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1 **The mercury-tolerant microbiota of the zooplankton *Daphnia* aids in**

2 **host survival and maintains fecundity under mercury stress**

3

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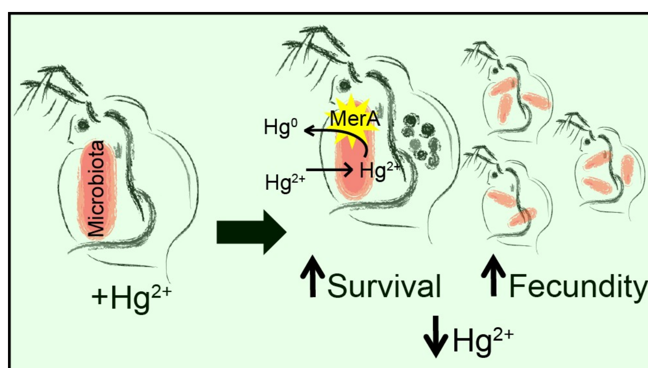
14 fleas, mercury stress, Yolo Bypass, symbiosis



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16 TOC Art

17 **ABSTRACT**

18 Many aquatic organisms can thrive in polluted environments by having the
19 genetic capability to withstand sub-optimal conditions. However, the
20 contributions of microbiomes under these stressful environments are poorly
21 understood. We investigated whether a mercury-tolerant microbiota can
22 extend its phenotype to its host by ameliorating host survival and fecundity
23 under mercury-stress. We isolated microbiota members from various clones
24 of *Daphnia magna*, screened for the mercury-biotransforming *merA* gene,
25 and determined their mercury tolerance levels. We then introduced the
26 mercury-tolerant microbiota, *Pseudomonas*-10, to axenic *D. magna* and
27 quantified its *merA* gene expression, mercury reduction capability and
28 measured its impact on host survival and fecundity. Expression of *merA* gene
29 was up-regulated in *Pseudomonas*-10, both in isolation and in host-
30 association with mercury exposure. *Pseudomonas*-10 is also capable of
31 significantly reducing mercury concentration in the medium. Notably,
32 mercury-exposed daphnids containing only *Pseudomonas*-10 exhibited
33 higher survival and fecundity than mercury-exposed daphnids supplemented
34 with parental microbiome. Our study showed that zooplankton, such as
35 *Daphnia*, naturally harbor microbiome members that are eco-responsive and
36 tolerant to mercury exposure and can aid in host survival and maintain host
37 fecundity in a mercury-contaminated environment. This study further
38 demonstrates that under stressful environmental condition, the fitness of the
39 host can depend on the genotype and the phenotype of its microbiome.

40

41 ## INTRODUCTION

42 *Daphnia* is a ubiquitous keystone zooplankton species found in many
43 aquatic ecosystems, capable of growing in both pristine and polluted
44 environments. It can withstand many environmental stressors, hence, is
45 widely used as a model organism for numerous research (1-7), including
46 studies on the effects of temperature fluctuations (8, 9), food availability (10-
47 12), predations (13-15), and exposure to metal pollutants (such as mercury,
48 cadmium, copper, arsenic) (16-23). To date, mercury studies on *Daphnia*
49 have focused mainly on the mechanisms of uptake, accumulation and
50 elimination in *D. magna* (16, 24-26). While host responses to stressors are
51 evaluated primarily, the responses and contributions of their microbiomes
52 are often overlooked. Aside from host genetics, microbiomes can also be an
53 important component in host survival in deleterious environments. For
54 instance, the gut microbiome of desert woodrats enhances the host's ability
55 to ingest plants with toxic secondary compounds (27), and the gut
56 microbiota of a coffee berry borer can detoxify the caffeine ingested by its
57 host, allowing the beetle to subsist on the otherwise toxic caffeine-laden
58 berries (28).

59 Mercury is a well-known metal contaminant that bioaccumulates and
60 biomagnifies in aquatic food webs (29-31). Many studies on mercury
61 pollution and its neurotoxicity have been reported (30, 32-34). Three major

62 species of mercury can be found in the environment: inorganic mercury
63 (Hg^{2+}), organic methylmercury (MeHg including the mono- and dimethyl
64 forms), and elemental mercury (Hg^0). Hg^0 and Hg^{2+} are commonly released
65 into the atmosphere via anthropogenic and natural sources, while deposition
66 of atmospheric mercury leads to mercury contamination of terrestrial and
67 aquatic ecosystems (31, 33, 35). Anaerobic microorganisms harboring *hgcAB*
68 genes (encoding a corrinoid-dependent protein and an associated ferredoxin
69 protein, respectively) have been shown to methylate mercury, producing the
70 highly bioaccumulative MeHg from inorganic Hg^{2+} (36-39). Demethylation of
71 MeHg to Hg^{2+}, on the other hand, can be facilitated by microorganisms
72 containing *merB* that encodes an organomercurial lyase. Inorganic Hg^{2+} can
73 be further converted into the less toxic, extremely volatile Hg^0 by
74 microorganisms expressing the *merA* gene that encodes a mercury
75 reductase (40-43). Therefore, microorganisms harboring these mercury-
76 biotransformation genes have the potential to impact mercury speciation,
77 cycling and concentration in the environment. Although mercury tolerance
78 and detoxification in many bacteria species have been reported (42-44),
79 most of these bacteria were identified in water columns, sediments and soil
80 (45-47). In addition, most studies on biotransformation of mercury in natural
81 ecosystems focused on bacteria found in specific geographical
82 environmental habitats (45-47), with only a few studies on mercury-
83 biotransforming bacteria found in animal hosts (48, 49). The contribution of

84 these bacteria on host mercury tolerance and survival, however, has never
85 been investigated.

86 In this study, we investigated the role of microbiomes on host fitness
87 under mercury stress. We first assessed the microbiome community
88 structure of *D. magna* collected from a seasonally mercury-polluted site (Yolo
89 Bypass, California) (50). We then isolated members of the microbiota,
90 determined their mercury tolerance levels, and measured *merA* gene
91 expression and mercury reduction of the mercury-tolerant microbiota
92 member (*Pseudomonas*-10) in isolation and in association with the daphnid
93 host. We found that *Daphnia* is an environmental reservoir of mercury-
94 tolerant bacteria that could potentially biotransform mercury into less-toxic
95 form by up-regulating the expression of *merA* gene upon exposure to
96 elevated levels of mercury and reducing the concentration of mercury in the
97 medium. Most importantly, we found that a daphnid microbiota member can
98 aid in the host survival and allow the host to produce viable offspring even
99 when exposed to mercury contamination.

100

101 **MATERIALS AND METHODS**

102 ***Daphnia* animal collection and husbandry.** *D. magna* CAY (**Ca**lifornia
103 **Y**olo-bypass) was sampled in the inlet ponds (38°31’45.4"N, 121°36’28.9"W)
104 that are part of the Yolo Bypass Wildlife Area (Davis, California, U.S.A). Other
105 *D. magna* clones, DE-K35-Iinb1 (Germany) and FI-Xinb3 (Finland) (51), are
106 part of the *Daphnia* collection in our laboratory. Daphnid husbandry was

107 carried out as previously published (52). Animals were routinely cultured in
108 artificial *Daphnia* medium (ADaM) (53) at 20℃ with 14:10 light:dark
109 photoperiod and fed daily with *Scenedesmus obliquus* (~$2x10^7$ cells).
110 Experiments with *D. magna* were also carried in the same media and
111 conditions.

112

113 **16S rRNA fragments high-throughput sequencing and data analysis**.
114 Total genomic DNA (gDNA) (n = 3) was extracted using DNeasy Blood and
115 Tissue Kit (Qiagen, Germantown, MD). Equimolar DNA concentration was
116 sent to Argonne National Laboratory Sequencing Core for library preparation
117 and sequencing of the 16S rRNA V4 region (PCR-amplified with 515F and
118 806R primers, Table S1), using MiSeq Illumina sequencing platform.
119 Additional details are provided under Supporting Information B.

120

121 *D. magna* sequences were demultiplexed and checked for chimeras,
122 with low quality sequence and short sequence reads (<150 bp) removed
123 prior to post-analysis with the Quantitative Insights Into Microbial Ecology
124 (QIIME 1.8) pipeline (54). Operational taxonomic units (OTUs) were clustered
125 at 97% sequence similarity with Uclust (55). Representative sequences from
126 the clustered OTUs were picked for taxonomic identification based on RDP
127 classifier 2.2 (56). Singletons, chloroplast and mitochondria sequences were
128 filtered out of the OTU table prior to alignment of OTU representative
129 sequences with PyNAST (54). Samples were rarified at a minimum of 10
130 sequences and a maximum depth of 12,030 sequences in steps of 10. For

131 microbiome composition analysis, assignment of bacterial taxon were
132 performed using BLAST 2.22.2 (57). Information on NCBI data availability of
133 the raw sequence reads are provided in Supporting Information E.
134
135 **Bacteria isolation and identification, growth conditions, and *merA***
136 **screening.** *D. magna* microbiota were isolated from various *D. magna*
137 clones: the newly collected CAY clone and clones in the laboratory collections
138 (FI-Xinb3 and DE-K35-Iinb1), as well as sediment samples from Yolo Bypass.
139 Individual *D. magna* were washed three times with 1 ml of sterile ADaM,
140 homogenized in 200 μl of sterile ADaM, plated on various agar media,
141 including LB, R2A (Teknova, Hollister, CA), 10x-diluted R2A with and without
142 N-acetylglucosamine supplement, and MacConkey (Teknova, Hollister, CA),
143 and incubated at room temperature (22-23℃) for several days. Sediment
144 samples were also plated on the same set of agar media and incubated at
145 room temperature for several days. Agar medium contains 1.5% (w/v)
146 granulated agar. Colonies exhibiting different phenotypes were repeatedly
147 streaked for single colonies. Pure microbiota isolates were cultured in R2A
148 liquid media and stored at -80℃ in autoclaved glycerol.
149 Genetic identification of bacterial isolates and *merA* screening were
150 carried out by sequencing the partial PCR-amplified 16S rRNA and *merA*
151 fragments. PCR amplification was carried out with MyTaq Red (Bioline,
152 Taunton, MA) using the following PCR program: initial denaturation at 95°C
153 for 2 min, followed by 35 cycles of 95°C for 30 sec, 50°C for 30 sec, 72°C for

154 2 min and a final extension step at 72°C for 5 min. Universal 16S primer set
155 8F and 1492R (58) and *merA* primer sets MERA5 and MERA1 (44) or merA-
156 128_F and merA-993_R, were used to amplify 16S rRNA and *merA* genes,
157 respectively. Additional details are provided in Supporting Information C.
158 Primers used in this study are listed in Table S1. GenBank accession
159 numbers for the partial 16S rRNA and *merA* sequences are found in the
160 Supporting Information. Details on 16S rRNA phylogenetic analysis is
161 provided in Supporting Information D.
162
163 **Bacteria mercury minimal inhibitory concentration (MIC) assays**.
164 Mercury MIC assays of twenty-seven bacterial isolates were carried out at
165 room temperature (22-23°C) in 96-well microtiter plates containing 200 μl of
166 R2A media with different concentrations of mercury (0.1, 0.2, 0.4, 0.6, 0.8
167 μM, and 1 μM to 15 μM, with 1 μM increment). The bacteria MIC was
168 determined using 1 μM to 15 μM first, followed by the lower mercury
169 concentrations (0.1, 0.2, 0.4, 0.6, 0.8 μM and 1 μM) for bacteria that have
170 MIC < 1 μM. Each well containing the defined mercury concentrations was
171 inoculated with 2 μl of bacterial inoculum and visible bacterial growth,
172 defined as increased in culture turbidity, was visually checked and measured
173 as increased in absorbance/optical density at 600 nm (OD_{600}). Cultures grown
174 in the absence of mercury were used as positive controls, while un-
175 inoculated R2A media were used as negative controls. The MIC is the lowest
176 mercury concentration where bacterial growth (culture turbidity) was not

177 observed with the naked eye (59), and the difference of OD_{600} values
178 between measured and negative control (un-inoculated R2A media) is less
179 than 0.01 (60). The MIC assays were repeated three times and the lowest
180 mercury concentration where *no growth* occurred (59) after 3 days of
181 incubation indicates the mercury MIC for that particular bacterial isolate.
182 Mercury stock solution (1,000 μg/ml mercury in 10% nitric acid) was
183 purchased from SPEX CertiPrep (Metuchen, NJ). OD_{600} was determined daily
184 using SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, CA).
185
186 **RNA extraction and cDNA synthesis.** Since *Pseudomonas*-10 exhibited
187 the highest mercury MIC (8 μM) among the *merA*-positive microbiota isolates
188 identified in this study (Table 1), this microbiota isolate was used to study
189 *merA* gene expression. To quantify *merA* gene expression in isolation,
190 *Pseudomonas*-10 was grown aerobically at room temperature (22-23℃) in LB
191 media until mid-exponential growth phase (OD_{600} = 0.3). Aliquots (10 ml) of
192 the cultures were transferred to new culture flasks, followed by no mercury
193 exposure (0 μM) or exposure to 2.5 μM or 5 μM of mercury for 15 min at room
194 temperature. After treatments, 2 ml-aliquots were pelleted by centrifugation
195 (21, 200 x *g*) at room temperature for 2 min. Cell pellets were immediately
196 resuspended in Trizol (Fisher Scientific, Hampton, NH) and stored at -80℃.
197 For measuring *merA* expression of daphnid-associated *Pseudomonas*-
198 10, *D. magna* CAY harboring *Pseudomonas*-10 exposed to 50 nM mercury for
199 4 h on Day 5 (Experiment 1, *n* = 55; Experiment 2, *n* = 50) or unexposed

200 (Experiment 1, *n* = 50; Experiment 2, *n* = 53) were harvested, washed once
201 with sterile ADaM, homogenized in Trizol and stored at -80℃. The mercury
202 LD_{50} for *D. magna* is 51.5 nM (Figure S3), hence, the mercury concentration
203 of 50 nM was chosen for the mercury stress experiments in *D. magna* CAY.
204 Gnotobiotic *D. magna* were generated as described for survival assays (52).

205 RNA was extracted with Direct-zol RNA Miniprep Plus (Zymo research,
206 Irvine, CA) according to manufacturer's instructions. Eluted total RNA was
207 further treated with DNase I (Promega, Madison, WI) for 30 min at 37℃,
208 followed by inactivation at 65℃ for 10 min, and purified using Direct-zol RNA
209 Miniprep Plus. RNA concentrations were estimated using either NanoDrop
210 spectrophotometer or Qubit Fluorometer (Fisher Scientific, Hampton, NH).
211 cDNA was synthesized using SuperScript III and random hexamers
212 (Invitrogen, Carlsbad, CA), according to manufacturer's instructions. For no-
213 host RNA samples, 200 ng of RNA was used as templates for cDNA synthesis.
214 For daphnid-associated RNA samples, depletion of daphnid host RNA was
215 first carried out using Dynabeads mRNA purification Kit (Fisher Scientific,
216 Hampton, NH), where the Dynabeads Oligo $(dT)_{25}$ binds to host RNA that
217 contains poly-A tail. Unbound bacterial RNA was collected and purified using
218 Direct-zol RNA Miniprep Plus. cDNA synthesis was then carried out using 2-3
219 μg of RNA as templates. Reactions without reverse transcriptase were used
220 as controls (NRT).

221

222 **Quantitative real-time PCR (qRT-PCR) and semi-quantitative PCR**
223 **(semi-qPCR).** qRT-PCR reactions (10 μl) were set up using PowerUp SYBR
224 Green Master Mix (Applied Biosystems, Foster City, CA), according to
225 manufacturer's instructions. Each reaction contains 2 μl of cDNA, 0.4 μM of
226 each specific primer (merA-Pse10_F and merA-Pse10_R or glnA_F and
227 glnA_R; Table S1) and 1X SYBR green master mix. qRT-PCR was performed
228 on an Applied Biosystems 7500 real-time PCR system with the following
229 thermal cycling steps: 50°C for 2 min, 95°C for 2 min, followed by 40 cycles of
230 95°C for 15 sec and 60°C for 1 min. Specificity of the *merA* and *glnA* primers
231 were tested with regular PCR and DNA gel electrophoresis, as well as in qRT-
232 PCR with a Dissociation Stage (melt curve analysis). No amplification of no
233 template control (NTC) and no reverse transcriptase (NRT) reactions served
234 as negative controls. Serially-diluted *Pseudomonas*-10 gDNA samples,
235 extracted with DNeasy Blood and Tissue Kit (Qiagen, Germantown, MD),
236 were used as positive controls and for generating calibration curves. The C_T
237 values of the samples were within the linear dynamic range of the calibration
238 curves for *glnA* and *merA*, with R^2 values of >0.99. All samples were
239 normalized to the expression of the glutamine synthetase (*glnA*)
240 housekeeping gene (61), and relative gene expression (fold change) is
241 calculated using $2^{-\Delta\Delta C_T}$ method (62). Data were log-transformed for statistical
242 analysis. The assay was carried out three times, each with three technical
243 replicates.

244 Semi-qPCR was carried out, similar to a protocol published previously
245 (63), to measure *merA* expression in daphnid-associated *Pseudomonas*-10.
246 The reactions (10 μl) were set up using MyTaq Red master mix, 3 μl of cDNA
247 and 1 μM of each specific primer (merA-Pse10_F and merA-Pse10_R or glnA_F
248 and glnA_R) with the following PCR program: initial denaturation at 95°C for
249 2 min, followed by 35 cycles of 95°C for 15 sec, 55°C for 20 sec, 72°C for 1
250 min and a final extension step at 72°C for 5 min. NRT and NTC were used as
251 negative controls. Amplified products (3 μl) were analyzed with 2% (w/v)
252 agarose gel electrophoresis and the gel images were captured using
253 ChemiDoc System (BioRad, Hercules, California). Fluorescence were
254 determined with Image Lab v5 software (Bio-Rad) and calculated by
255 subtracting background fluorescence of the corresponding NRT reactions for
256 both *merA* and *glnA*, followed by normalization to the corresponding *glnA*
257 samples. The assay was carried out twice, each with three technical
258 replicates. Statistical methods for comparison between treatments are
259 described in Supporting Information D.

260

261 ***D. magna* mercury lethal dose 50 (LD_{50}), survival and fecundity**
262 **assays.** Mercury LD_{50} of *D. magna* was determined in non-axenic daphnids
263 cultured in 80 ml of ADaM medium containing 10, 50, 75 and 100 nM
264 mercury. Daphnids cultured in ADaM without mercury (0 μM mercury) served
265 as controls. The mercury LD_{50} experiments were carried out in 10% nitric
266 acid-washed experimental jars (with 0.22-μM filter caps) containing 4

267 daphnids per experimental jar (*n* = 4 for each treatment) and were fed with
268 non-axenic *S. obliquus* (~2x10^7 cells per jar). LD_{50} was determined using 4-
269 parameter logistic regression (Figure S3). The LD_{50} of *D. magna* CAY after 2
270 days of mercury exposure is 51.5 nM, and as such 50 nM of mercury was
271 used as the applied concentration in the experiments that involved mercury
272 exposure in *D. magna* CAY. The LD_{50} assays were carried out with four
273 replicates.

274 Survival assays were carried out twice in 10% nitric acid-washed
275 experimental jars (with 0.22-μM filter caps) containing 80 ml of sterile ADaM
276 with one daphnid per jar and fed with axenic *S. obliquus* (~8x10^7 cells/jar).
277 Bacteria-free eggs were generated with antibiotic-treatment (52) using the
278 third egg clutches of reproductively synchronized F2 adults and were
279 separated into 3 groups: Bacteria-free (Bac-Free), bacteria supplemented
280 with parental microbiome (Bac-Suppl) and *Pseudomonas*-10-infected (Pse-
281 Inf). *Pseudomonas*-10 was used as it exhibits the highest MIC (8 μM) among
282 the *merA*-positive microbiota isolates identified in this study (Table 1).
283 Mercury stress (50 nM) was introduced at Day 5. Survival were monitored
284 daily for a period of 18 days. Fecundity assays were carried out twice, set up
285 similarly as the survival assays, in 80 ml of sterile ADaM with one daphnid
286 per jar and fed with axenic *S. obliquus* (~8x10^7 cells/jar). Number of live
287 hatchlings was counted and removed from the experimental jars daily for a
288 period of 18 days. Mercury stress (50 nM) was added on Day 5. At the end of
289 the experiments, sterility of the daphnids in Bac-Free group and the

290 presence of bacteria in Bac-Suppl and Pse-Inf groups were verified by PCR
291 using 327F and 936R primer set (Table S1), targeting bacterial 16S rRNA
292 gene (52). In addition, crushed daphnids from each experimental group were
293 also plated on LB or R2A agar media (Sigma, St. Louis, MO) to verify that
294 there was no bacterial growth from the Bac-Free group, mixed bacterial
295 growth from Bac-Suppl group (indicated by the growth of bacteria exhibiting
296 various morphotypes), and pure bacterial growth from Pse-Inf group. The
297 identity of *Pseudomonas*-10 from the Pse-Inf groups was further confirmed
298 via sequencing of the 16S rRNA using 8F and 1492R (58).

299

300 **Mercury biotransformation by *Pseudomonas*-10.** Total mercury (Hg^{2+}
301 and Hg^0, MeHg and other Hg species) concentrations in the ADaM medium of
302 *Pseudomonas*-10 only (Pse), bacteria-free daphnids (Bac-Free) and daphnids
303 infected with *Pseudomonas*-10 (Pse-Inf) were determined by cold vapor
304 atomic absorption spectrometry using a DMA-80 (Milestone, Inc.), calibrated
305 with aqueous standards and accuracy checked against standard reference
306 material BCR-414 (EPA Standard Method 7473) (64). Experimental jars were
307 set up similar to the survival assays, except that each experimental jar
308 contains 5 daphnids. Experimental jars containing ADaM medium (80 ml)
309 only served as controls. Experimental jars of the Pse group contain ADaM
310 medium (80 ml) and *Pseudomonas*-10 (200 μl of ADaM-washed bacterial
311 culture suspension diluted to OD = ~0.6). Mercury (50 nM) was added to the
312 experimental jars on Day 5. Samples were collected and filtered through

313 0.22 μm filter on Day 5, before (No Hg) and after (D5) addition of mercury,
314 and on Day 8 (D8). Mercury samples were stored at 4°C until analysis. Total
315 mercury in the samples were measured directly with cold vapor atomic
316 absorption spectrometry without any pretreatment. Data were obtained from
317 at least 3 replicates for each condition: ADaM medium (n = 3), Pse (n = 3),
318 Bac-Free (n = 4), Pse-Inf (n = 4).

319

320 **Statistical Analysis.** Data were presented as mean values with standard
321 errors, except for box plots where medians (horizontal lines within the box)
322 were shown with 25% to 75% quartiles (whiskers). For experiments
323 measuring the differences in gene expression and mercury reduction
324 between treatments, data were log-transformed (when necessary) to fit the
325 assumption of normal distribution and were tested for homogeneity of
326 variance prior to performing one-way ANOVA with Tukey's HSD post-hoc test
327 or Student's t test using JMP 14. Statistical analysis for differences in survival
328 rates was conducted using Mantel Cox log-rank test in GraphPad Prism 5.01,
329 with Bonferroni corrected p-value for multiple comparisons. Kruskal-Wallis
330 test and Wilcoxon each pair test was used to compare differences in
331 fecundity using JMP 14.0.

332

333 **RESULTS AND DISCUSSION**

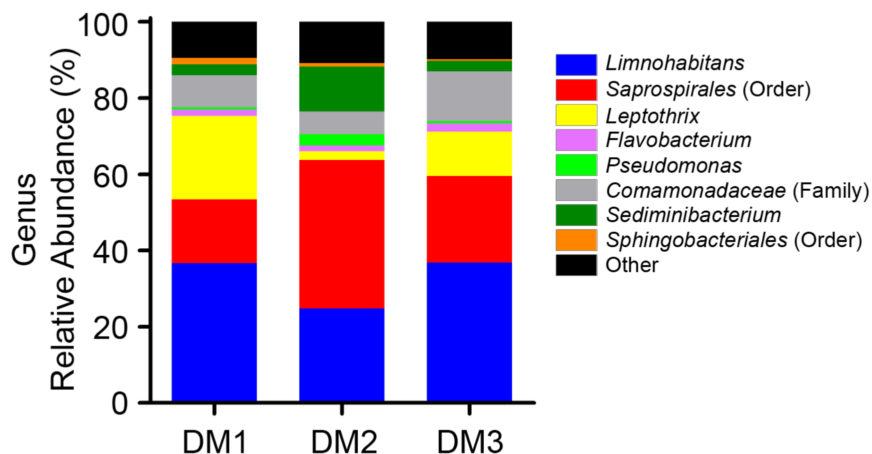
334 **Microbiome composition of *D. magna* CAY**

335 To investigate whether *D. magna* CAY (collected from **Ca**lifornia **Y**olo
336 Bypass) harbors mercury-tolerant and *merA*-positive bacteria, we first
337 determined the composition of the *D. magna* CAY microbiome via 16S rRNA
338 amplicon sequencing. A total of 1,295 OTUs were identified from the samples
339 (n = 3). After removing singletons, mitochondrial and chloroplast sequences,
340 416 OTUs remained and were assigned into 73 genera, 32 families, 10
341 orders, 9 classes and 1 phylum (Table S2). Only bacteria with ≥1% average
342 relative abundance (from 3 samples) were included in the comparison shown
343 in Figure 1. The top three most abundant microbiota in *D. magna* CAY are
344 bacteria from the genus *Limnohabitans* (32.8%, average abundance), the
345 order *Saprospirales* (26.2%), and the genus *Leptothrix* (11.9%). Other
346 identified microbiota members that are ≥1% (average abundance) include
347 bacteria from the family *Comamonadaceae* (9.2%), the genera
348 *Sediminibacterium* (5.8%), *Flavobacterium* (1.8%), *Pseudomonas* (1.3%), and
349 the order *Sphingobacteriales* (1%). Since *Pseudomonas* has been shown to
350 be pathogenic to *D. magna* (65-67), it is interesting that *Pseudomonas* was
351 found to be >1% in the microbiota of *D. magna* CAY. The presence of
352 *Pseudomonas* as part of *Daphnia* microbiome has also been reported in
353 different *Daphnia* species (51, 68, 69), suggesting that these *Pseudomonas*
354 microbiome members may be non-pathogenic, as opposed to the isolates (*P.*
355 *aeruginosa* PAO1 and strain DD1) used in the reported studies.

356 Notably, the microbial composition and structure of *D. magna* CAY
357 reported here is similar with the microbiomes of other *D. magna* coming

358 from different geographical locations (51, 68, 70), such as the dominance of
359 *Limnohabitans* and Saprospirales group for instance, suggesting active
360 selection of microbiome by the daphnid host. *Limnohabitans* have been
361 reported to increase fecundity in the *Daphnia* host (71) and are abundant in
362 host's filter apparatus (72). Indeed, host genetics have been shown to play a
363 role in structuring and maintaining different microbiota community
364 abundances in *Daphnia* (51) and in *Drosophila* (73-75). Collectively, our data
365 indicates that the newly collected *D. magna* CAY clones harbor microbiota
366 similar to other published *D. magna* clones and reinforces the idea that host
367 factors influence the microbial composition of the *Daphnia* microbiome.

368



369

370 **Figure 1. Relative abundance of major taxonomic groups of**
371 **microbiota from *D. magna* CAY.** Relative abundance of microbiota
372 members that are ≥1% (average abundance of all 3 samples). Genera with
373 less than 1% are grouped as "Other". Some of the microbiota were identified
374 only at the family and order levels using BLAST. *D. magna* samples (*n* = 3):
375 DM1, DM2 and DM3.

376

377 **Mercury minimal inhibitory concentration (MIC) and *merA* screening**
378 **of microbiota isolates**

379 Many bacteria are capable of mercury detoxification, commonly
380 through the expression of *mer* operons (42-44). One of the central players of
381 this widespread mercury-detoxification system is MerA, a mercuric reductase
382 that is capable of reducing a more reactive, cationic form of mercury (Hg^{2+})
383 to a relative inert, volatile monoatomic mercury vapor (Hg^0), which can
384 diffuse through the cell membrane (40-42). Cross-referencing the 73
385 assigned genera found in *D. magna* CAY with NCBI databases, 41 genera
386 were found to contain species that putatively harbor *merA* genes (Table S2).
387 We therefore hypothesize that *Daphnia* could harbor *merA*-positive bacteria
388 capable of detoxifying mercury through biotransformation. To test this
389 hypothesis, we isolated pure cultures of the microbiota members from the
390 newly collected *D. magna* CAY and two published *D. magna* clones (DE-K35-
391 Iinb1 and FI-Xinb3) in the laboratory collection as well as their environments
392 (ADaM culture media and the sediment from Yolo Bypass sampling site). We
393 then determined the mercury MICs of individual isolated bacteria and
394 screened them for the presence of *merA* gene. We isolated 27 bacteria from
395 different *D. magna* clones and their environments (Table 1) and determined
396 their identities and phylogeny using partial 16S rRNA sequences. The well-
397 resolved phylogenetic tree showed the phylum/class groupings of *D. magna*
398 CAY microbiota isolates (Figure 2). Several of the bacterial isolates

399 (*Acidovorax, Acinetobacter, Blastomonas, Exiguobacterium*,
400 *Hydrogenophaga*, and *Pseudomonas*) have homologous 16s rRNA sequences
401 identified in the *D. magna* CAY microbiome (Figure 1 and Table S2). In
402 addition, some of these isolated bacteria (*Acidovorax, Acinetobacter,*
403 *Aeromonas, Hydrogenophaga* and *Pseudomonas*) were also identified as
404 microbiome members of other *Daphnia* species (68, 69, 76). We were not
405 able to isolate and culture the dominant microbiota member, *Limnohabitans,*
406 despite numerous attempts using several different media and methods of
407 isolation.

408 Table 1 also shows the level of mercury tolerance (reported as MIC) of
409 the isolated *D. magna* microbiota. Highest mercury MIC was observed with
410 *Acidovorax*-4 (11 μM), followed by *Curtobacterium*-20 (11 μM) and
411 *Pseudomonas*-10 (8 μM). Other microbiota isolates exhibited low to medium
412 MICs ranging from 0.6 μM to 6 μM. To date, most bacteria tested for mercury
413 tolerance are either free-living bacteria or bacteria found in the sediments
414 (45, 77-79). Host-associated bacteria that are mercury-tolerant have been
415 reported only in a few hosts, including fish gut bacteria grown in media
416 containing 12.5 μM of mercury (48), bacteria isolated from nodules of
417 leguminous plants and marine sponges exhibiting MICs of 30 μM and >100
418 μM of mercury, respectively (49, 80). Hence, our study and that of others
419 indicate that host-associated microbiomes are also potential sources of
420 mercury-detoxifying bacteria.

421 To determine if mercury-tolerant bacteria contain *merA* genes, we
422 screened all 27 bacteria isolates for the presence of *merA* using published
423 primer sets (Table S1) (44, 77, 81). Initial screening identified partial *merA*
424 genes in *Acinetobacter*-1 and *Hydrogenophaga*-8 using primer set MERA5
425 and MERA1 (44); other primer sets did not yield amplified *merA* fragments.
426 Using the partial *merA* sequences from *Hydrogenophaga*-8 and
427 *Acinetobacter*-1, we designed a primer set, merA-128_F and merA-993_R
428 (Table S1), targeting the conserved sequence regions (Figure S1) and further
429 identified three more bacteria isolates containing *merA*. In all, *merA*
430 fragments were amplified from five microbiota isolates: three Gamma-
431 proteobacteria isolates (*Pseudomonas*-10, *Pseudomonas*-23, and
432 *Acinetobacter*-1) and two Beta-proteobacteria isolates (*Variovorax*-11 and
433 *Hydrogenophaga*-8). Homology sequence searches using BLAST showed high
434 sequence similarity to known *merA* sequences (Table S3 and Figure S1),
435 indicating that *Pseudomonas*-10, *Pseudomonas*-23, *Acinetobacter*-1,
436 *Variovorax*-11, and *Hydrogenophaga*-8 harbor *merA* genes. It is noteworthy
437 that all these five *merA*-positive isolates also exhibited moderate to high
438 mercury MICs, ranging from 3 to 8 μM (Table 1), hinting that these bacteria
439 isolates likely contain functional *merA* genes. The ability of primer set merA-
440 128_F and merA-993_R in amplifying *merA* fragments from both Beta-
441 proteobacteria and Gamma-proteobacteria, indicates that it can be used for
442 *merA* screening of environmental samples.

443 Although many other isolated microbiota exhibited high mercury
444 tolerance or belong to genera that putatively contain *merA*-positive species,
445 we were unable to amplify their *merA* gene, including *Acidovorax*-4 and
446 *Curtobacterium*-20. In addition, while *Pseudomonas*-10 and -23 harbor the
447 *merA* gene, we were unable to detect the presence of *merA* in the
448 congeneric *Pseudomonas*-28. It is possible that these bacteria isolates
449 contain nucleotide polymorphisms in the binding regions of the designed
450 *merA* primer sets, or that these particular species/strains do not contain
451 *merA* but exhibit mercury tolerance by other yet-to-be identified
452 mechanisms. We are continuing our effort in using new *merA* primer sets to
453 screen our remaining microbiota isolates, as well as screening for the
454 presence of *merB*, and will be part of another study.

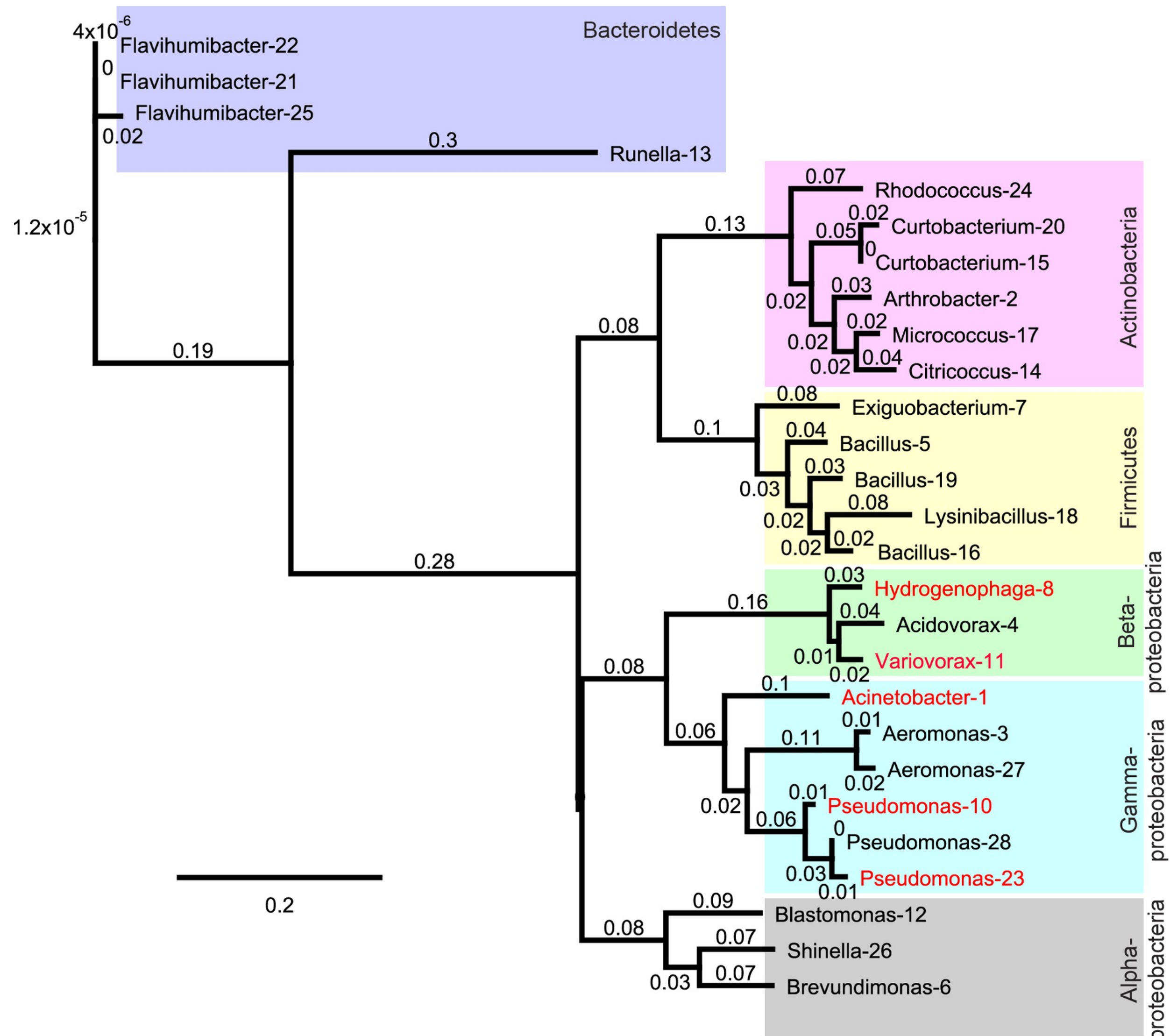
455 MerA exists as a homodimer protein and three major conserved
456 regions/residues critical for MerA activity have been identified (42). At the C-
457 terminus, a stretch of residues containing two cysteines forms a redox-active
458 site, while the N-terminus short cysteine pair aids in Hg^{2+} binding to the C-
459 terminal redox-active site of the corresponding monomer. In addition, two
460 tyrosine residues are also involved in Hg^{2+} binding (42). While most of the
461 conserved active regions/residues are beyond the identified *merA* sequences
462 of the microbiota isolates, the first Hg^{2+}-binding tyrosine is found in
463 *Pseudomonas*-10, *Hydrogenophaga*-8 and *Acinetobacter*-1 (Figure S1). The
464 well-resolved MerA tree also showed divergence of MerA between
465 *Pseudomonas* isolates and the other three microbiota isolates (Figure S1).

466 This is not surprising since *merA* found in *Pseudomonas* has been suggested
467 to be more distantly related to the *merA* of other Gram-negative bacteria
468 (82). While the partial MerA sequences between *Pseudomonas*-10 and -23
469 determined in this study are identical, they exhibited distinct mercury
470 tolerance levels. Possible sequence variations may exist in their C- and N-
471 terminus active sites, which may explain the tolerance differences between
472 these two *Pseudomonas* isolates. Together, our data showed that *Daphnia*
473 microbiomes contain mercury-tolerant bacteria that harbor *merA* genes,
474 which imply of their potential to biotransform mercury from the toxic Hg^{2+} to
475 less toxic Hg^0 species.
476
477 **Table 1.** List of identified *D. magna* microbiota isolates, mercury MIC and
478 *merA* gene screening.

16S rRNA Identification[a]	Source[b]	Mercury MIC[c] (μM)	*merA*[d]
Acidovorax-4	FI-Xinb3	11	
Curtobacterium-20	CAY	11	
Pseudomonas-10	DE-K35-Iinb1	8	+
Acinetobacter-1	DE-K35-Iinb1 (ADaM)	6	+
Brevundimonas-6	DE-K35-Iinb1	6	
Pseudomonas-28	CAY	6	
Hydrogenophaga-8	DE-K35-Iinb1	5	+
Variovorax-11	FI-Xinb3	5	+
Bacillus-19	CAY	5	
Rhodococcus-24	CAY	5	
Blastomonas-12	CAY	4	
Runella-13	CAY	4	
Bacillus-16	CAY	4	
Bacillus-5	FI-Xinb3	3	
Micrococcus-17	CAY	3	
Pseudomonas-23	CAY	3	+

Shinella-26	CAY	3
Aeromonas-27	CAY	3
Arthrobacter-2	DE-K35-Iinb1	2
Aeromonas-3	FI-Xinb3	2
Citricoccus-14	Sediment, Yolo Bypass	2
Flavihumibacter-21	CAY	2
Curtobacterium-15	CAY	1
Flavihumibacter-25	CAY	1
Exiguobacterium-7	DE-K35-Iinb1	0.8
Flavihumibacter-22	CAY	0.8
Lysinibacillus-18	Sediment, Yolo Bypass	0.6

480 [a] Numbers after the genus names indicated the laboratory collection
481 numbers, so as to distinguish between isolates from the same genera.
482 [b] Bacteria were isolated either from *D. magna* crushed body (CAY, DE-K35-
483 Iinb1 or Fl-Xinb3 clones), Yolo Bypass sediment (where the *D. magna* CAY
484 clones were collected) or the ADaM culture medium.
485 [c] Lowest mercury concentration with no bacterial growth after 3 days of
486 incubation at room temperature (22-23℃).
487 [d] Presence of *merA* gene determined by PCR and sequencing, indicated with
488 + and shaded.
489
490
491
492
493
494



495

496 **Figure 2**. **16S rRNA phylogenetic tree of isolated *D. magna***
497 **microbiota.** 16S rRNA sequences of the 27 isolates were first aligned using
498 MUSCLE, followed by tree-construction using maximum likelihood method
499 (PhyML), with Jukes-Cantor substitution model and 250 bootstrap replicates.
500 *merA*-positive microbiota isolates identified in this study (Table 1) are in red.
501 Numbers after the bacteria name represent laboratory collection ID number.
502 Bootstrap values and scale bar are indicated as substitution per site.

503

504 **Expression of bacterial *merA* gene in isolation and in association
505 with *D. magna* after mercury exposure**

506 To test the functionality of the *merA* gene, we exposed exponentially
507 growing *Pseudomonas*-10 to 2.5 μM and 5 μM of mercury for 15 min and
508 compared *merA* expression between mercury-exposed and unexposed
509 cultures (0 μM). *Pseudomonas*-10 was chosen as it exhibits the highest
510 mercury MIC (8 μM) among the *merA*-positive microbiota isolates identified in
511 this study (Table 1). Using qRT-PCR, relative expression of *merA* was found
512 to be more than 300-fold higher in cells exposed to mercury compared to
513 unexposed cells (one-way ANOVA, F = 130.79, $p = 0.00001$ Figure 3). We
514 also visualized the qRT-PCR amplified products with agarose gel
515 electrophoresis and quantify the relative fluorescence (Figure S2). As
516 expected, higher fluorescence was detected in samples exposed to mercury
517 compared to the unexposed samples. Expression of *merA* is not significantly
518 different between cells exposed to 2.5 μM and 5 μM mercury (Tukey's HSD
519 Post-hoc test, $p > 0.05$, Figure 3 and Figure S2). Similarly, increases in *merA*
520 gene expressions have also been reported in other *Pseudomonas* strains,
521 where over 10-fold and 30-fold increases were observed with 2 μM and 5 μM
522 mercury exposure (respectively) in *Pseudomonas stutzeri* OX (83), and more
523 than 2-fold increase with 50 μM of mercury exposure in *Pseudomonas* strain
524 ATH-43 (78). Our data show that the mercury-tolerant and *merA*-positive
525 *Pseudomonas*-10 isolate is capable of upregulating *merA* gene expression
526 upon mercury exposure.

527 We further investigated if similar upregulation of *merA* also occurs in
528 *Pseudomonas*-10 when in association with the *D. magna* host upon exposure
529 to mercury. We first determined the lethal dose 50 (LD_{50}) of mercury in *D.*
530 *magna* CAY. To do this, we exposed 5-day-old non-axenic daphnids to
531 various concentration of mercury for 2 days and monitored their survival.
532 Using 4-parameter logistic regression, we determined that the LD_{50} of
533 mercury is 51.5 nM (Figure S3) and as such, 50 nM was chosen as the
534 applied mercury concentration for the mercury stress experiments in *D.*
535 *magna*. We infected bacteria-free *D. magna* CAY with *Pseudomonas*-10,
536 allowed bacteria-host association to establish for 5 days, exposed the
537 *Pseudomonas*-10-infected daphnids to 50 nM of mercury for 4 hours,
538 harvested total RNA and determined *merA* gene expression. Several
539 attempts using qRT-PCR to determine *merA* expression in host-associated
540 *Pseudomonas*-10 did not yield reliable results, likely due to low abundance of
541 bacterial cDNA and interference from the daphnid host cDNA, despite
542 attempts to deplete host RNA before cDNA synthesis. As such, we carried out
543 semi-quantitative RT-PCR (semi-qPCR) by analyzing the amplified *merA* and
544 *glnA* (housekeeping gene for normalization) on agarose gel and determined
545 band intensities of the amplified products (Figure 4). Upon exposure to 50
546 nM of mercury, *merA* expression in daphnid-associated *Pseudomonas*-10 was
547 upregulated approximately 31-fold, when compared to daphnid-associated
548 *Pseudomonas*-10 without mercury treatment. This data indicates that
549 *Pseudomonas*-10 is capable of upregulating *merA* expression in response to

550 mercury stress even when in association with the daphnid host. The lower
551 expression level of *merA* in daphnid-associated *Pseudomonas*-10 (31-fold),
552 when compared to *Pseudomonas*-10 in isolation (>300-fold), is likely due to
553 the lower mercury concentration used in host-associated *Pseudomonas*-10
554 exposure.

555

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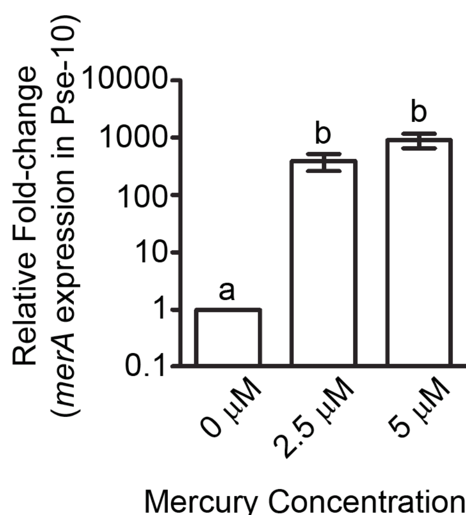
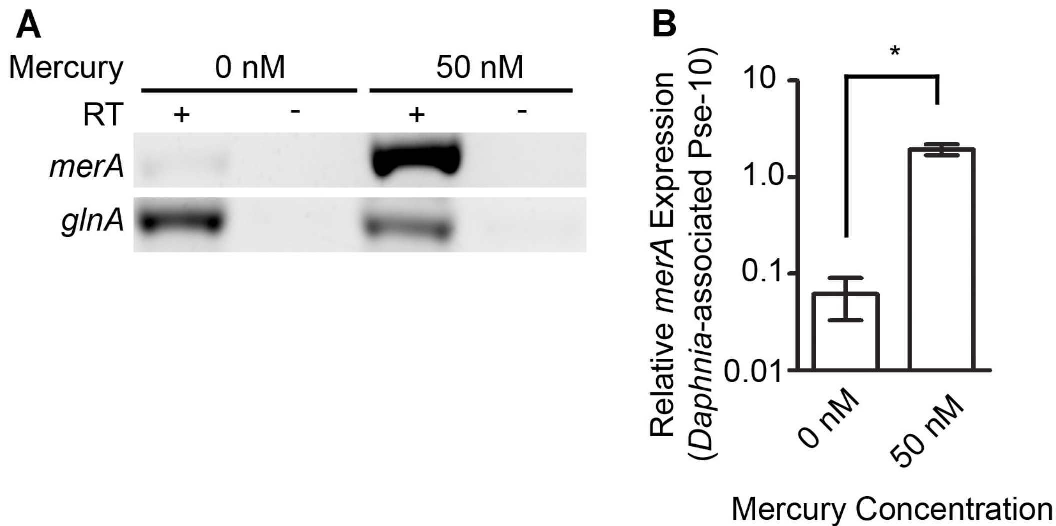


557

558 **Figure 3**. ***merA* expression in the microbiota isolate *Pseudomonas*-**
559 **10.** Relative fold-change of *merA* expression in *Pseudomonas*-10 (Pse-10)
560 bacterial cultures exposed to 0, 2.5 and 5 μM of mercury in LB media,
561 determined from 3 independent experiments with 3 technical replicates.
562 Data were log-transformed and was analyzed using one-way ANOVA with
563 Tukey's HSD Post-hoc test. Column with different letters are significantly
564 different ($p<0.05$).

565



566

567 **Figure 4**. **Expression of *merA* in the daphnid-associated**
568 ***Pseudomonas*-10. (A)** Agarose gel electrophoresis of amplified *merA* and
569 *glnA* (housekeeping gene for normalization). **(B)** Relative *merA* expression in
570 host-associated *Pseudomonas*-10 exposed to 50 nM mercury, determined
571 from 2 independent experiments with 3 technical replicates. *, p = 0.0001
572 (Student's t test). RT, reverse transcriptase.

573

574 ***Pseudomonas*-10 aids in host survival and maintains host fecundity**
575 **under mercury stress.**

576 Since host-associated *Pseudomonas*-10 exhibited increased *merA*
577 expression upon mercury exposure, we investigated whether *Pseudomonas*-
578 10 can contribute to host survival and fecundity under mercury stress. We
579 infected *D. magna* CAY with *Pseudomonas*-10 (Pse-Inf), allowed the daphnids
580 to grow for 5 days before exposing the daphnids to 50 nM mercury, and
581 compared their survival rates to bacteria-free (Bac-Free) and parental-
582 microbiome supplemented *D. magna* CAY (Bac-Suppl) over a period of 18

583 days (Figure 5A). We positively verified the absence of bacteria in Bac-Free
584 group, the presence of *Pseudomonas* in Pse-Inf group and the presence of
585 various bacterial morphotypes in Bac-Suppl group at the end of the
586 experiment.

587 A significant difference in survival rates was observed between
588 daphnid groups (Mantel-Cox log-rank test, χ^2 = 26.29, p = 0.003). In the
589 absence of mercury stress, Bac-Suppl daphnids (94%, n = 29/31) exhibited
590 similar survival rates with Pse-Inf daphnids (87%, n = 26/30, χ^2 = 0.68, p =
591 0.41) and significantly higher survival rates than Bac-Free daphnids (73%, n
592 = 22/30, χ^2 = 4.16, p = 0.04) at the end of the assay at Day 18. This
593 suggests that *Pseudomonas*-10 is a mutualistic symbiont and contributes to
594 *D. magna* survival. Similar report also showed that a *Pseudomonas* strain
595 isolated from a *D. magna* host is beneficial to the development of *D. magna*
596 resting eggs at warm conditions (84).

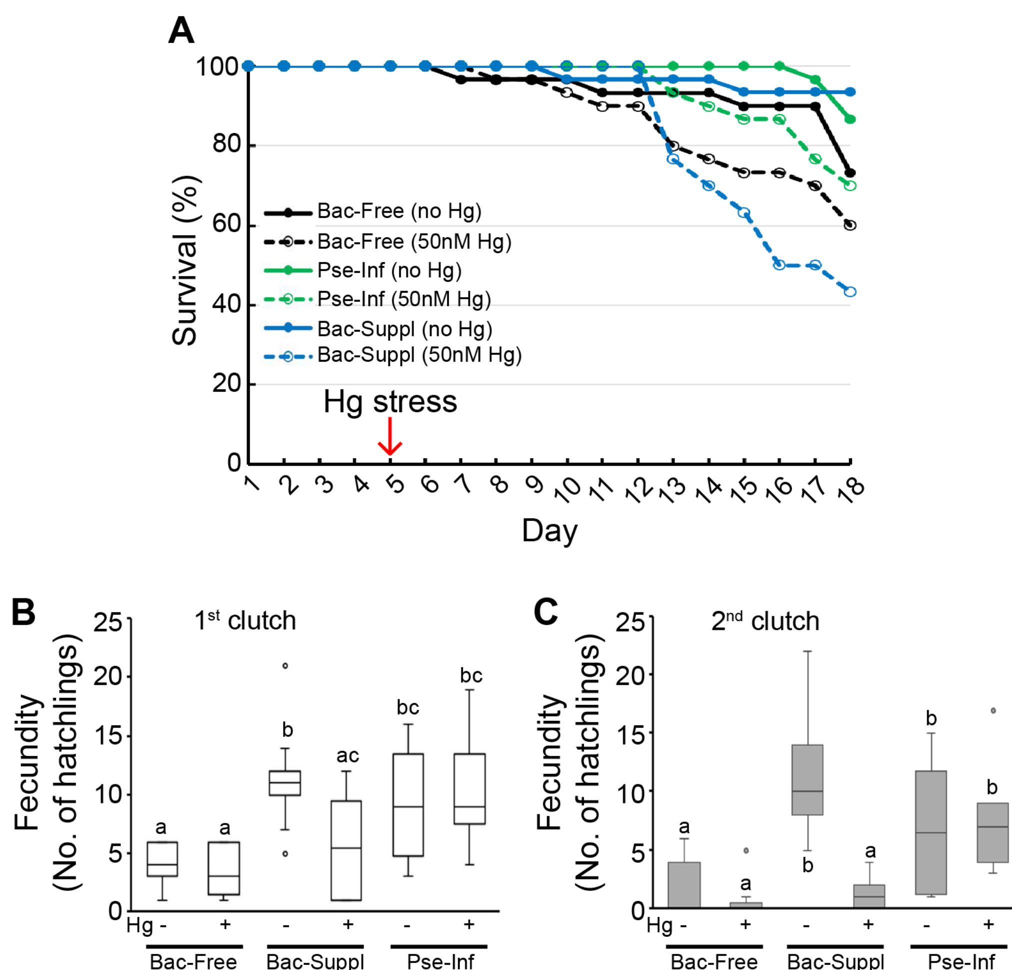
597 Under mercury stress, lower survival rates were observed for the three
598 mercury-treated groups when compared to their counterparts at the end of
599 the assay (Day 18): Bac-Suppl + Hg (43%, n = 13/30), Pse-Inf + Hg (70%, n
600 = 21/30) and Bac-Free + Hg (60%, n = 18/30). Daphnids from the Bac-Suppl
601 + Hg were most severely affected under mercury stress (χ^2 = 17.22, p <
602 0.0001) while the survival rates of Pse-Inf + Hg daphnids were statistically
603 similar with Pse-Inf daphnids (χ^2 = 2.18, p = 0.14). Survival rates of Bac-Free
604 daphnids were also statistically similar under mercury and without mercury
605 stress (χ^2 = 1.61, p = 0.21). Notably, under mercury stress, daphnids

606 harboring *Pseudomonas*-10 (Pse-Inf + Hg) exhibited significantly higher
607 survival rates than Bac-Suppl + Hg (χ^2 = 6.19, p = 0.01). These results
608 suggest that the mercury-tolerant *Pseudomonas*-10 is capable of
609 augmenting host survival under mercury stress, likely by biotransforming
610 mercury into less toxic form, in addition to the daphnid's ability to eliminate
611 mercury through excretion, egestion, molting, and neonate production (25).
612 Intriguingly, under mercury stress, daphnids supplemented with parental
613 microbiome (Bac-Suppl + Hg) exhibited the lowest survival (43% at Day 18
614 n=13/30). Similar results were obtained in a repeated experiment (Figure
615 S4). Possibly, the microbiota composition of the Bac-Suppl daphnids is low in
616 *merA*-positive bacteria, hence the low survival under mercury exposure, as
617 compared to the higher abundance of *merA*-positive *Pseudomonas*-10 in Pse-
618 Inf daphnids. It is also likely that under mercury stress, harboring various
619 kinds of microbiota can have a detrimental effect on the health of the
620 daphnids. Indeed, Bac-Suppl + Hg daphnids also exhibited significantly lower
621 survival rates than BacFree + Hg daphnids (χ^2 = 14.49, p = 0.0001), implying
622 that harboring many bacteria is beneficial under normal conditions but can
623 be a burden under stressful environmental conditions. This is similarly
624 observed on grasses with and without fungal endophytes, where the
625 endophyte-free plants significantly fared better than endophytic-laden plants
626 in terms of root:shoot ratio under extreme limiting-nutrient conditions (85).

627 To further investigate the contributions of *Pseudomonas*-10 in host
628 fitness, we carried out fecundity assays and compare the number of live

629 hatchlings from first and second clutches of all daphnid groups with and
630 without mercury stress (Figure 5B, C and Figure S5). The six groups
631 significantly vary in the number of first and second clutch hatchlings
632 (Kruskal-Wallis test, χ^2 = 26.97 and 37.56, respectively, $p < 0.0001$),
633 suggesting differences between treatments. Under no mercury stress, Bac-
634 Suppl daphnids have significantly higher number of first and second clutch
635 hatchlings than Bac-Free daphnids (Wilcoxon Each Pair test, $p = 0.0004$ and
636 $p = 0.0003$, respectively), but did not differ with Pse-Inf daphnids ($p = 0.31$
637 and $p = 0.15$, respectively). This data confirms that *Pseudomonas*-10 is a
638 beneficial symbiont and positively contributes to daphnid host fitness under
639 normal environmental condition. Intriguingly, untreated Bac-Suppl and Pse-
640 inf daphnids produced similar number of first and second clutch hatchlings
641 as the Pse-Inf + Hg treated daphnids ($p > 0.05$), suggesting that the
642 fecundity of the daphnids harboring mercury-tolerant *Pseudomonas*-10 is
643 maintained under mercury stress, and is comparable to that of unstressed
644 daphnids. On the other hand, under mercury stress, hatchlings production of
645 Bac-Suppl + Hg treated daphnids, were significantly lower than Bac-Suppl
646 daphnids ($p = 0.007$, Figure 5A) and this decrease in fecundity is even more
647 pronounced in the second clutch ($p < 0.0001$, Figure 5C). Moreover, Bac-
648 Suppl + Hg have similar number of hatchlings with Bac-Free and Bac-Free +
649 Hg daphnids ($p > 0.05$). This indicates that prolonged exposure to mercury
650 results in lower fecundity of daphnids harboring several microbiota
651 associates. Interestingly, Bac-Suppl + Hg treated daphnids have

652 significantly lower number of second clutch hatchlings than Pse-Inf + Hg
653 daphnids (p = 0.0011). This further confirms that mercury-tolerant
654 *Pseudomonas*-10 can maintain the fecundity of the daphnid host under
655 mercury exposure, likely by up-regulating its *merA* expression and reducing
656 the toxicity of mercury, thereby allowing the host to withstand and survive,
657 and maintain clonal reproduction in mercury-contaminated environment.



658

659 **Figure 5. Survival and fecundity of gnotobiotic *D. magna* CAY**
660 **harboring different microbiota under mercury stress. (A)** Survival of
661 bacteria-free daphnids (Bac-Free), bacteria-supplemented daphnids
662 harboring parental microbiota (Bac-Suppl), and *Pseudomonas*-10 infected

663 daphnids (Pse-Inf) with (50 nM Hg) and without (no Hg) mercury exposure.
664 Mercury stress (Hg stress) was introduced at Day 5, indicated with a red
665 arrow. Bac-Free (no Hg), *n* = 30; Bac-Free (50 nM Hg), *n* = 30; Bac-Suppl (no
666 Hg), *n* = 31; Bac-Suppl (50 nM Hg), *n* = 30; Pse-Inf (no Hg), *n* = 30; Pse-Inf
667 (50 nM Hg), *n* =30. Survival assays were repeated twice (Figure S4), but only
668 one representative experiment is shown here. **(B)** Fecundity of Bac-Free,
669 Bac-Suppl and Pse-Inf daphnids with (50 nM Hg) and without (no Hg) mercury
670 stress. Boxes show the 25% to 75% quartiles, medians are shown as
671 horizontal lines (within the box), and maximum and minimum values are
672 shown as whiskers. Columns with the same letter are not significantly
673 different, analyzed using Kruskal-Wallis test with Wilcoxon each pair test for
674 pairwise comparisons. Bac-Free (no Hg), n = 8; Bac-Free (50 nM Hg), n = 9;
675 Bac-Suppl (no Hg), *n* =15; Bac-Suppl (50 nM Hg), *n* =10; Pse-Inf (no Hg), *n* =
676 9; Pse-Inf (50 nM Hg), *n* = 9. Fecundity assays were repeated twice (Figure
677 S5).
678
679 ***Pseudomonas*-10 is capable of biotransforming mercury**
680 Since the *merA*-positive and mercury-tolerant *Pseudomonas*-10 can up-
681 regulate *merA* expression, both in isolation and in host-association, and is
682 capable of aiding host survival and fecundity under mercury stress, we
683 wanted to investigate its ability to biotransform and reduce mercury (Figure
684 6). In a separate experiment, we measured the reduction or loss of mercury
685 by *Pseudomonas*-10 in isolation (Pse) and in association with the daphnids

686 (Pse-Inf) and compared with the Bac-Free daphnids to tease apart the
687 contribution of each respective partner to mercury reduction. We did not
688 include Bac-Suppl in this study because it harbors several microbiota of
689 unknown mercury biotransformation potentials. As expected, before the
690 addition of mercury on Day 5 (No Hg), negligible background concentrations
691 of mercury were detected. Upon addition of 50nM mercury on Day 5 (D5), we
692 detected initial mercury concentrations ranging from 11.6 to 15.6 nM in all
693 groups (ADaM control, Pse, Bac-Free and Pse-Inf). The significant mercury
694 loss after initial mercury addition could be attributed to the attachment of
695 mercury to the walls of the processing vessels. Nonetheless, the
696 concentration of mercury in ADaM only control group between D5 and D8
697 showed no significant differences (Tukey's HSD, $p > 0.05$), as well as in D5
698 samples of all treatments (Tukey's HSD, $p > 0.05$), indicating that the loss of
699 mercury due to attachment to processing vessels is consistent among
700 samples. Intriguingly, after 3 days (Day 8), a drastic 4.7-fold decrease in
701 mercury concentrations (from 15.5 nM to 3.3 nM) was detected in the Pse
702 group, which contained only ADaM medium and *Pseudomonas*-10 (Tukey's
703 HSD, $p < 0.0001$). Mercury loss in the ADaM only control group between D5
704 and D8 is not significant, indicating that the reduction of mercury from
705 solution in Pse group was due to the presence of *Pseudomonas*-10. This
706 strongly suggests that *Pseudomonas*-10 biotransformed and reduced
707 mercury in the solution, most likely with the use of its MerA enzyme, which is
708 known to be the common mechanism for the reduction and

709 biotransformation of Hg^{2+} to elemental Hg^0 (42). We also observed significant
710 reduction of mercury in the Bac-Free group after 3 days (12.9 nM to 6.3 nM,
711 2.0-fold), suggesting that the daphnids are capable of mercury uptake from
712 their environment. This is not surprising as assimilation of mercury by
713 *Daphnia* has been reported (25). Similar reduction of mercury was also
714 detected in the Pse-Inf group after 3 days (15.6 nM to 5.8 nM, 2.7-fold), but
715 mercury reduction was not significantly different from that of Bac-Free
716 group. Hence, we could not conclusively show that *Pseudomonas*-10
717 significantly contributes to mercury reduction when it is in association with
718 the daphnid host, despite its mercury biotransformation capability in
719 isolation.

720 Host genetics indeed play a major role on metal stress tolerance via
721 metallothionein genes, which act as metal-responsive systems upon metal
722 exposure. *D. magna* contain three putative genes encoding the metal-
723 binding metallothionein proteins in their genome (86, 87) and these proteins
724 are known to play an important role in metal detoxification and homeostasis
725 in many organisms, likely through binding and sequestration of various
726 metals, including mercury (88, 89). While the expression of these
727 metallothionein genes may enable the *Daphnia* host to tolerate mercury
728 stress, we showed in this study that the *merA*-positive and mercury-tolerant
729 microbiota *Pseudomonas*-10 can also reduce mercury in isolation and
730 contributes to host survival and fecundity under mercury stress, through
731 upregulation of *merA* and thus likely reducing the mercury stress

732 experienced by the host. To definitively show the contribution of
733 *Pseudomonas*-10 in biotransforming mercury inside the host, and to further
734 investigate its mechanistic role during host association, daphnids containing
735 knock-outs of the host metallothionein genes and a *Pseudomonas*-10 strain
736 harboring a *merA*-knockout, needs to be generated and will be part of
737 another study.

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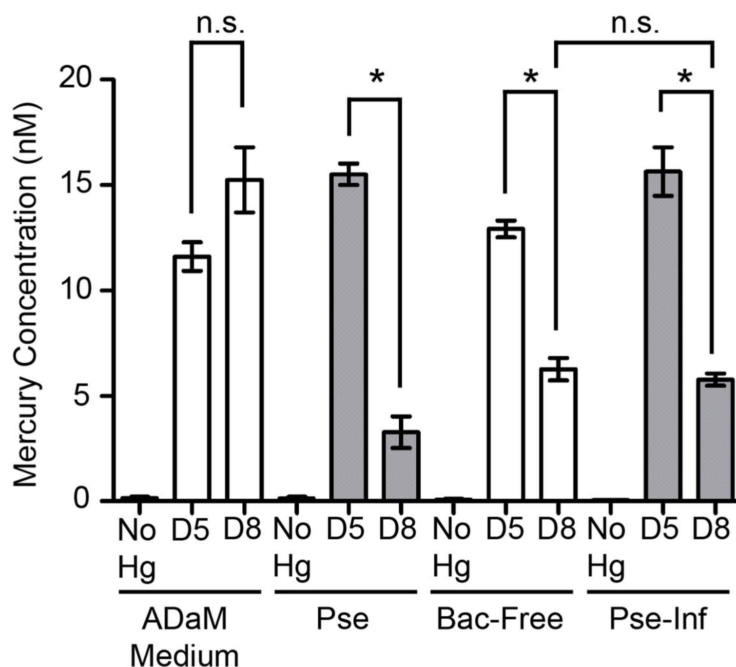

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741

742 **Figure 6**. **Mercury biotransformation by *Pseudomonas*-10.** Mercury
743 concentrations (nM of Hg^{2+}) measured in the ADaM medium control, with
744 *Pseudomonas*-10 only (Pse), bacterial-free daphnids (Bac-Free) and daphnids
745 infected with *Pseudomonas*-10 (Pse-Inf) on Day 5, before (No Hg) and after
746 (D5) addition of mercury, and on Day 8 (D8). Experimental jars containing

747 ADaM medium only served as controls. * indicates significant difference ($p <$

748 0.0001), analyzed using one-way ANOVA with Tukey's HSD Post-hoc test,

749 while n.s. indicates no significant difference ($p>0.05$).

750

751 Our study highlights one of the mechanisms on how an aquatic

752 organism like *Daphnia* can potentially withstand and survive environmental

753 stresses. Just like many hosts, *Daphnia* harbor several groups of bacteria

754 (with different relative abundances) in their microbiome consortium, most of

755 them with unknown functions; some microbial members maybe mutualistic,

756 commensals or parasitic depending on existing environmental conditions, or

757 the microbiome consortium may be operating as ecological guilds, with

758 various microbial members having specific contribution to the functioning of

759 the ecosystem (i.e. the host). Under normal conditions, the microbial

760 consortium collectively increase *D. magna* survival and fitness as reported

761 here and in another study (51). But under stressful environmental conditions,

762 the collective beneficial contribution of the microbial consortium to the host

763 can break down (as shown in this study) and the fitness of the host may

764 depend on the genetic and phenotypic traits of a specific microbiome

765 member/s that can positively respond to the given environmental condition.

766 *Pseudomonas* and other *merA*-containing bacteria only constitute a minor

767 percentage of the microbiome consortium in *D. magna*. In this study, we did

768 not investigate if prolonged exposure to mercury will induce changes in

769 microbiota composition or if it can lead to the enrichment of *merA*-positive

bacteria in *Daphnia*. It would be interesting to investigate the flexibility of the symbiosis under stressful environmental conditions.

In conclusion, this study is one of the few that shows the extension of the microbiome genetic and phenotypic traits to the fitness trait of the host, resulting in a holobiont phenotype that can withstand stressful environmental condition. The microbiome of *Daphnia* should therefore be taken into consideration specifically in ecotoxicological research where *Daphnia* is commonly used as a testing animal.

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Disclosures

The authors declare no competing financial interest.

793

794 **Supporting Information**

795 Additional materials and experimental methods, Genbank accession

796 numbers, list of primers (Table S1), assigned taxon of *D. magna* CAY

797 microbiota (Table S2), *merA* Blastn matches (Table S3), MerA protein

798 alignment and tree (Figure S1), *merA* expression in *Pseudomonas*-10 (Figure

799 S2), *Daphnia* mercury LD_{50} assay (Figure S3), replicates of survival (Figure

800 S4) and fecundity (Figure S5) are available as Supporting Information.

801

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