium acetate 3M pH 5.2
249 and isopropanol were added to the DNA solution and the tubes were stored
250 overnight at -20 °C. The following day, DNA was purified by two rounds of 70 %
251 ethanol precipitation and suspended in water. The extractions were then incubated
252 at 4 °C overnight and then stored at -20 °C.

253 All DNA extractions were conducted over a period of 6 days with samples
254 from the different clone by treatment combinations randomly distributed between
255 the days and one reagent-only negative control extraction included every day. DNA
256 from the sediment samples and from one negative control was extracted on a
257 different day using a commercial kit (PowerSoil® DNA Extraction kit; MO BIO
258 Laboratories, cat. 12888-100).

259 We sequenced amplicons of the V3-V4 variable region of the bacterial 16S
260 rRNA gene using the Illumina MiSeq platform. Amplicons were generated using
261 NEBNext High Fidelity PCR Master Mix (New England Biolabs catalog#M0541L) for
262 27 cycles in 25 μl reactions containing 3% DMSO. The primers used were 341F (5'-
263 TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGA-3') and 785R (5'-

264 GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCAGA-3') with Illumina adapter
265 sequences and 0-3 bp random frameshifts. PCR product was purified with Ampure
266 beads at a 0.6x ratio of beads to PCR product, amplified for 9 cycles with Nextera XT
267 v2 indexing primers, and purified again. Libraries were quantified with Qubit and
268 quantitative PCR, normalized, and pooled, followed by additional bead purification
269 to remove remaining short fragments before sequencing on the Illumina MiSeq
270 (reagent kit v3, 300 bp paired-end reads). The same library pool was used for two
271 MiSeq runs; after checking that there was no statistical difference in community
272 composition between the runs (Adonis analysis of Bray-Curtis dissimilarity between
273 samples, p=0.394), the data from the two runs were merged.
274
275 *Sequence quality control*
276 Raw reads were quality controlled with FastQC (Babraham Institute, UK).
277 Paired reads were merged (FLASH v1.2.9), primers trimmed (Cutadapt v1.5), and
278 quality filtered (PRINSEQ-lite v0.20.4). OTU clustering including abundance sorting
279 and chimera removal was performed using the UPARSE workflow (Edgar 2013).
280 Only those OTUs represented by 5 or more reads in the run were included.
281 Taxonomic assignment was performed using UTAX against the GreenGenes v13/5
282 database. We analyzed samples with more than 5000 total reads. This left 214
283 samples; numbers of replicates for each combination of variables are reported in
284 Table 1.
285 Since individual *Daphnia* contain low bacterial biomass, we considered the
286 issue of reagent contamination with bacterial DNA (Salter et al. 2014). Samples were
287 processed in haphazard order, so erroneous sequences originating from reagent
288 contamination were expected to be distributed randomly and not confounded with
289 any treatment or genotype. For our research question, we were interested in
290 patterns of diversity and changes in composition in response to experimental
291 factors rather than in the presence or absence of any particular strain. For all
292 analyses, we first tested for processing batch effects and stratified the main analysis
293 by batch if they were significant.

294 For statistical analyses in which host clone was a fixed effect, we excluded
295 clone NO-V-7, since it did not have at least 3 replicates in each treatment; we
296 included this clone in analyses where clone was treated as a random effect. We
297 examined the effects of experimental factors on both overall diversity and the
298 community composition of each animal's microbiota using standard ecological
299 diversity indices and ordination methods. To evaluate the effect of animal behavior
300 on microbiota, we used as proxies for individual behavior either the mean browsing
301 intensity index of the clone or the browsing intensity of the individual co-housed
302 with the sequenced individual in the same jar ("jar-mate").
303 Analyses were carried out in R (3.4.3), using the packages phyloseq (1.22.3),
304 vegan (2.4.6), plyr (1.8.4), dplyr (0.7.4), DESeq2 (1.18.1), nlme (3.1.131), lme4
305 (1.1.15), metacoder (0.2.0.9012), and ggplot2 (2.2.1).
306
307 **Results**
308
309 *Browsing intensity, animal microbiota, and sediment bacteria*
310 Consistently with previous studies (Arbore et al. 2016), browsing behavior
311 intensity varied among *Daphnia* clones (Fig. 2). Clone and treatment, but not their
312 interaction, had a significant effect on browsing behavior (analysis of variance:
313 clone F=12.717, df=11, p<0.0001; treatment F=4.100, df=2, p=0.018;
314 clone*treatment F=1.274, df=22, p=0.193). The average browsing intensity of
315 animals from the NET treatment was lower than that of animals in the SED and AUT
316 treatments (Fig. S1). The total phenotypic variance for browsing behavior explained
317 by clone, after controlling for the treatment effect, corresponded to 36.5% (95% CI
318 = [13.2, 55.3%], p = 0.0002). Clone but not treatment had a significant effect on body
319 size, so we assume that access to (and type of) sediment did not substantially affect
320 nutrition and growth over the timeframe of the experiment (analysis of variance:
321 clone F=8.08, df=11, p < 0.001; treatment F=2.01, df=2, p=0.137; clone*treatment
322 F=1.43, df=22, p=0.103). Individual body size was uncorrelated with behavior
323 (analysis of variance: F=0.346, df=1, p=0.56; Fig. S2).

324 A total of 370 OTUs were found among the animal samples; of these, 318
325 were found in less than 10% of samples. (See Fig. S3A-C for taxonomic heat trees of
326 OTUs with presence/absence information) (Foster et al. 2016). Consistently with
327 multiple previous studies of *Daphnia* microbiota (Peerakietkhajorn et al. 2015; Qi et
328 al. 2009; Callens et al. 2015; Eckert & Pernthaler 2014), the most abundant bacterial
329 species was a single strain (OTU_1) of *Limnohabitans* sp (Betaproteobacteria,
330 Comamonadaceae), with a mean relative abundance across all clones of 0.39 (s.e.m.
331 0.02). Interestingly, a second strain of *Limnohabitans* (OTU_2) was a dominant
332 strain only in the three clones originating from clones bred in the laboratory as part
333 of a genetic breeding design (QTL panel; 0.32 mean relative abundance among
334 individuals of clones IXF1, F2-82, F2-918; 0.0016 mean relative abundance in
335 remaining clones). As expected, the sediment originating from the SED treatment
336 had much higher bacterial species richness than that originating from the AUT
337 treatment (Fig. S4).
338
339 *Effects of treatment and clone on alpha diversity*
340 Both *Daphnia* clone and treatment, but not their interaction, had significant
341 effect on the Shannon and inverse Simpson alpha diversity indices (Table 2). For
342 further analyses, we focused on the Shannon index, because it takes into account not
343 only species richness but also evenness (with additional species given more weight
344 as they become more abundant). The Shannon index displayed no significant effect
345 of processing batch (df=5, F=1.42, p=0.22). Shannon diversity estimates for the 12
346 clones arranged in order of increasing average browsing intensity and the three
347 groups (AUT, NET and SED) are shown in Fig. 3 (species richness and inverse
348 Simpson index are shown in Fig. S5A-B). Unexpectedly, the highest average alpha
349 diversity in most clones (9/12) was observed in the AUT treatment group, despite
350 their exposure to less-diverse sediment than the SED group. Therefore, diversity of
351 animal microbiota does not directly reflect diversity of bacteria in the environment.
352 To specifically investigate the effect of direct access to the same bacteria-rich
353 sediment, we compared the NET and SED treatment groups' diversity as a function
354 of clonal average behavior in each group (Fig. 4A). The difference in mean Shannon

355 diversity between SED and NET animals was greatest at the highest average clonal
356 level of browsing intensity (Fig. 4B; linear regression p=0.055). A similar tendency
357 could be seen when the browsing intensity of each individual's jar-mate was used as
358 the proxy for individual behavior (Fig. S6). Shannon diversity significantly depended
359 on the interaction between treatment and clonal average browsing intensity in a
360 linear mixed-effects model with clone included as a random effect (Table 3); the
361 same was true when treatment-specific clonal average behavior was used as the
362 behavior proxy, but not when individual jar-mate behavior was used (Table S1).
363
364 *Community composition and acquisition of bacteria from sediment*
365 To examine shifts in bacterial community composition in response to
366 environmental treatments, we Hellinger-transformed the bacterial abundances by
367 taking the square root of the relative abundance of each taxon in each sample to
368 reduce the influence of rare taxa, and then calculated pairwise Bray-Curtis distances
369 between samples. The average distance to the centroid (dispersion) was lower in
370 the NET group than in the AUT and the SED groups (Fig. 5), suggesting that access to
371 sediment increases variability of microbiota regardless of the composition of the
372 sediment. To see whether the different sediments resulted in systematically
373 different microbiota composition, we excluded the NET group and performed
374 principal coordinates analysis (PCoA) (Fig.6). Adonis analysis stratified by
375 processing batch showed that both treatment and clone had a significant effect
376 (treatment: R^2=0.05, p=0.001; clone: R^2=0.16, p=0.001), but not their interactions
377 (treatment:clone p=0.63). However, clones also showed significant differences in
378 dispersion (p<0.001).
379 Having confirmed that differences in the sediment environment resulted in
380 differences in animal microbiota composition, we next explored the extent to which
381 environment-specific bacteria contributed to these differences. We used DESeq2 to
382 determine which bacteria were significantly more present in natural sediment than
383 autoclaved sediment (n=3 each). 115 OTUs were calculated to be significantly
384 differentially present between the two sediment types; of these, 48 had at a
385 log2-fold increase of at least 8 in natural sediment compared to autoclaved

386 sediment. We refer to these as natural-sediment-derived taxa. The 8-fold threshold
387 was chosen based on inspecting the data; similar results were seen when sediment-
388 derived bacteria were defined by a log2-fold change of 5 or 10; see Fig. S7. Only one
389 of these OTUs was found in a majority of animals, and the median number of
390 animals in which a given OTU was found was 6.5. We therefore concluded that
391 animals likely acquired environmental bacteria randomly rather than selectively
392 from the environment. Accordingly, we looked at the total relative abundance of all
393 natural sediment–derived bacteria in each individual.
394 The relative abundance of natural-sediment-derived bacteria was generally
395 low in both the AUT and NET treatment groups, and increased with browsing
396 intensity in the SED treatment group (Fig. 7), with a significant interaction effect
397 between treatment and clonal average browsing intensity (Table 4). Treatment-
398 specific clonal average behavior showed the same significant interaction effect with
399 treatment, but the interaction effect was not significant when jar-mate behavior was
400 used as the behavior proxy. Among the set of clones examined here, an appreciably
401 high relative abundance of sediment-derived bacteria was detectable mainly in
402 clones with a browsing intensity index higher than mean 4.4 (clones IXF1, NO-V-7,
403 DE-KA-F28, CZ-N2-6, CZ-N1-1, F2-918). The mean relative abundance of sediment-
404 derived bacteria in the SED treatment in the pooled animals from these clones was
405 0.14 (s.e.m. 0.026), whereas it was 0.048 (s.e.m. 0.0097) in the lower-browsing
406 clones. Across all clones in the AUT and NET treatment groups, the average relative
407 abundance of sediment-derived bacteria was 0.051, nearly identical to that of the
408 low-browsing clones in the SED conditions.
409
410 **Discussion**
411 Our results have several implications for studies of animal-associated
412 microbiota in diverse environmental settings. First, we confirm the intuition that
413 environmental sources of bacteria affect the diversity of animal microbiota, but not
414 because more diverse environments always create more diverse microbiota; rather,
415 the animals we exposed to the less species-rich autoclaved sediments had higher
416 overall diversity in their microbiota than those exposed to untreated, bacterial-

417 species-rich sediment. We hypothesize that this might be due to competitive
418 interactions between *Daphnia* microbiota and the particular microbes found in
419 these sediments. The untreated sediments may contain bacteria that can
420 outcompete multiple strains of "native," preexisting *Daphnia* microbiota. If this were
421 the case, then browsing in sediment could have multiple opposing effects on overall
422 microbiota diversity: on the one hand, it would bring daphnids into contact with
423 more diverse bacteria, but on the other hand those bacteria could reduce existing
424 microbiota diversity. In the NET treatment, animals might be exposed to some
425 sediment-derived bacteria in the water column but lack access to the full diversity of
426 bacteria in the sediment. We also saw that having access to either sediment
427 increased the variability of community composition as measured by multivariate
428 dispersion. These results suggest that having access to multiple habitats with
429 different bacterial communities can affect the diversity and composition of an
430 animal's microbiota. Therefore, fine-scale heterogeneity in a host's habitat might be
431 a relevant aspect to take into account when examining effects of environment on
432 animal microbiota. This is especially important when considering ecological
433 immunology, because disease-causing bacteria in the environment may cause short-
434 term risk but also long-term fitness benefit via processes like immune priming
435 (Kaltenpoth & Engl 2014; Olszak et al. 2012).
436 Our data further suggest that the diversity of *Daphnia*-associated microbiota
437 in a particular environment may to some extent be mediated by genotype-specific
438 sediment browsing intensity. This was apparent as the net barrier made the greatest
439 difference in microbial alpha diversity in high-browsing host clones. However, this
440 effect may be partially obscured by several factors: the hypothesized competitive
441 exclusion effects we allude to above, and also non-behavior-related host genotype
442 effects on microbiota diversity. While host genotype had an effect on microbial
443 diversity, the highest- and lowest-browsing clones in our study had similar
444 microbial alpha diversity overall. It was only in evaluating the difference between
445 presence and absence of the barrier that an effect of browsing on diversity could be
446 seen. We conclude that the effect of environmental bacteria on host-associated
447 microbiota is not additive. The clearest effect of environmental bacteria on host-

448 associated microbiota was not on alpha diversity, but relative abundances of certain
449 taxa.
450 Clones with low average browsing intensity had no greater amount of
451 sediment-specific bacteria than animals exposed to autoclaved sediment or
452 prevented from browsing, whereas those with high browsing intensity could reach
453 over 60% of reads from environment-derived bacteria in some individuals. While
454 many studies of animal microbiota rightly concern themselves with distinguishing
455 between truly "host-associated" microbiota versus "transient environmental"
456 microbiota, these results raise the possibility that the amount of environmental
457 microbes found in association with an animal could itself be a host-genotype-
458 specific feature of the microbiome.
459 A key question for understanding symbiosis that future studies should
460 address is whether browsing behavior affects community composition by simple
461 exposure to more colonizing bacteria, or by more frequent replenishment of
462 bacterial taxa that would not otherwise persist in association with the host (Blum et
463 al. 2013). For example, browsing frequently enough may replenish bacteria that
464 would otherwise be lost when the animal molts. In this study, we made no
465 assumptions about the type of the interactions between the sediment-associated
466 bacteria and the *Daphnia*, but still were able to see that presence of sediment-
467 associated bacteria affected the bacteria that *Daphnia* were carrying.
468 It would also be interesting to investigate whether carriage of bacteria on
469 *Daphnia* from the sediment into the water column affects bacterial dynamics in the
470 larger environment; previous studies have shown that movement of *Daphnia*
471 between benthic and limnetic environments represents a mechanism of bacterial
472 dispersal in the environment (Grossart et al. 2010). Studies using classification
473 methods more sensitive than 16S-based taxonomy may be necessary to
474 unambiguously distinguish and assign sources to different bacterial strains.
475
476 **Conclusion**
477 We show that at least some characteristics of host-associated microbial
478 community composition result from genotype-by-microhabitat interactions,

479 specifically ones resulting from genotype-specific variation in behavior. Behavior
480 may thus be considered a genetic factor that shapes microbial exposure in a given
481 environment. Overall, these results provide further evidence that environment,
482 behavior, genetics, disease risk, and microbial community composition are
483 interrelated in potentially complex ways. Our observations indicate a need for more
484 integrative eco-immunology studies, in which the interfaces between behavioral
485 ecology, microbial community ecology and evolution of immune function are
486 explored. Studies can take advantage of the experimental tractability of the
487 *Daphnia*-microbiota system to further investigate these relationships in mechanistic
488 detail.
489

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493 providing sediment. Illumina sequencing was performed at the Genetic Diversity
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496

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499

500 **Author contributions**
501 AAM and RA conceived the study and performed the experiment with input from
502 DE. RA performed behavioral assays and analysis. AAM and RA prepared sequencing
503 libraries. JCW performed quality control and OTU clustering of sequence data. AAM
504 analyzed sequencing data. AAM and RA wrote the paper. DE and JCW revised the
505 paper.
506

507 **Data deposition**
508 Sequence data will be deposited in the European Nucleotide Archive of the EBI
509 upon publication. Data tables and code used for analysis can be found on Github at

510 https://github.com/amusheg/Daphnia-microbiota-behavior and will be deposited
511 in Dryad upon publication.
512
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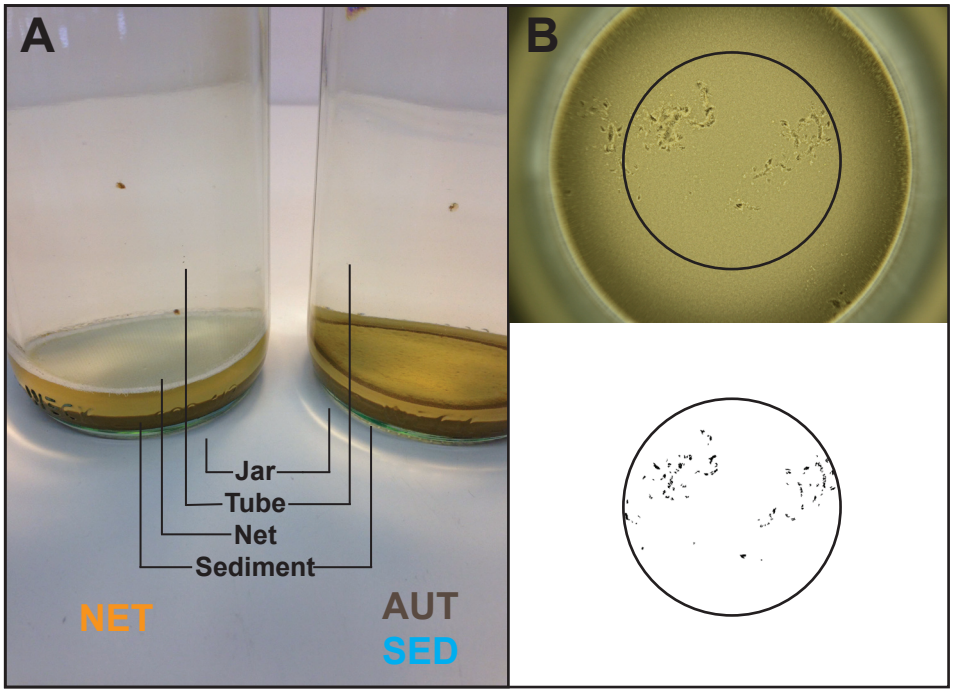
602

603 **Figure legends**

604 **Figure 1: Experimental set-up and browsing behavior assay.** A: the jars used in
605 the experiment had a bottom layer of fine loess and contained two animals each; the
606 animals were prevented from browsing on untreated sediments by a net placed 5
607 mm above the sediment surface (NET, right) or were allowed to browse on
608 autoclaved sediments (AUT) or untreated sediments (SED) (left). B: traces left by
609 one animal browsing on a sediment surface for 30 minutes and the same picture
610 after processing for quantification of the browsing behavior.



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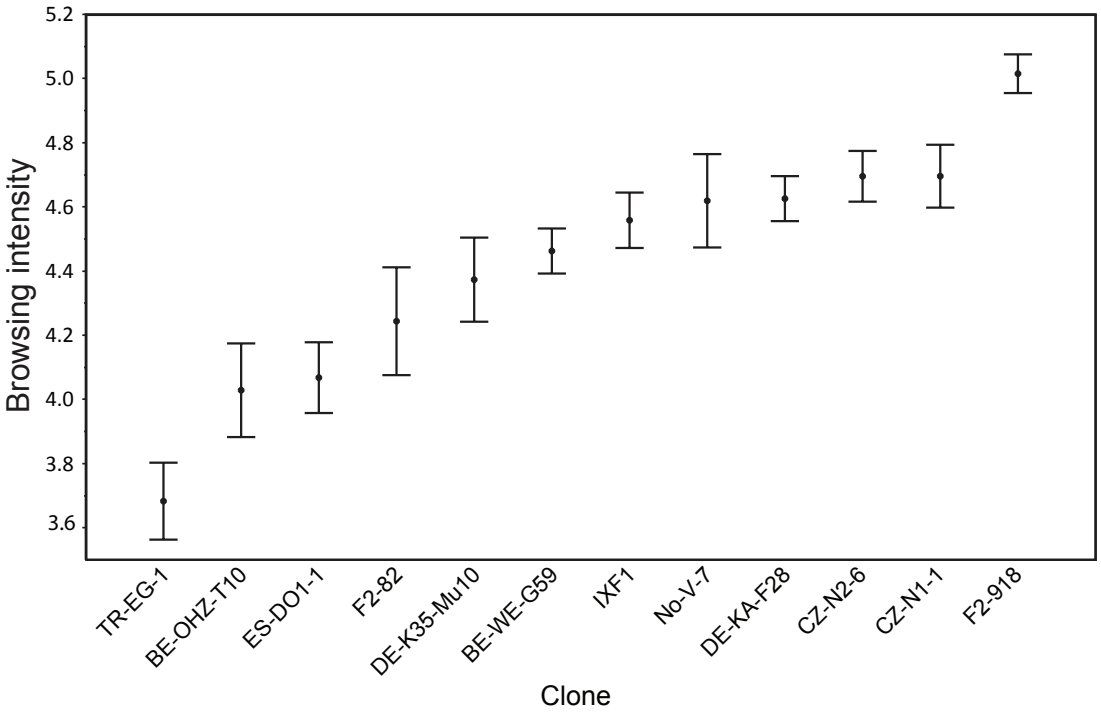
614 **Figure 2: Browsing intensity of 12 *D. magna* clones (mean and SE).** Browsing
615 intensity was defined as the Log_{10} of the area of the browsing traces left by
616 individual replicate animals browsing on a sediment surface for 30 minutes (see Fig.
617 1).



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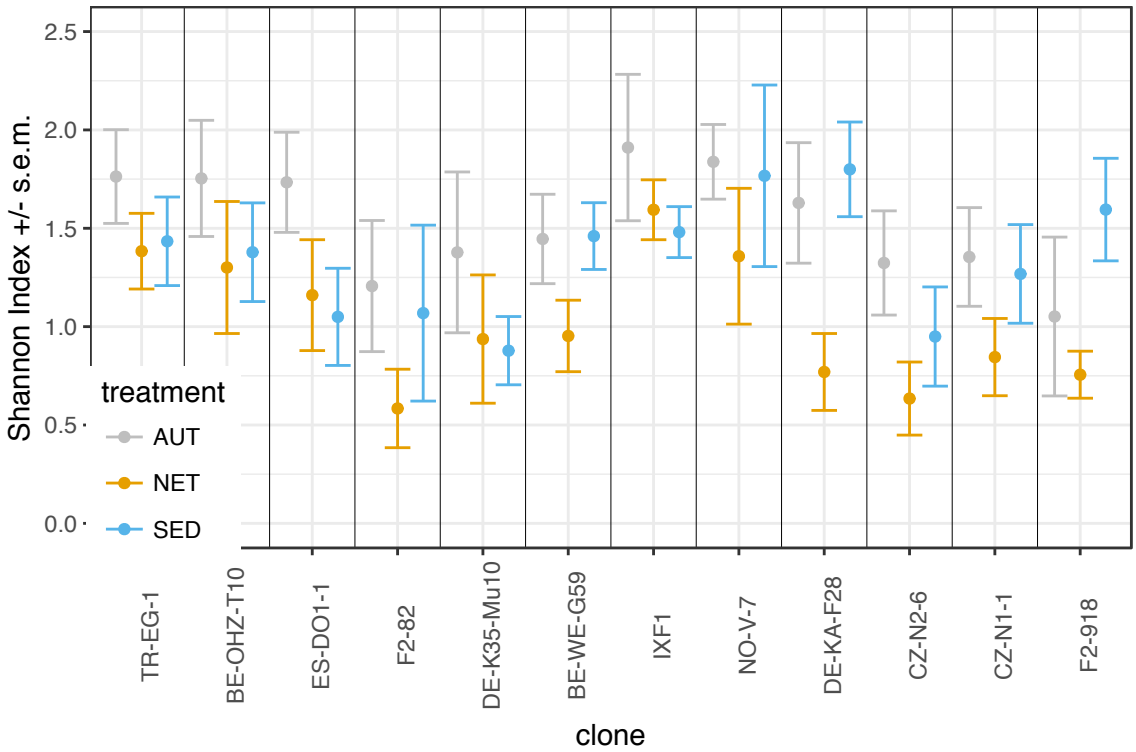
621 **Figure 3: Microbiota diversity (Shannon index) of *Daphnia* clones under three**
622 **different treatment conditions (AUT, NET and SED)**. AUT: Exposure to
623 autoclaved sediment; NET: prevented exposure to untreated sediment; SED:
624 exposure to untreated sediments. Error bars represent standard error of the mean.
625 Clones are arranged left-right by increasing average clone browsing intensity.



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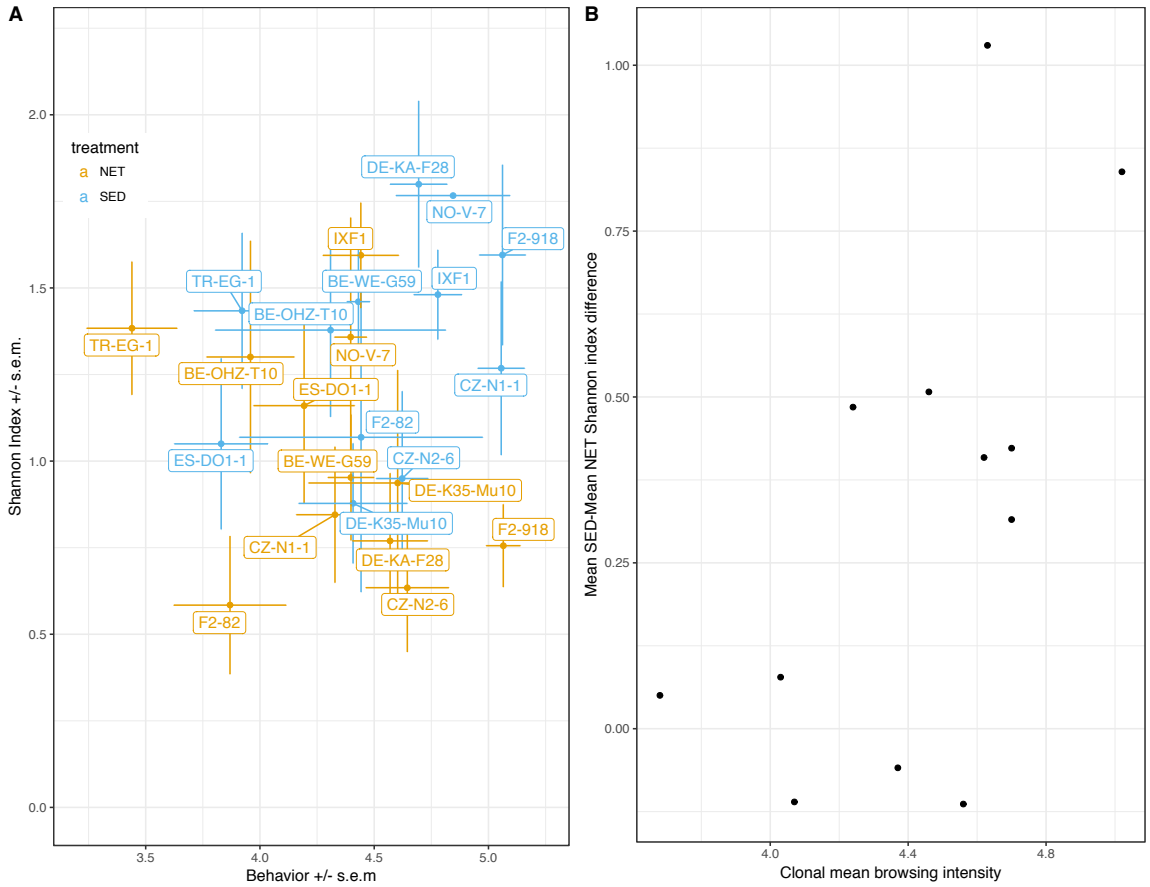
628 **Figure 4: Average browsing intensity and average microbiota diversity in the**
629 **NET and SED treatments**. A: average clone browsing intensity and average clone
630 microbiota diversity in the NET and SED treatments. Average browsing intensities
631 were calculated based on samples whose jar-mates passed the sequence quality
632 control (N=214, Table 1). B: average clone browsing intensity and the difference
633 between average Shannon diversity in the SED treatment and average diversity in
634 the NET treatment. Here, average browsing intensities were calculated based on the
635 complete set of samples (N=228, i.e. all assayed jar-mates). Error bars represent
636 standard error of the mean.



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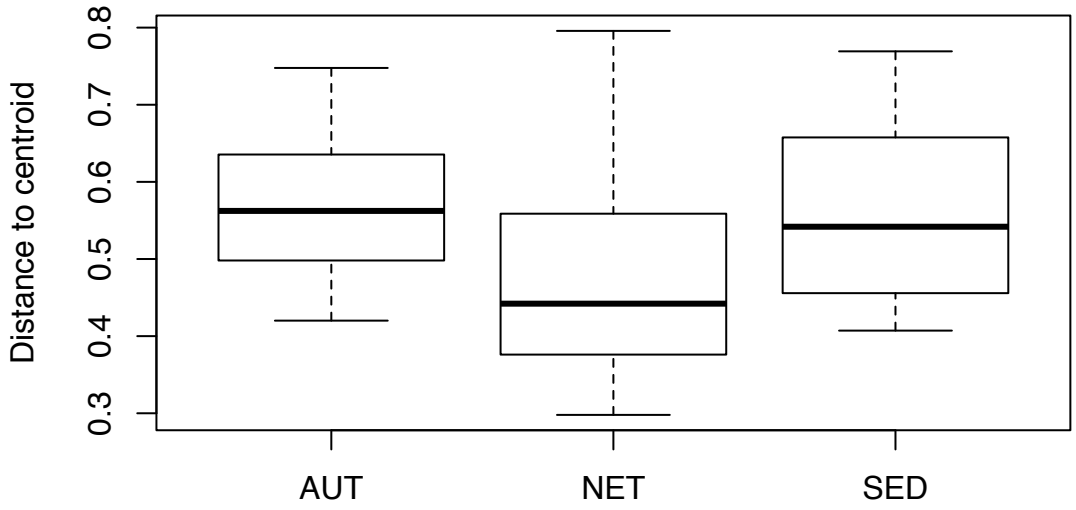
640 **Figure 5: Within-group dispersion of community similarity.** The median
641 distance to the centroid is lower in the NET treatment group than in the others
642 (Permutation test of multivariate dispersion p<0.0001, 999 permutations), meaning
643 that NET communities are less variable than AUT or SED microbiotas.



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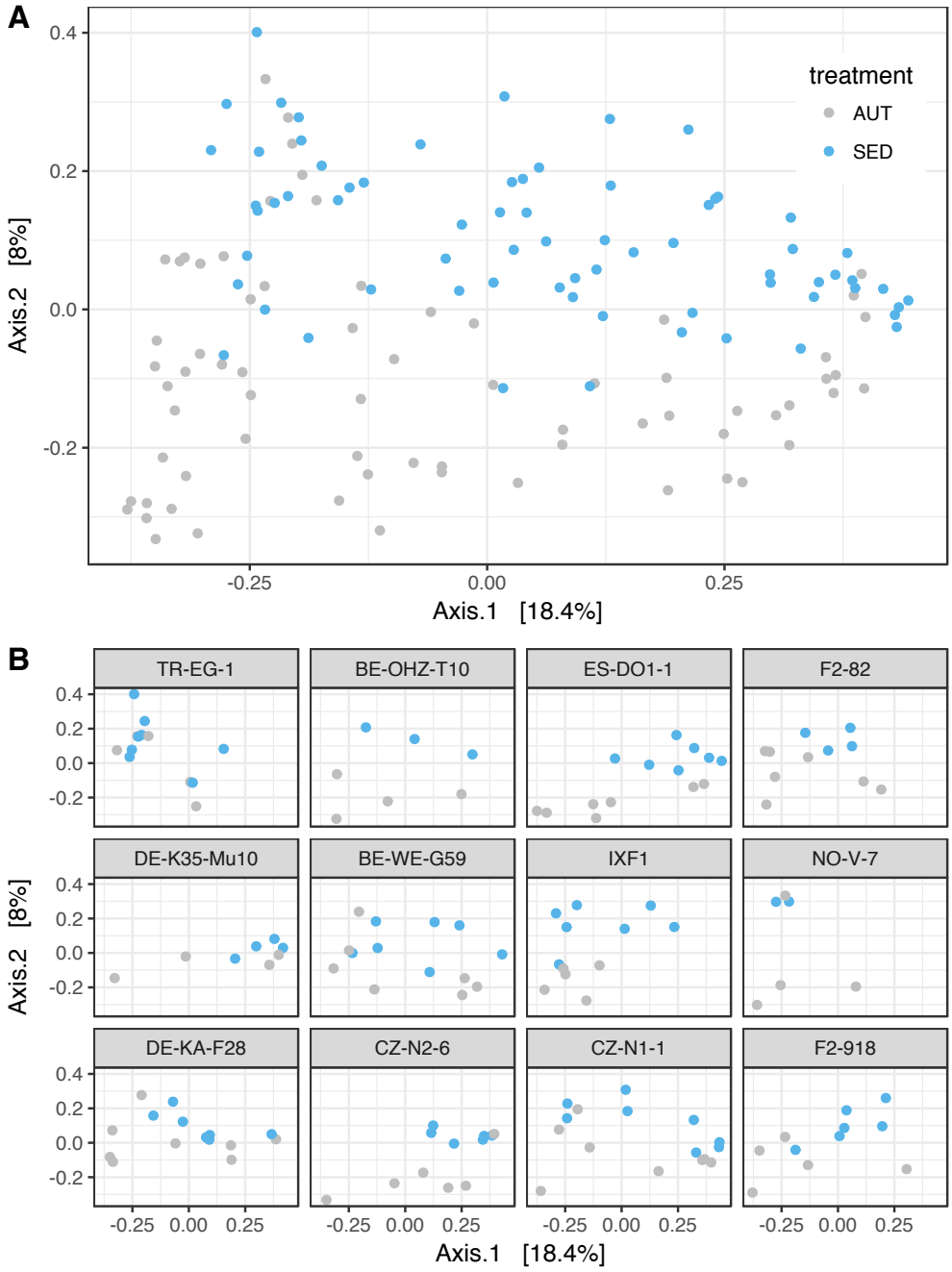
647 **Figure 6: Similarity of bacterial community composition in the AUT and SED**
648 **treatments.** A: first and second axis of a principal coordinates analysis (PCoA) of
649 bacterial community composition based on Hellinger-transformed Bray-Curtis
650 dissimilarities. B: first and second axis of a principal coordinates analysis (PCoA) of
651 bacterial community composition by *Daphnia* clone.



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653 **Figure 7: Analysis of sediment-derived bacteria.** Proportion of sediment-derived
654 bacteria in the microbiota of animals from AUT, NET and SED treatments. Sediment-
655 derived bacteria were identified by comparing autoclaved and untreated sediment
656 samples (log2-fold increase of at least 8 in natural sediment compared to autoclaved
657 sediment). Error bars correspond to standard errors of the mean. Clones are
658 arranged left-right by increasing average clone browsing intensity.
659

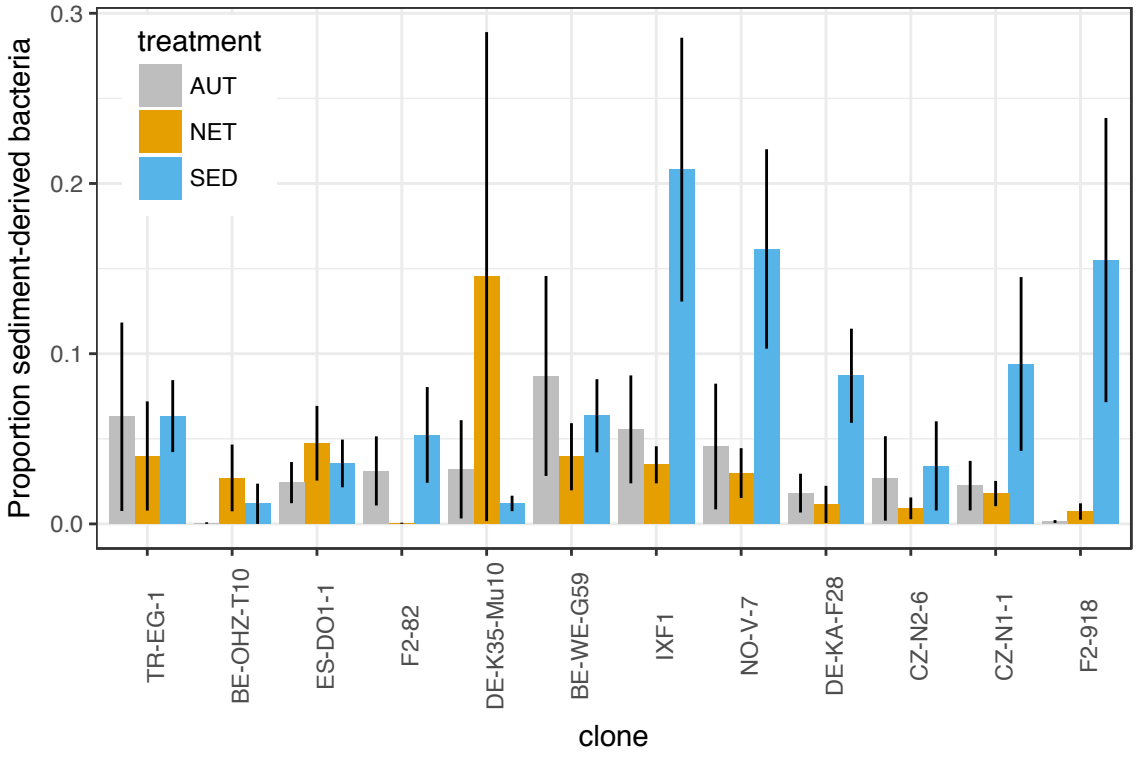

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Table 1. Names, number of individual replicates included in the microbiota analyses in the three treatments (AUT, NET and SED) and origin information of the 12 *Daphnia magna* clones used in this study. AUT: Exposure to autoclaved sediment; NET: prevented exposure to untreated sediment; SED: exposure to untreated sediments.

Clone ID	N (AUT)	N (NET)	N (SED)	Country	Latitude, N	Longitude E/W	Source	Description
BE-OHZ-T10	**4**	**5**	**3**	Belgium	50º50'00"N	4º39'00"E		
CZ-N1-1	**8**	**8**	**8**	Czech Rep.	48°46'31.14"N	16°43'24.70"E		
CZ-N2-6	**6**	**7**	**6**	Czech Rep.	48°46'31.14"N	16°43'24.70"E		
DE-K35-Mu10	**4**	**3**	**4**	Germany	48°12'23.93"N	11°42'34.98"E	*D. magna* Diversity panel	A geographically diverse collection of clones maintained asexually in the laboratory since 2012
DE-KA-F28	**8**	**7**	**7**	Germany	50°56'02"N	6°55'41"E		
ES-DO1-1	**7**	**6**	**7**	Spain	36°58'42.1"N	6°28'39.5"W		
TR-EG-1	**5**	**7**	**8**	Turkey	39°49' 25"N	32°49' 50"E		
BE-WE-G59	**7**	**8**	**7**	Belgium	51°04'04"N	3°46'25"E		
No-V-7	**4**	**3**	**2**	Norway	67°41'13.06"N	12°40'19.09E		

Clone ID				Description	Source	Description
IXF1	**5**	**8**	**7**	F1 clone	*D. magna* QTL panel	An intercross F2 recombinant panel maintained asexually in the laboratory since 2006/2007
F2-82	**7**	**7**	**4**	F2 clone		
F2-918	**5**	**6**	**6**	F2 clone		

Table 2. Results of analyses of variance of different alpha diversity indices. All treatments are included; clone NO-V-7 is excluded.

	Richness	Shannon	Inverse Simpson
Clone	F=0.40, df=10, **p=.944**	F=2.43, df=10, **P=.00997 ***	F=1.98, df=10, **p=.0383 ***
Treatment	F=4.91, df=2, **p=.00842 ***	F=12.21, df=2, **p<.0001 ***	F=13.25, df=2, **p<.0001 ***
Clone:Treatment	F=0.75, df=20, **p=.770**	F=0.78, df=20, **P=.734**	F=0.65, df=20, **P=.8124**

Table 3. Effect on Shannon index. NET and SED treatments only, all clones included. Linear mixed-effects model with treatment, clonal average browsing intensity and clonal average size as fixed effects and clone as random effect.

	numDF	denDf	F-value	p-value
(Intercept)	1	129	270.12	<.0001
Treatment	**1**	**129**	**12.26**	**0.0006 ***
Clone average behavior	1	9	0.275	0.613
Clone average size	1	9	2.568	0.144
Treatment:Clone average behavior	**1**	**129**	**4.677**	**0.0324 ***
Treatment:Clone average size	1	129	0.0980	0.755

Table 4. Effect on relative abundance of sediment-derived bacteria. All treatments and all animals included. Linear mixed-effects model with treatment and clonal average browsing intensity as fixed effects and clone as random effect.

	numDF	denDf	F-value	p-value
(Intercept)	1	198	53.999	<.0001
Treatment	**2**	**198**	**6.500**	**0.0018***
Clone average behavior	1	10	0.490	0.4998
Treatment:Clone average behavior	**2**	**198**	**4.185**	**0.0166***