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 (1). 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 . 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 (2) or within social groups (3), but the diversity of microbiota typically changes over time depending on the microbes available in the environment (4, 5). In some cases, environmentally acquired microbes are even essential for the completion of postembryonic development (6, 7). Thus microbes from the

47 environment can be co-opted as part of the microbiota, or can affect host health
48 during a transient occupation (8).
49 Environmental effects on microbiota community structure have been
50 extensively documented (9, 10) and studies on model organisms have started to
51 shed light on the relative importance of environmental and host genetic factors in
52 determining microbiota composition (11, 12). Recently, the focus has been moving
53 towards a better understanding of the mechanisms of bacterial aqusition from the
54 environment. Host genetics have been shown to play a role in the establishment of
55 microbial associations through microbial recognition, immune selection, and
56 determination of the biochemical niche (12). Importantly, these processes select
57 microbes after the host has come in contact with bacterial communities in the
58 environment. The initial encounter may be a key phase of the host's colonization by
59 microbes. If host genetics influence interaction with the environment, for example
60 through the expression of behavioral variation, it may influence the initial
61 encounters with environmental bacteria and thus affect the composition of the host
62 microbiota.
63 Many animals utilize different habitats according to behavioral strategies
64 collectively termed habitat selection. If habitats differ in their microbial
65 communities, host behavior influencing habitat choice and the microbiome may
66 influence each other. Hosts may have evolved strategies to ensure or avoid
67 encounter with beneficial and pathogenic microorganisms. Avoidance behaviors of
68 harmful bacteria are well documented, and behavior is considered one of the first
69 lines of defense against infectious disease. For example, the nematode
70 *Caenorhabditis elegans* actively avoids pathogenic bacteria and the genetic
71 determinants of this behavior have been worked out (13). The opposite case, where
72 a host's behavior is involved in the acquisition of beneficial bacteria from the
73 environment, has received less attention, despite speculation about the role of
74 human behaviors such as outdoor play in preventing autoimmune diseases (14).
75 The overall effects of host habitat choice behavior on microbiome composition have
76 not, to our knowledge, been explored in any system. An analysis of natural genetic
77 variation in behavioral traits potentially influencing microbiota acquisition is

78 therefore timely (15). If variation in behavior affects the composition of the host's
79 microbial community, then behavior could underlie some genotype-environment
80 interaction effects on microbiota. The goal of this study was to examine the effect of
81 genetic variation in host behavior on microbiota composition in different
82 environments using the freshwater planktonic crustacean *Daphnia magna*.
83 Recently, it has been shown that *D. magna* microbiota play a major role in
84 host fitness (16), that both host clonal line and environmental factors are
85 determinants of microbiota community structure (17) and that genotype-specific
86 microbiomes can mediate daphnids' adaptive traits (18). However, little is known
87 about the mechanisms by which the host acquires microbiota from the environment.
88 A specific behavior, termed sediment browsing, mediates the interaction between
89 *D. magna* and bottom sediments of ponds and lakes (19, 20). During browsing, the
90 animals swim along the sediment surface, stirring up particles, and then ingest the
91 particles by filter feeding. Besides representing valuable food reservoirs, sediments
92 are likely important environmental sources of bacteria. Therefore, the physical
93 contact with the sediments resulting from browsing might present both disease
94 risks and benefits from increased contact with bacteria. Previous work found
95 evidence of genetic variation for the levels of browsing activity in *D. magna* (21).
96 We performed a laboratory experiment in which we analyzed the browsing
97 behavior and microbiota of 12 genetically distinct *D. magna* clones allowed to
98 browse in sediment. The animals were exposed to three different treatment
99 conditions, where they had access to either previously autoclaved (i) or untreated
100 ("natural" and therefore microbe-rich) sediments (ii), or where their access to
101 natural sediment was prevented (iii) (Figure 1A). We hypothesized that *D. magna*
102 clones exhibiting more intense browsing behavior would have more diverse
103 microbiota in conditions where they had access to bacteria-rich sediment, whereas
104 the microbiome would be less affected by the bacterial environment in genotypes
105 that browsed less. In this experiment, we made no assumptions about whether
106 bacteria found in the sediments were beneficial, harmful, or neutral for the host, nor
107 whether they colonized *Daphnia* stably or transiently; therefore, the patterns
108 observed here could be applicable to studies of disease, microbiota, or general

109 environmental microbial community dynamics. Our analysis illustrates how a
110 behavioral trait can mediate the interplay between genetic and environmental
111 variation in the establishment of host-microbe associations.
112
113 **Methods**
114
115 *Overview of the experiment*
116 In this study, we combined the analysis of animals with constitutive (genetic)
117 differences in browsing behavior with manipulations of the environment that
118 affected animals' access to the sediments. Animals were either exposed to natural
119 sediment, to autoclaved sediment, or to natural sediment blocked by a permeable
120 net barrier (Figure 1A). In order to analyze both the browsing behavior of the
121 animals and their microbiota, we placed two animals in each jar; of these pairs of
122 animals, after 6 days of exposure to the different treatments, one animal was used to
123 assay browsing behavior while the other was used for microbiota analyses.
124
125 *Experimental animals*
126 *D. magna* reproduces by cyclical parthenogenesis. Clonal populations can be
127 generated and propagated in the laboratory through asexual reproduction. Here, we
128 refer to such genetically identical individuals as "replicates" or "animals" while we
129 refer to different genetic lines as "clones." In this study we used 12 *D. magna* clones
130 from our stock collection, originating from different populations (Table 1). The
131 animals were propagated from stock cultures maintained in the laboratory in
132 standardized conditions and without any effort to modify their microbiota. The
133 browsing behavior of these clones has been assessed before (21) and was shown to
134 differ among genotypes.
135 All animals used in this study were females. Prior to the experiment, every
136 clone was propagated in individual replicates for three generations in order to
137 minimize variation due to maternal effects. These animals were kept individually in
138 100-ml glass jars filled with 80 ml of ADaM (*Daphnia* medium (22)) randomly
139 distributed within trays in incubators with a 16:8 light/dark cycle and constant

140 temperature of 20 °C. To establish every generation, the animals were isolated at 4
141 days old and fed daily with chemostat-grown green algae *Scenedesmus* sp: 1 x 10^6
142 algae cells/animal until day 5, 2 x 10^6 until day 8, 2.5 x 10^6 until day 10, 3 x 10^6 until
143 day 12, and 5 x 10^6 onwards. The animals were transferred to fresh medium when
144 they were 12 days old and thereafter every day.
145 For the experiment, we used animals from the 4th generation of each of the
146 12 clones. These animals were kept in groups of 8 siblings belonging to one clutch of
147 one mother. At 4 days old (± 1 day), 6 animals from every clutch were randomly
148 assigned in pairs to individual jars divided into the three different treatments (split
149 brood design); each such jar containing a pair of animals was an experimental
150 replicate. In total, we included in the experiment 540 animals (270 pairs)
151 corresponding to 15 pairs of clone BE-OHZ-T10, 18 pairs of clones DE-K35-Mu10
152 and NO-V-7, 21 pairs of clone F2-918, and 24 pairs of each of the remaining clones.
153 Variation in replicate numbers resulted from differences in availability of female
154 offspring at the time that the treatments were established.
155
156 *Experimental design*
157 The experiment was conducted in cylindrical glass jars (height = 20 cm;
158 diameter = 6.5 cm) (Fig. 1A) kept in cardboard boxes on shelves in a climate room
159 (16:8 light/dark cycle at 20 °C), loosely covered with transparent plastic film and
160 top-illuminated with neon lights. In this way, light only entered the jars from the
161 top. All the experimental jars were first filled with 400 ml of medium. 15 ml of a
162 suspension of loess (fine silt) was then carefully deposited on the bottom using a
163 serological pipette. The loess was previously collected from a soil stock, suspended
164 in water, passed through a 200 µm filter and washed to remove very fine particles.
165 After two days of sedimentation, the loess formed a 1 cm layer at the bottom of the
166 jar. Then, an acrylic tube (height = 21 cm; diameter = 5 cm) was inserted into the
167 jars and kept in position with a plastic ring fixed to the opening of the jar, so that its
168 lower end was positioned close to the sediment surface. In one treatment (NET), the
169 acrylic tube was closed with a 500 µm net at the lower end (suspended 5 mm above
170 the sediment surface) preventing animals from direct contact with the sediment

171 (Fig.1A left). In the other two treatments (AUT and SED), the acrylic tubes had no
172 net so that the animals had free access to the sediment (Fig.1A right). In the AUT
173 treatment, the loess was previously autoclaved while in the SED and the NET
174 treatment the loess was left untreated ("natural"). (After autoclaving, AUT sediment
175 was handled in the same way as natural sediment, i.e. exposed to nonsterile media
176 and laboratory environment.) After inserting the tubes, the jars were left
177 undisturbed for two days before the animals were introduced in order to allow the
178 sediment to settle. Immediately before the experiment, the sediments of three jars
179 of each of the SED and the AUT treatment were sampled and frozen at -20 °C; these
180 sampled jars were not used further.
181 Two animals from the same clutch were carefully introduced into the inner
182 tube of each jar. The 264 jars, each containing one pair of animals, were evenly
183 distributed among the treatments and their positions in the incubator room were
184 randomized. The animals remained in the experimental jars for 6 days. During this
185 time, the animals were carefully fed twice daily with 2.5 x 10^6 algal cells. At day 6, all
186 animals were collected and one member of every pair was assigned to the
187 behavioral assay (see below) and the other was frozen for later DNA extraction. 32
188 pairs of animals were lost or damaged during the experiment and were excluded
189 from further analyses. At the end of the experiment, 3 sediment samples from the
190 NET treatment and 3 sediment samples from the AUT treatment were collected and
191 frozen at -20°C.
192
193 *Behavioral analysis*
194 The animals for the behavioral assay were transferred individually from the
195 sediment jars to 100-ml glass jars filled with medium and kept in an incubator and
196 fed daily with 5 x 10^6 algal cells. The behavior assay was conducted over two days
197 when the animals were 12 to 14 days old with all replicates for the different clone
198 by treatment combinations evenly distributed across time. The behavior assay was
199 performed as described previously (21). Briefly, we measured the traces left by
200 individual replicate animals on a smooth surface layer of sediment (loess) at the
201 bottom of tall cylindrical glass jars (20 cm tall, 6.5 cm diameter; Fig. 1B) during 30

202 minutes. The sediment surface was photographed before animals were released
203 (time 0), using a ring light to ensure uniform illumination. The jar was then
204 transferred into a cardboard tube and illuminated from the top with a neon light
205 and one animal was introduced in each jar. After exactly 30 minutes, the animal was
206 removed and the sediment surface was again photographed (time 1), in the same
207 position and under the same light conditions. Using the software ImageJ
208 (http://rsb.info.nih. gov/ij/), the pictures were converted to grey scale and a central
209 circular area was cropped to exclude shadows from the edge of the jar (Fig. 1B).
210 Pictures were processed such that the browsing traces of the animals on the
211 sediment surface resulted black areas against a white background. Then the number
212 of black pixels was quantified. Pictures taken at time 0 were used to correct the
213 values calculated for the browsing traces when irregularities on the sediment
214 surface were detected (i.e. in cases the picture of time 0 was not entirely white). The
215 pixel values were then log-transformed ([$\log_{10}(X+1000)$]; 1000 corresponds
216 approximately to the number of pixels of one individual browsing trace). During the
217 assay, four animals were accidentally damaged while handling and were excluded
218 from the analyses. The body lengths of the animals used for behavior analysis were
219 measured after the behavioral assay.
220 The adjusted intra-class correlation coefficient for the browsing behavior
221 (equivalent to broad sense heritability) was calculated with a linear mixed effect
222 (LMM) model, with treatment as a fixed effect and clone as a random effect (R
223 software package rptR developmental version; (23)). Confidence intervals and
224 statistical significance were calculated using parametric bootstrapping with 5000
225 iterations and a randomization procedure with 5000 permutations.
226
227 *DNA extraction, library preparation and sequencing*
228 The animals assigned to the microbiota analysis were transferred
229 individually from the sediment treatment jars to 40 mL of autoclaved ADaM for
230 about 2 hours to dilute carryover of unattached bacteria. Then, the animals were
231 transferred into 1.5 ml Eppendorf tubes, the ADaM was removed and the tubes were
232 stored at -20 °C until DNA extraction.

233 DNA was extracted from single animals using a cetyltrimethylammonium
234 bromide (CTAB)-based protocol. The animals were ground with a sterile pestle in
235 1.5 ml Eppendorf tubes in a 10 mg/ml lysozyme solution and mixed at 850 rpm and
236 37 °C for 45 minutes. Then, a 20 mg/ml solution of proteinase K was added and the
237 tubes mixed at 850 rpm and 55 °C for 1 hour. After an RNase treatment (20 mg/ml)
238 at room temperature for 10 minutes, a preheated 2X solution of CTAB was added
239 and the tubes mixed at 300 rpm and 65 °C for 1 hour. After two rounds of
240 chloroform isoamyl alcohol (CIA) purification (1 volume CIA; 8 minutes
241 centrifugation at 12,0000 rpm and 15 °C), a solution of sodium acetate 3M pH 5.2
242 and isopropanol were added to the DNA solution and the tubes were stored
243 overnight at -20 °C. The following day, DNA was purified by two rounds of 70 %
244 ethanol precipitation and suspended in water. The extractions were then incubated
245 at 4 °C overnight and then stored at -20 °C.
246 All DNA extractions were conducted over a period of 6 days with samples
247 from the different clone by treatment combinations randomly distributed between
248 the days and one reagent-only negative control extraction included every day. DNA
249 from the sediment samples and from one negative control was extracted on a
250 different day using a commercial kit (PowerSoil® DNA Extraction kit; MO BIO
251 Laboratories, cat. 12888-100).
252 We sequenced amplicons of the V3-V4 variable region of the bacterial 16S
253 rRNA gene using the Illumina MiSeq platform. Amplicons were generated using
254 NEBNext High Fidelity PCR Master Mix (New England Biolabs catalog#M0541L) for
255 27 cycles in 25 µl reactions containing 3% DMSO. The primers used were 341F (5'-
256 TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGA-3') and 785R (5'-
257 GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCAGA-3') with Illumina adapter
258 sequences and 0-3 bp random frameshifts. PCR product was purified with Ampure
259 beads at a 0.6x ratio of beads to PCR product, amplified for 9 cycles with Nextera XT
260 v2 indexing primers, and purified again. Libraries were quantified with Qubit and
261 quantitative PCR, normalized, and pooled, followed by additional bead purification
262 to remove remaining short fragments before sequencing on the Illumina MiSeq

263 (reagent kit v3, 300 bp paired-end reads). The same library pool was used for two
264 MiSeq runs; after checking that there was no statistical difference in community
265 composition between the runs (Adonis analysis of Bray-Curtis dissimilarity between
266 samples, p=0.394), the data from the two runs were merged.
267
268 *Sequence quality control*
269 Raw reads were quality controlled with FastQC (Babraham Institute, UK).
270 Paired reads were merged (FLASH v1.2.9), primers trimmed (Cutadapt v1.5), and
271 quality filtered (PRINSEQ-lite v0.20.4). OTU clustering including abundance sorting
272 and chimera removal was performed using the UPARSE workflow (24). Only those
273 OTUs represented by 5 or more reads in the run were included. Taxonomic
274 assignment was performed using UTAX against the GreenGenes v13/5 database. We
275 analyzed samples with more than 5000 total reads. This left 214 samples; numbers
276 of replicates for each combination of variables are reported in Table 1.
277 Since individual *Daphnia* contain low bacterial biomass, we considered the
278 issue of reagent contamination with bacterial DNA (25). Samples were processed in
279 haphazard order, so erroneous sequences originating from reagent contamination
280 were expected to be distributed randomly and not confounded with any treatment
281 or genotype. For our research question, we were interested in patterns of diversity
282 and changes in composition in response to experimental factors rather than in the
283 presence or absence of any particular strain. For all analyses, we first tested for
284 processing batch effects and stratified the main analysis by batch if they were
285 significant.
286 For statistical analyses in which host clone was a fixed effect, we excluded
287 clone NO-V-7, since it did not have at least 3 replicates in each treatment; we
288 included this clone in analyses where clone was treated as a random effect. We
289 examined the effects of experimental factors on both overall diversity and the
290 community composition of each animal's microbiota using standard ecological
291 diversity indices and ordination methods. To evaluate the effect of animal behavior
292 on microbiota, we used as proxies for individual behavior either the mean browsing

293 intensity index of the clone or the browsing intensity of the individual co-housed
294 with the sequenced individual in the same jar ("jar-mate").
295 Analyses were carried out in R (3.4.3), using the packages phyloseq (1.22.3),
296 vegan (2.4.6), plyr (1.8.4), dplyr (0.7.4), DESeq2 (1.18.1), nlme (3.1.131), lme4
297 (1.1.15), metacoder (0.2.0.9012), and ggplot2 (2.2.1).
298
299 **Results**
300
301 *Browsing intensity, animal microbiota, and sediment bacteria*
302 Consistently with previous studies (Arbore et al. 2016), browsing behavior
303 intensity varied among *Daphnia* clones (Fig. 2). Clone and treatment, but not their
304 interaction, had a significant effect on browsing behavior (analysis of variance:
305 clone F=12.717, df=11, p<0.0001; treatment F=4.100, df=2, p=0.018;
306 clone*treatment F=1.274, df=22, p=0.193). The average browsing intensity of
307 animals from the NET treatment was lower than that of animals in the SED and AUT
308 treatments (Fig. S1). The total phenotypic variance for browsing behavior explained
309 by clone, after controlling for the treatment effect, corresponded to 36.5% (95% CI
310 = [13.2, 55.3%], p = 0.0002). Clone but not treatment had a significant effect on body
311 size, so we assume that access to (and type of) sediment did not substantially affect
312 nutrition and growth over the timeframe of the experiment (analysis of variance:
313 clone F=8.08, df=11, p < 0.001; treatment F=2.01, df=2, p=0.137; clone*treatment
314 F=1.43, df=22, p=0.103). Individual body size was uncorrelated with behavior
315 (analysis of variance: F=0.346, df=1, p=0.56; Fig. S2).
316 A total of 370 OTUs were found among the animal samples; of these, 318
317 were found in less than 10% of samples. (See Fig. S3A-C for taxonomic heat trees of
318 OTUs with presence/absence information) (26). Consistently with multiple previous
319 studies of *Daphnia* microbiota (27–30), the most abundant bacterial species was a
320 single strain (OTU_1) of *Limnohabitans* sp (Betaproteobacteria, Comamonadaceae),
321 with a mean relative abundance across all clones of 0.39 (s.e.m. 0.02). Interestingly,
322 a second strain of *Limnohabitans* (OTU_2) was a dominant strain only in the three
323 clones originating from clones bred in the laboratory as part of a genetic breeding

324 design (QTL panel; 0.32 mean relative abundance among individuals of clones IXF1,
325 F2-82, F2-918; 0.0016 mean relative abundance in remaining clones). As expected,
326 the sediment originating from the SED treatment had much higher bacterial species
327 richness than that originating from the AUT treatment (Fig. S4).
328
329 *Effects of treatment and clone on alpha diversity*
330 Both *Daphnia* clone and treatment, but not their interaction, had significant
331 effect on the Shannon and inverse Simpson alpha diversity indices (Table 2). For
332 further analyses, we focused on the Shannon index, because it takes into account not
333 only species richness but also evenness (with additional species given more weight
334 as they become more abundant). The Shannon index displayed no significant effect
335 of processing batch (df=5, F=1.42, p=0.22). Shannon diversity estimates for the 12
336 clones arranged in order of increasing average browsing intensity and the three
337 groups (AUT, NET and SED) are shown in Fig. 3 (species richness and inverse
338 Simpson index are shown in Fig. S5A-B). Unexpectedly, the highest average alpha
339 diversity in most clones (9/12) was observed in the AUT treatment group, despite
340 their exposure to less-diverse sediment than the SED group. Therefore, diversity of
341 animal microbiota does not directly reflect diversity of bacteria in the environment.
342 To specifically investigate the effect of direct access to the same bacteria-rich
343 sediment, we compared the NET and SED treatment groups' diversity as a function
344 of clonal average behavior in each group (Fig. 4A). The difference in mean Shannon
345 diversity between SED and NET animals was greatest at the highest average clonal
346 level of browsing intensity (Fig. 4B; linear regression p=0.055). A similar tendency
347 could be seen when the browsing intensity of each individual's jar-mate was used as
348 the proxy for individual behavior (Fig. S6). Shannon diversity significantly depended
349 on the interaction between treatment and clonal average browsing intensity in a
350 linear mixed-effects model with clone included as a random effect (Table 3); the
351 same was true when treatment-specific clonal average behavior was used as the
352 behavior proxy, but not when individual jar-mate behavior was used (Table S1).
353
354 *Community composition and acquisition of bacteria from sediment*

355 To examine shifts in bacterial community composition in response to
356 environmental treatments, we Hellinger-transformed the bacterial abundances by
357 taking the square root of the relative abundance of each taxon in each sample to
358 reduce the influence of rare taxa, and then calculated pairwise Bray-Curtis distances
359 between samples. The average distance to the centroid (dispersion) was lower in
360 the NET group than in the AUT and the SED groups (Fig. 5), suggesting that access to
361 sediment increases variability of microbiota regardless of the composition of the
362 sediment. To see whether the different sediments resulted in systematically
363 different microbiota composition, we excluded the NET group and performed
364 principal coordinates analysis (PCoA) (Fig.6). Adonis analysis stratified by
365 processing batch showed that both treatment and clone had a significant effect
366 (treatment: R^2=0.05, p=0.001; clone: R^2=0.16, p=0.001), but not their interactions
367 (treatment:clone p=0.63). However, clones also showed significant differences in
368 dispersion ($p<0.001$).
369 Having confirmed that differences in the sediment environment resulted in
370 differences in animal microbiota composition, we next explored the extent to which
371 environment-specific bacteria contributed to these differences. We used DESeq2 to
372 determine which bacteria were significantly more present in natural sediment than
373 autoclaved sediment (n=3 each). 115 OTUs were calculated to be significantly
374 differentially present between the two sediment types; of these, 48 had at a
375 log2-fold increase of at least 8 in natural sediment compared to autoclaved
376 sediment. We refer to these as natural-sediment-derived taxa. The 8-fold threshold
377 was chosen based on inspecting the data; similar results were seen when sediment-
378 derived bacteria were defined by a log2-fold change of 5 or 10; see Fig. S7. Only one
379 of these OTUs was found in a majority of animals, and the median number of
380 animals in which a given OTU was found was 6.5. We therefore concluded that
381 animals likely acquired environmental bacteria randomly rather than selectively
382 from the environment. Accordingly, we looked at the total relative abundance of
383 reads from all natural sediment–derived bacteria in each individual.
384 The relative abundance of natural-sediment-derived bacteria was generally
385 low in both the AUT and NET treatment groups, and increased with browsing

386 intensity in the SED treatment group (Fig. 7), with a significant interaction effect
387 between treatment and clonal average browsing intensity (Table 4). Treatment-
388 specific clonal average behavior showed the same significant interaction effect with
389 treatment, but the interaction effect was not significant when jar-mate behavior was
390 used as the behavior proxy. Among the set of clones examined here, an appreciably
391 high relative abundance of sediment-derived bacteria was detectable mainly in
392 clones with a browsing intensity index higher than mean 4.4 (clones IXF1, NO-V-7,
393 DE-KA-F28, CZ-N2-6, CZ-N1-1, F2-918). The mean relative abundance of sediment-
394 derived bacteria in the SED treatment in the pooled animals from these clones was
395 0.14 (s.e.m. 0.026), whereas it was 0.048 (s.e.m. 0.0097) in the lower-browsing
396 clones. Across all clones in the AUT and NET treatment groups, the average relative
397 abundance of sediment-derived bacteria was 0.051, nearly identical to that of the
398 low-browsing clones in the SED conditions.
399
400 **Discussion**
401 Our results have several implications for studies of animal-associated
402 microbiota in diverse environmental settings. First, we confirm the intuition that
403 environmental sources of bacteria affect the diversity of animal microbiota, but not
404 because more diverse environments always create more diverse microbiota; rather,
405 the animals we exposed to the less species-rich autoclaved sediments had higher
406 overall diversity in their microbiota than those exposed to untreated, bacterial-
407 species-rich sediment. We hypothesize that this might be due to competitive
408 interactions between *Daphnia* microbiota and the particular microbes found in
409 these sediments. The untreated sediments may contain bacteria that can
410 outcompete multiple strains of "native," preexisting *Daphnia* microbiota. If this were
411 the case, then browsing in sediment could have multiple opposing effects on overall
412 microbiota diversity: on the one hand, it would bring daphnids into contact with
413 more diverse bacteria, but on the other hand those bacteria could reduce existing
414 microbiota diversity. In the NET treatment, animals might be exposed to some
415 sediment-derived bacteria in the water column but lack access to the full diversity of
416 bacteria in the sediment. An experiment designed to explicitly test this hypothesis

417 would be required to determine whether there are competitive interactions
418 between exogenous sediment-derived bacteria and those typically carried by
419 *Daphnia* in the laboratory; it would also be interesting to see how these competitive
420 effects interact with early colonization events in young *Daphnia.*
421 We also saw that having access to either sediment increased the variability
422 of community composition as measured by multivariate dispersion. These results
423 suggest that having access to multiple habitats with different bacterial communities
424 can affect the diversity and composition of an animal's microbiota. Therefore, fine-
425 scale heterogeneity in a host's habitat might be a relevant aspect to take into
426 account when examining effects of environment on animal microbiota. This is
427 especially important when considering ecological immunology, because disease-
428 causing bacteria in the environment may cause short-term risk but also long-term
429 fitness benefit via processes like immune priming (31, 32).
430 Our data further suggest that the diversity of *Daphnia*-associated microbiota
431 in a particular environment may to some extent be mediated by genotype-specific
432 sediment browsing intensity. This was apparent as the net barrier made the greatest
433 difference in microbial alpha diversity in high-browsing host clones. However, this
434 effect may be partially obscured by several factors: the hypothesized competitive
435 exclusion effects we allude to above, and also non-behavior-related host genotype
436 effects on microbiota diversity. While host genotype had an effect on microbial
437 diversity, the highest- and lowest-browsing clones in our study had similar
438 microbial alpha diversity overall. The only way to conclusively determine that
439 differences between the microbiotas of different genotypes are mediated by host
440 behavior independently of other host traits would be to genetically manipulate
441 behavior on an otherwise identical genetic background; we approximate this in our
442 experiment with the treatment where *Daphnia* are blocked from sediment
443 browsing, contrasted with the treatment where they are allowed to browse freely.
444 Our cautious conclusions about the effect of behavior on microbiome are based on
445 examining the contrast between these treatments within each genotype, not based
446 on the observation of genotype-dependent differences alone. It was only in
447 evaluating the difference between presence and absence of the barrier that an effect

448 of browsing on diversity could be seen. We conclude that the effect of environmental
449 bacteria on host-associated microbiota is not additive. The clearest effect of
450 environmental bacteria on host-associated microbiota was not on alpha diversity,
451 but relative abundances of certain taxa

452 Clones with low average browsing intensity had no greater amount of
453 sediment-specific bacteria than animals exposed to autoclaved sediment or
454 prevented from browsing, whereas those with high browsing intensity could reach
455 over 60% of reads from environment-derived bacteria in some individuals. While
456 many studies of animal microbiota rightly concern themselves with distinguishing
457 between truly "host-associated" microbiota versus "transient environmental"
458 microbiota, these results raise the possibility that the amount of environmental
459 microbes found in association with an animal could itself be a host-genotype-
460 specific feature of the microbiome.

461 Another key question is whether browsing behavior affects community
462 composition by simple exposure to more colonizing bacteria, or by more frequent
463 replenishment of bacterial taxa that would not otherwise persist in association with
464 the host (33). For example, browsing frequently enough may replenish bacteria that
465 would otherwise be lost when the animal molts. In this study, we made no
466 assumptions about the type of the interactions between the sediment-associated
467 bacteria and the *Daphnia*, but still were able to see that presence of sediment-
468 associated bacteria affected the bacteria that *Daphnia* were carrying.

469 It would also be interesting to investigate whether carriage of bacteria on
470 *Daphnia* from the sediment into the water column affects bacterial dynamics in the
471 larger environment; previous studies have shown that movement of *Daphnia*
472 between benthic and limnetic environments represents a mechanism of bacterial
473 dispersal in the environment (34). Studies using classification methods more
474 sensitive than 16S-based taxonomy may be necessary to unambiguously distinguish
475 and assign sources to different bacterial strains.

476

477 **Conclusion**

We show that at least some characteristics of host-associated microbial community composition result from genotype-by-microhabitat interactions, specifically ones resulting from genotype-specific variation in behavior. We show this using an experimental treatment that externally manipulated behavior, but genetically manipulating behavior to confirm these results would be a natural next step when the molecular tools to do so become available. Behavior could thus be considered a genetic factor that shapes microbial exposure in a given environment. Overall, these results provide further evidence that environment, behavior, genetics, disease risk, and microbial community composition are interrelated in potentially complex ways. Our observations indicate a need for more integrative eco-immunology studies, in which the interfaces between behavioral ecology, microbial community ecology and evolution of immune function are explored. Studies can take advantage of the experimental tractability of the *Daphnia*-microbiota system to further investigate these relationships in mechanistic detail.

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Author contributions

AAM and RA conceived the study and performed the experiment with input from DE. RA performed behavioral assays and analysis. AAM and RA prepared sequencing libraries. JCW performed quality control and OTU clustering of sequence data. AAM analyzed sequencing data. AAM and RA wrote the paper. DE and JCW revised the paper.

509

510 **Data deposition**

511 Sequence data will be deposited in the European Nucleotide Archive of the EBI
512 upon publication. Data tables and code used for analysis can be found on Github at
513 https://github.com/amusheg/Daphnia-microbiota-behavior and will be deposited
514 in Dryad upon publication.

515

516 **References**

517 1. McFall-Ngai M, Hadfield MG, Bosch TCG, Carey H V, Domazet-Loso T, Douglas
518 AE, Dubilier N, Eberl G, Fukami T, Gilbert SF, Hentschel U, King N, Kjelleberg S,
519 Knoll AH, Kremer N, Mazmanian SK, Metcalf JL, Nealson K, Pierce NE, Rawls JF,
520 Reid A, Ruby EG, Rumpho M, Sanders JG, Tautz D, Wernegreen JJ. 2013.
521 Animals in a bacterial world, a new imperative for the life sciences. Proc Natl
522 Acad Sci U S A 110:3229–3236.

523 2. Funkhouser LJ, Bordenstein SR. 2013. Mom knows best: the universality of
524 maternal microbial transmission. PLoS Biol 11:e1001631.

525 3. Tung J, Barreiro LB, Burns MB, Grenier JC, Lynch J, Grieneisen LE, Altmann J,
526 Alberts SC, Blekhman R, Archie EA. 2015. Social networks predict gut
527 microbiome composition in wild baboons. Elife 2015:1–18.

528 4. Rajilic-Stojanovic M, Heilig HGHJ, Molenaar D, Kajander K, Surakka A, Smidt H,
529 De Vos WM. 2009. Development and application of the human intestinal tract
530 chip, a phylogenetic microarray: Analysis of universally conserved phylotypes
531 in the abundant microbiota of young and elderly adults. Environ Microbiol
532 11:1736–1751.

533 5. Shin H, Pei Z, Martinez KA, Rivera-Vinas JI, Mendez K, Cavallin H, Dominguez-
534 Bello MG. 2015. The first microbial environment of infants born by C-section:
535 the operating room microbes. Microbiome 3:59.

536 6. Cheesman SE, Neal JT, Mittge E, Seredick BM, Guillemin K. 2011. Epithelial cell
537 proliferation in the developing zebrafish intestine is regulated by the Wnt
538 pathway and microbial signaling via Myd88. Proc Natl Acad Sci U S A 108
539 Suppl:4570–7.

540 7. Heijtz RD, Wang S, Anuar F, Qian Y, Bjorkholm B, Samuelsson A, Hibberd ML,
541 Forssberg H, Pettersson S, Diaz Heijtz R, Wang S, Anuar F, Qian Y, Björkholm
542 B, Samuelsson A, Hibberd ML, Forssberg H, Pettersson S. 2011. Normal gut
543 microbiota modulates brain development and behavior. Proc Natl Acad Sci
544 108:3047–3052.
545 8. Voss JD, Leon JC, Dhurandhar N V., Robb FT. 2015. Pawnobiome: manipulation
546 of the hologenome within one host generation and beyond. Front Microbiol
547 6:1–5.
548 9. Fan L, Liu M, Simister R, Webster NS, Thomas T. 2013. Marine microbial
549 symbiosis heats up: the phylogenetic and functional response of a sponge
550 holobiont to thermal stress. ISME J 7:991–1002.
551 10. Seedorf H, Griffin NW, Ridaura VK, Reyes A, Cheng J, Rey FE, Smith MI, Simon
552 GM, Scheffrahn RH, Woebken D, Spormann AM, Van Treuren W, Ursell LK,
553 Pirrung M, Robbins-Pianka A, Cantarel BL, Lombard V, Henrissat B, Knight R,
554 Gordon JI. 2014. Bacteria from diverse habitats colonize and compete in the
555 mouse gut. Cell 159:253–266.
556 11. Campbell JH, Foster CM, Vishnivetskaya T, Campbell AG, Yang ZK, Wymore A,
557 Palumbo A V, Chesler EJ, Podar M. 2012. Host genetic and environmental
558 effects on mouse intestinal microbiota. ISME J 6:2033–2044.
559 12. Spor A, Koren O, Ley R. 2011. Unravelling the effects of the environment and
560 host genotype on the gut microbiome. Nat Publ Gr 9:279–290.
561 13. Meisel JD, Kim DH. 2014. Behavioral avoidance of pathogenic bacteria by
562 Caenorhabditis elegans. Trends Immunol 35:465–470.
563 14. Rook GA. 2013. Regulation of the immune system by biodiversity from the
564 natural environment: an ecosystem service essential to health. Proc Natl Acad
565 Sci USA 110:18360–18367.
566 15. Ezenwa VO, Gerardo NM, Inouye DW, Medina M, Xavier JB. 2012. Animal
567 Behavior and the Microbiome. Science (80-) 338:198–199.
568 16. Sison-Mangus MP, Mushegian AA, Ebert D. 2015. Water fleas require
569 microbiota for survival, growth and reproduction. ISME J 9:59–67.
570 17. Sullam KE, Pichon S, Schaer TMM, Ebert D. 2017. The Combined Effect of

571 Temperature and Host Clonal Line on the Microbiota of a Planktonic
572 Crustacean. Microb Ecol.
573 18. Macke E, Callens M, De Meester L, Decaestecker E. 2017. Host-genotype
574 dependent gut microbiota drives zooplankton tolerance to toxic
575 cyanobacteria. Nat Commun 8.
576 19. Horton P, Rowan M, Webster K, Peters R. 1979. Browsing and grazing by
577 cladoceran filter feeders. Can J Zool Can Zool 57.
578 20. Decaestecker E, De Meester L, Ebert D. 2002. In deep trouble: Habitat
579 selection constrained by multiple enemies in zooplankton. Proc Natl Acad Sci
580 99:5481–5485.
581 21. Arbore R, Andras JP, Routtu J, Ebert D. 2016. Ecological genetics of sediment
582 browsing behaviour in a planktonic crustacean. J Evol Biol 29:1999–2009.
583 22. Kluttgen B, Dulmer U, Engels M, Ratte HT. 1994. ADaM, an artificial freshwater
584 for the culture of zooplankton. Water Res 28:743–746.
585 23. Nakagawa S, Schielzeth H. 2010. Repeatability for Gaussian and non-Gaussian
586 data: A practical guide for biologists. Biol Rev 85:935–956.
587 24. Edgar RC. 2013. UPARSE: highly accurate OTU sequences from microbial
588 amplicon reads. Nat Methods 10:996–8.
589 25. Salter SJ, Cox MJ, Turek EM, Calus ST, Cookson WO, Moffatt MF, Turner P,
590 Parkhill J, Loman NJ, Walker AW. 2014. Reagent and laboratory contamination
591 can critically impact sequence-based microbiome analyses. BMC Biol 12:87.
592 26. Foster ZSL, Sharpton T, Grunwald NJ. 2016. MetacodeR: An R package for
593 manipulation and heat tree visualization of community taxonomic data from
594 metabarcoding. bioRxiv 071019.
595 27. Peerakietkhajorn S, Kato Y, Kasalický V, Matsuura T, Watanabe H. 2015.
596 Betaproteobacteria Limnohabitans strains increase fecundity in the
597 crustacean Daphnia magna: Symbiotic relationship between major
598 bacterioplankton and zooplankton in freshwater ecosystem. Environ
599 Microbiol 18:2366–2374.
600 28. Qi W, Nong G, Preston JF, Ben-Ami F, Ebert D. 2009. Comparative
601 metagenomics of Daphnia symbionts. BMC Genomics 10:172.

602 29. Callens M, Macke E, Muylaert K, Bossier P, Lievens B, Waud M, Decaestecker E.
603 2015. Food availability affects the strength of mutualistic host–microbiota
604 interactions in Daphnia magna. ISME J 10:911–920.
605 30. Eckert EM, Pernthaler J. 2014. Bacterial epibionts of Daphnia: a potential
606 route for the transfer of dissolved organic carbon in freshwater food webs.
607 ISME J 8:1808–1819.
608 31. Kaltenpoth M, Engl T. 2014. Defensive microbial symbionts in Hymenoptera.
609 Funct Ecol 28:315–327.
610 32. Olszak T, An D, Zeissig S, Vera MP, Richter J, Franke A, Glickman JN, Siebert R,
611 Baron RM, Kasper DL, Blumberg RS. 2012. Microbial exposure during early
612 life has persistent effects on natural killer T cell function. Science 336:489–93.
613 33. Blum JE, Fischer CN, Miles J, Handelsman J. 2013. Frequent Replenishment
614 Sustains the Beneficial Microbiome of Drosophila melanogaster. MBio 4:1–8.
615 34. Grossart H-P, Dziallas C, Leunert F, Tang KW. 2010. Bacteria dispersal by
616 hitchhiking on zooplankton. Proc Natl Acad Sci U S A 107:11959–11964.
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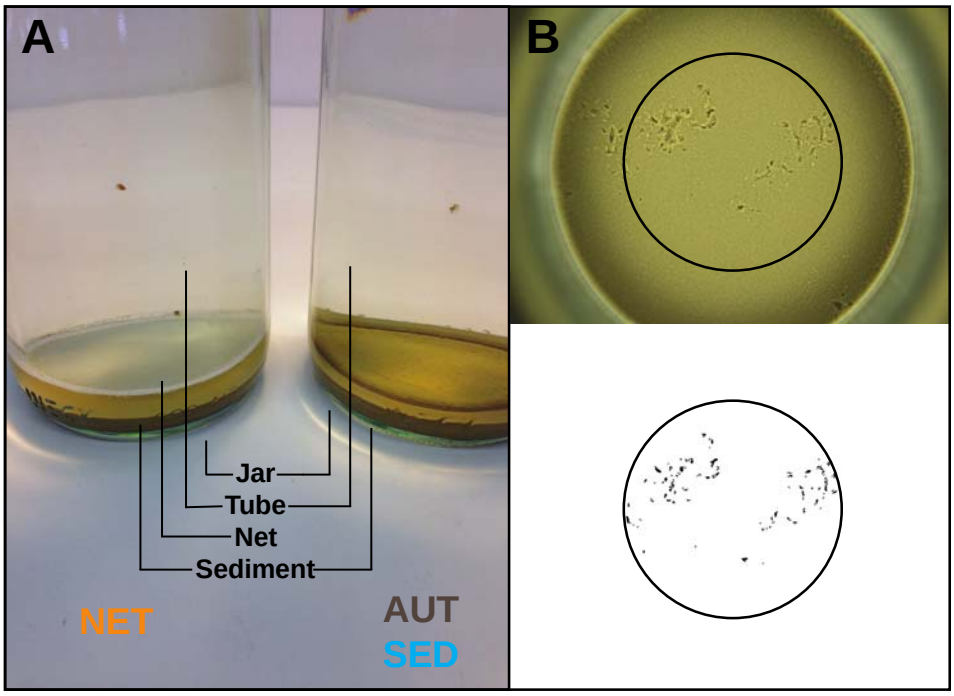
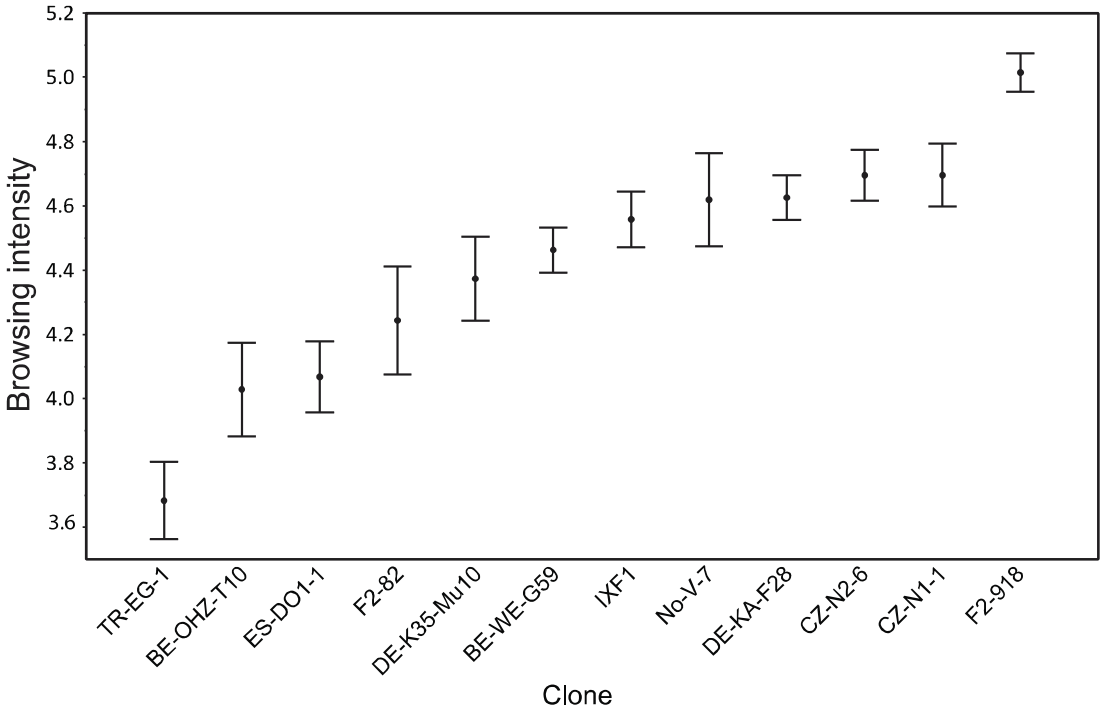
Figure legends

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



631 **Figure 2: Browsing intensity of 12 *D. magna* clones (mean and SE).** Browsing
632 intensity was defined as the Log_{10} of the area of the browsing traces left by
633 individual replicate animals browsing on a sediment surface for 30 minutes (see Fig.
634 1).



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

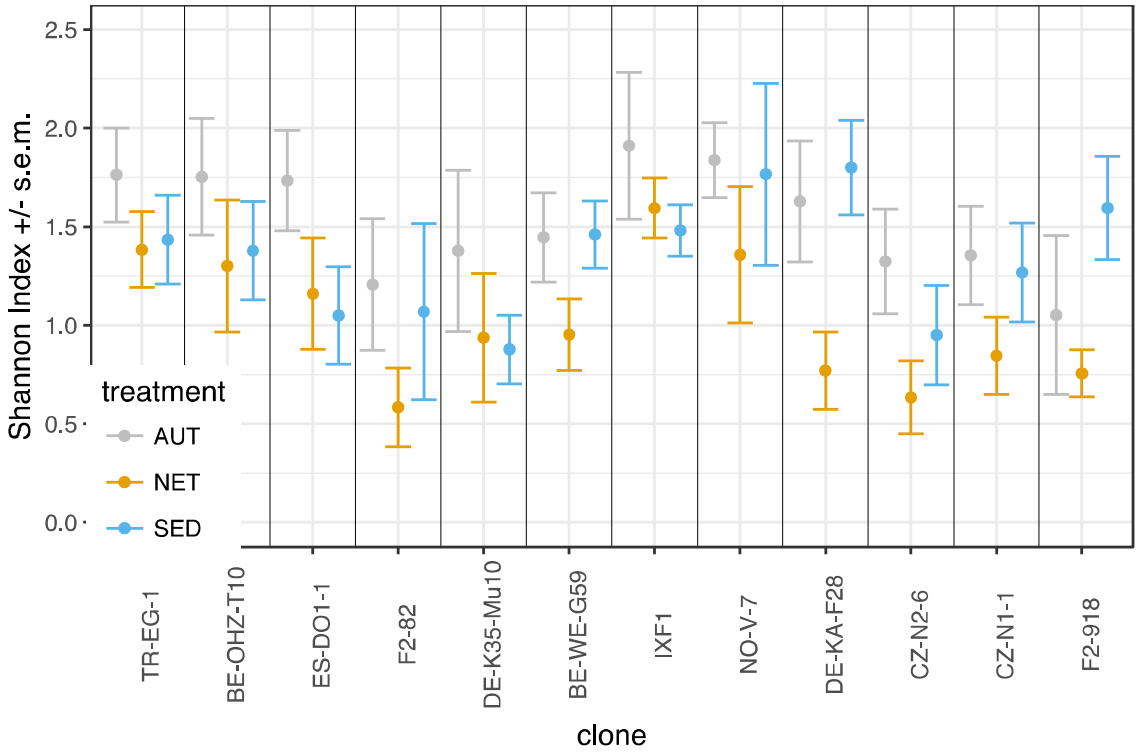

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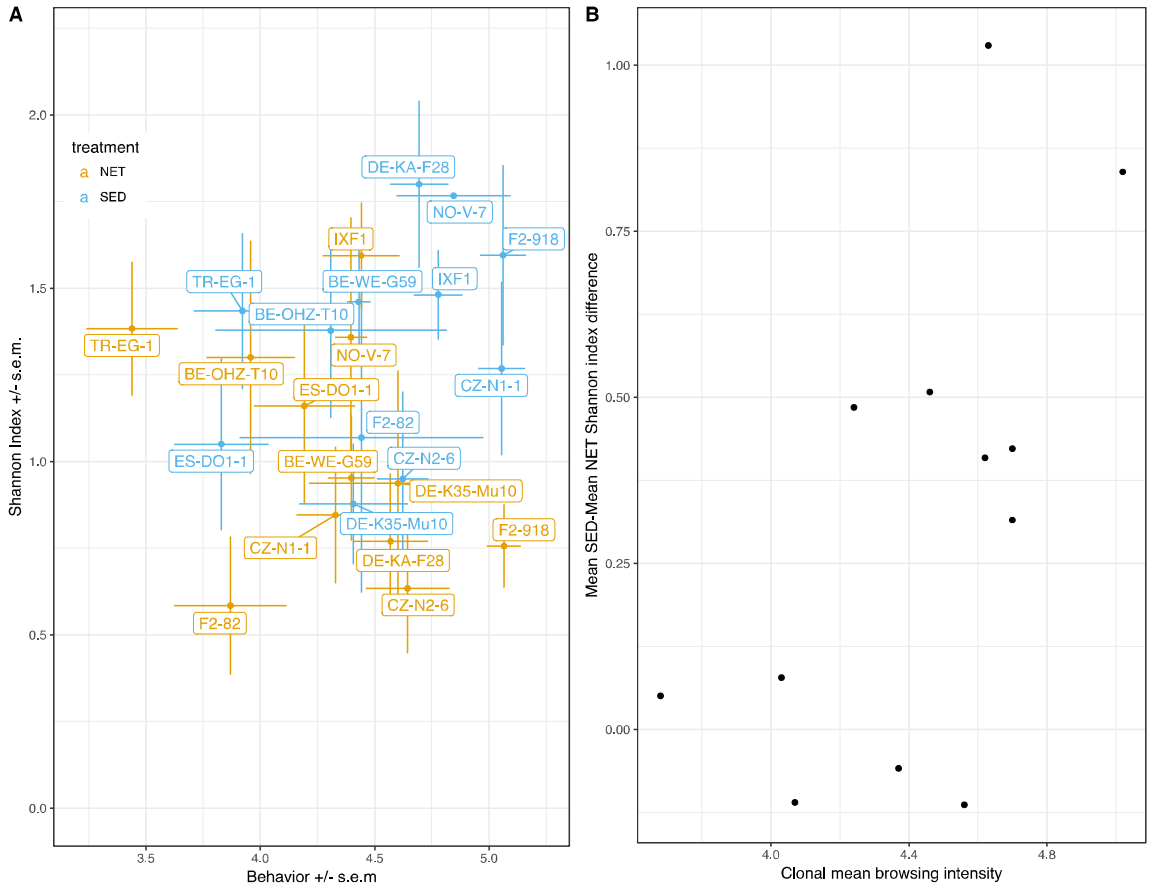
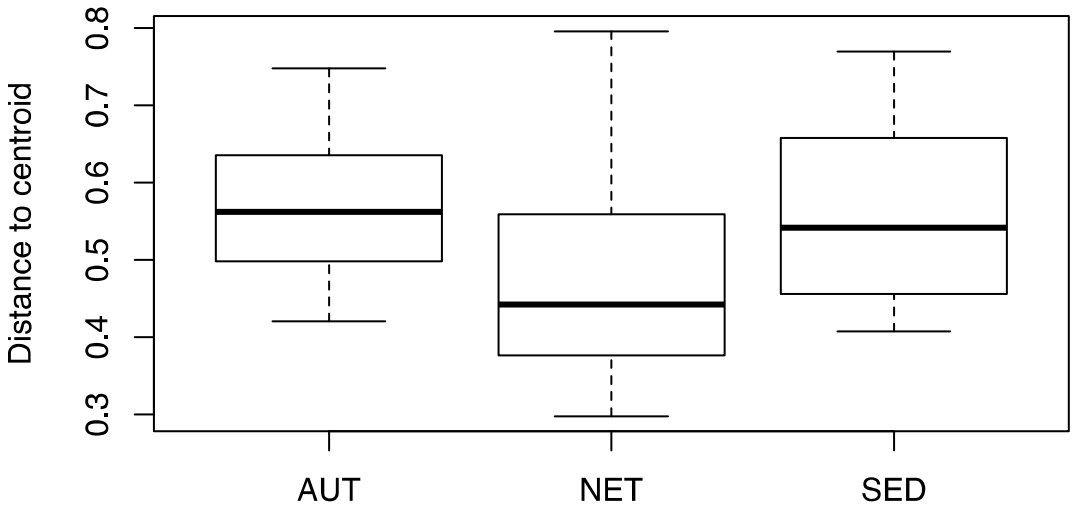
Figure 4: Average browsing intensity and average microbiota diversity in the NET and SED treatments. A: average clone browsing intensity and average clone microbiota diversity in the NET and SED treatments. Average browsing intensities were calculated based on samples whose jar-mates passed the sequence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



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



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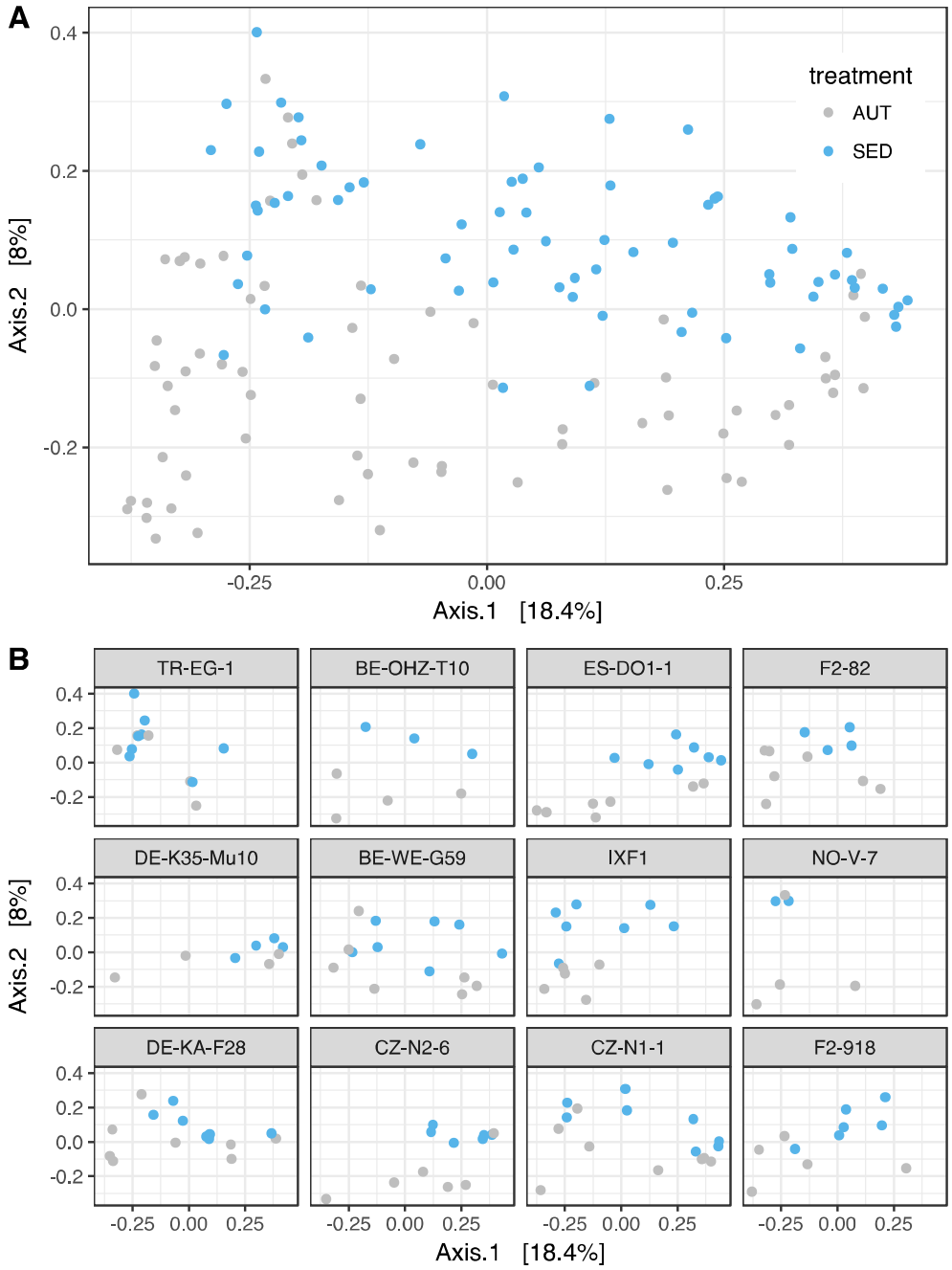
664 **Figure 6: Similarity of bacterial community composition in the AUT and SED**
665 **treatments.** A: first and second axis of a principal coordinates analysis (PCoA) of
666 bacterial community composition based on Hellinger-transformed Bray-Curtis
667 dissimilarities. B: first and second axis of a principal coordinates analysis (PCoA) of
668 bacterial community composition by *Daphnia* clone.



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670 **Figure 7: Analysis of sediment-derived bacteria.** Proportion of sediment-derived
671 bacteria in the microbiota of animals from AUT, NET and SED treatments. Sediment-
672 derived bacteria were identified by comparing autoclaved and untreated sediment
673 samples (log2-fold increase of at least 8 in natural sediment compared to autoclaved
674 sediment). Error bars correspond to standard errors of the mean. Clones are
675 arranged left-right by increasing average clone browsing intensity.
676

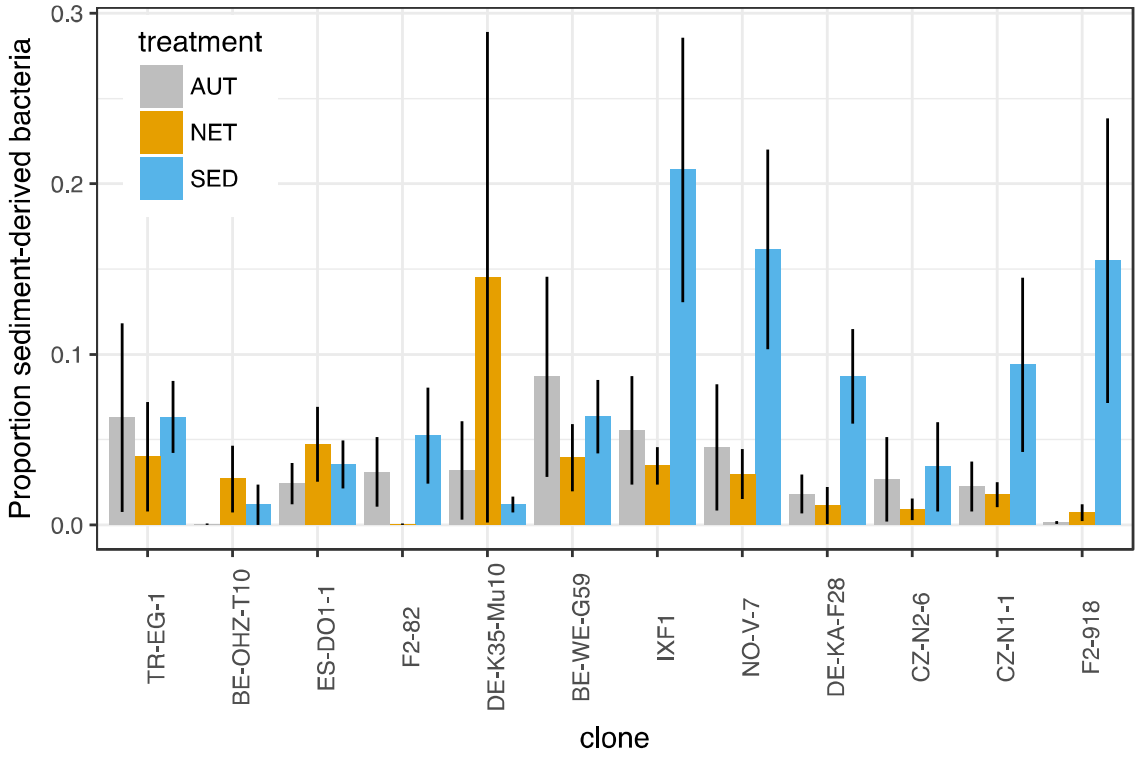

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Table 1. Names, number of individual replicates included in the microbiota analyses in the three treatments (AUT, NET and SED) and origin information of the 12 *Daphnia magna* clones used in this study. AUT: Exposure to autoclaved sediment; NET: prevented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