Tributyltin exposure leads to increased adiposity and reduced abundance of leptogenic bacteria in the zebrafish intestine

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ABSTRACT

The chemical obesogen tributyltin (TBT) is known to promote fat storage in adipose tissue through direct action on vertebrate cells. TBT also has direct toxic effects on microorganisms, raising the possibility that TBT may also promote fat storage in vertebrates by altering their microbiota. Here we show that exposure of conventionally-reared post-embryonic zebrafish to TBT results in increased adiposity, reduced body size, and altered intestinal microbiota composition including reduced relative abundance of *Plesiomonas* bacteria. To test if those microbiota alterations affected host adiposity, we exposed conventionally-reared zebrafish to intestinal bacterial strains representative of TBT-altered taxa. We found that introduction of a *Plesiomonas* strain into conventionally-reared zebrafish was sufficient to reduce adiposity and alter intestinal microbiota composition. Using new long-term gnotobiotic zebrafish husbandry methods,

we found that colonization of germ-free zebrafish with *Plesiomonas* was sufficient to reduce host adiposity. Together these results show the leptogenic activity of *Plesiomonas* on zebrafish hosts, indicating that the ability of TBT to increase adiposity *in vivo* may be due in part to TBT-mediated modification of the abundance of leptogenic bacteria like *Plesiomonas*. These findings underscore how complex reciprocal interactions between animals and their microbial and chemical environments can influence energy balance and metabolic health.

IMPORTANCE

Obesogens are environmental chemicals that promote fat storage and are generally thought to exert this effect directly on animal cells. Using zebrafish, we show that the obesogen tributyltin can also promote fat storage by acting upon intestinal microbiota via reduction of bacteria that are sufficient to reduce fat storage.

INTRODUCTION

Energy balance is a central aspect of animal physiology. When energy intake surpasses energy expenditure (i.e., positive energy balance), excess energy is stored as triglycerides in white adipocyte cells constituting adipose tissue (1, 2). Obesity is a condition of excessive accumulation of adipose tissue relative to body size. Obesity is a growing global public health challenge (3, 4) and a major risk factor for other non-communicable diseases (5, 6). Identification of environmental factors promoting sustained positive energy balance and expansion of adipose tissue is therefore an important goal (7). The intestinal microbiota has emerged as a key environmental factor

49 modulating adipose tissue and body size. Mice reared germ-free (GF) in the absence of
50 microorganisms have reduced adipose tissue (8) and body size (9) compared to mice
51 colonized with their normal microbiota. Moreover, transplantation of fecal microbial
52 communities into GF recipient mice from identical twins discordant for obesity (10), or
53 from individuals with or without a form of malnutrition called kwashiorkor (11),
54 recapitulated the respective phenotypes of the human host. Thus, both the presence
55 and specific composition of gut microbiota have the potential to alter energy storage in
56 adipose tissue and body size in vertebrates.

57 Interaction between the gut microbiota and their vertebrate hosts can be further
58 impacted by xenobiotics (i.e., chemicals not produced by metabolisms naturally
59 occurring in organisms and the environment) (12). Some xenobiotics can alter the
60 composition of intestinal microbiotas and alter host physiology (13-16). Also, xenobiotics
61 can be chemically transformed by intestinal microbiota thus modifying their toxicity,
62 bioavailability, and efficacy when used as therapeutics (17-20).

63 One class of xenobiotic chemicals particularly relevant to energy balance are
64 obesogens, which are defined by their ability to promote adipose tissue accumulation
65 and alter metabolism (21, 22). The organotin compound tributyltin (TBT) is a
66 prototypical obesogen. TBT was once broadly used in antifouling paints to inhibit growth
67 of organisms on the submerged surfaces of ships, and consequently accumulated in
68 marine sediments and organisms prior to the ban on its use (23, 24). Although TBT
69 toxicity was first identified as causing imposex in neogastropods (25, 26), it was later
70 shown to act as an obesogen through *in vivo* studies in multiple tetrapod species (27-
71 31). The effects of TBT in fishes remain less understood, but include increased visceral

72 adipose tissue in the rare minnow (*Gobiocypris rarus*) (32) and inhibited fat mobilization
73 from adipose tissue in response to starvation in zebrafish (*Danio rerio*) (33, 34).
74 Importantly, these *in vivo* studies were conducted in conventionally-reared (CR) animals
75 colonized with their normal microbiota. Thus, it remains unclear whether TBT promotes
76 adiposity via direct effects on host tissues or indirectly via their associated microbiota.
77 Indeed, TBT directly promotes adipocyte differentiation in vertebrate cells *in vitro*, likely
78 acting upon peroxisome proliferator-activated (PPAR) γ and retinoid X (RXR) receptors
79 (27, 28, 35-40). The possibility that TBT might directly affect microbial physiology is
80 supported by studies in bacteria, fungi, and other environmental microbial communities
81 (41-49). Variable levels of microbial susceptibility to TBT have been linked to
82 differences in TBT adsorption, bioaccumulation, enzymatic transformation, efflux and
83 other adaptations (47, 50-58). It is therefore possible that TBT could also directly impact
84 the composition and activity of host-associated microbial communities. Indeed, recent
85 studies showed TBT exposure affects gut microbiota composition in rats and mice (59-
86 61). However, the specific role of TBT-modified microbes on host adiposity remains
87 untested, and such TBT-microbiota interactions in other vertebrates are yet unexplored.
88 In this study, we used the zebrafish to test if the host-associated microbiota and
89 TBT interact to modulate host body size and adiposity. We and others have previously
90 defined the composition of the conventional zebrafish gut microbiota (62-64) and
91 developed short-term gnotobiotic methods for larval zebrafish to reveal impacts of
92 specific microbiota members on host physiology (62, 65-67). We and others also
93 showed that zebrafish develop white adipose tissues during later post-embryonic stages
94 that display extensive homologies with those of mammals (68-71). Using TBT and

95 microbial exposures under conventional as well as new long-term gnotobiotic husbandry
96 we developed for this work, we tested the hypothesis that TBT affects vertebrate
97 adiposity by modifying microbiota composition.
98
99 **RESULTS**
100 ***Tributyltin exposure affects body size and adiposity***
101 We conducted a repeated cross-sectional study to evaluate the impact of
102 tributyltin chloride (TBT) exposure on post-embryonic zebrafish (Fig. 1A). We raised
103 zebrafish embryos conventionally until 18 days post-fertilization (dpf) when the intestinal
104 microbiota had been established (64, 72). We then transferred the experimental cohort
105 to a modified flow through aquaculture system (mFTS, see Methods), where we initiated
106 exposure to TBT (0.01, 0.1, and 1 μg/l) or DMSO vehicle control in tank water at 22 dpf.
107 These TBT concentrations were selected based on ranges observed in the environment
108 and in animal tissues (73-76). Because adipose tissue development in zebrafish
109 depends on body size, particularly on standard length (SL) (69, 70), we size-matched
110 fish prior to exposure. We sampled fish from individual tanks at 7, 14, and 21 days post-
111 exposure (dpE) to measure SL (77) and adiposity (defined as the ratio of the total two-
112 dimensional area of adipose tissues per total body area excluding fins) (78), before
113 dissecting their intestines for 16S rRNA gene sequencing analysis of the intestinal
114 microbiota. We also sampled the tank water to evaluate concomitant effects of TBT on
115 the aquatic microbiota (Fig. 1A, see Methods).
116 We detected no significant differences in zebrafish SL or adiposity between
117 treatment groups (0 dpE; Fig. 2A and B) or replicate tanks (data not shown) prior to

118 exposure. Although no differences in adiposity between the control and all three TBT
119 concentrations were observed at 7 or 14 dpE, exposure to 1 μg/l TBT did cause an
120 increase in adiposity compared to controls at 21 dpE (P = 0.0120; Fig. 2B and D). In
121 addition to greater adiposity, we observed a reduction of SL in zebrafish exposed to 1
122 μg/l of TBT at 14 and 21 dpE (P = 0.0294 and P = 0.0105, respectively; Fig. 2A and C).
123 Other measurements of body size including height at anterior of anal fin (HAA) (77) and
124 total body area were only significantly reduced at 21dpE and only in the 1 μg/l TBT
125 treatment group (P = 0.0042 and P = 0.0083; Fig. S1). Therefore, 1 μg/l TBT inhibits
126 body growth and promotes adiposity in zebrafish after 21 days of exposure.

127 Since zebrafish display substantial inter-individual variation in SL even when
128 age-matched (Fig. 2A), we combined data from 7, 14, and 21 dpE to assess if TBT
129 treatment impacted the overall developmental relationship between SL and total
130 adipose tissue area (69, 70). A linear relationship between SL and adipose tissue area
131 was maintained in all treatment groups (all $P \geq 0.0853$), but the slope of the 1 μg/l TBT
132 group was significantly greater than DMSO vehicle control (P = 0.0005; Figure 2E).
133 Together, this indicates that exposure to 1 μg/l TBT alters the developmental
134 relationship between SL and adipose tissue accumulation.

135 ***Exposure to TBT is not a major determinant of intestinal or aquatic microbiota***
136 ***composition but does alter the relative abundance of specific bacterial taxa***

137 Having observed the effects of TBT on adiposity and body size, we tested if
138 chemical exposure also affected zebrafish intestinal or tank water microbiotas. We
139 performed 16S rRNA gene sequencing of whole intestines and tank water sampled
140 during TBT exposure (Fig. 1A). Intestinal and aquatic microbiota composition were

141 significantly different from each other at all time points and TBT concentrations (all $P \leq$
142 0.003; Table S1). Within each TBT concentration, the composition of intestinal
143 microbiota at each time point was significantly different from all other time points (all $P \leq$
144 0.028; Table S1). Therefore, sample type and developmental age were major
145 determinants of intestinal microbiota composition. In contrast, TBT exposure had a
146 relatively minor effect on intestinal and aquatic microbial community composition. We
147 found only two instances during the study in which the composition of intestinal
148 communities from zebrafish treated with TBT significantly differed from those in vehicle
149 control tanks: at 7 dpE when compared to 0.1 μg/l TBT (P = 0.0030; Table S1) and at
150 14 dpE when compared to 1 μg/l TBT (P = 0.0430; Table S1). Notably, it was at 14 dpE
151 that we first observed SL differences in zebrafish treated with 1 μg/L TBT. However, a
152 significant microbiota composition difference in zebrafish treated with 1 μg/l TBT was no
153 longer detected at the end of the experiment at 21 dpE, when both adiposity and body
154 size phenotypes were evident (Table S1, Fig. 2, and Fig. S1). Also, we found that
155 aquatic communities from control tanks were only significantly different compared to 1
156 μg/l or DMSO at 7 and 21 dpE (P = 0.028 and P = 0.029, respectively; Table S1).
157 Comparison of indices and estimators of α-diversity between controls and all TBT
158 concentrations revealed no differences in the total number of OTUs, Chao1, Shannon or
159 Faith's phylogenetic indices. Together, these data indicate that TBT does not markedly
160 alter the overall composition or diversity of the microbial communities in zebrafish
161 intestine or their tank water.
162 Although TBT exposure did not influence the overall composition of microbial
163 communities, we reasoned that it may affect individual microbial taxa. We therefore

164 applied linear discriminant analysis effect size analysis using LEfSe (79). LEfSe
165 identified biomarker taxa through pairwise comparisons of the relative abundance of
166 OTUs in microbial communities from zebrafish treated with DMSO or with 1 μg/l TBT
167 (the only TBT concentration that rendered body size and adiposity phenotypes) in every
168 timepoint of the exposure. We discovered 51 biomarker taxa at the genus level in the
169 zebrafish intestine (Table S2) and 79 in water (Table S3) associated with either of the
170 selected chemical treatments at any of the time points sampled (7, 14, and 21 dpE).
171 Further, only 4/51 biomarkers in intestinal communities and 9/79 in aquatic communities
172 were found in more than one of the time points sampled (Tables S2 and S3), in accord
173 with our observation above that age is a major determinant of community composition
174 (Table S1). Furthermore, only 7/51 biomarkers in the intestine were also identified as
175 biomarkers in the aquatic communities, in accord with the intestinal and aquatic
176 microbial communities being distinct (Table S1). Finally, most biomarkers in the
177 intestine (47/51) and water (67/79) were only significantly altered at 14 or 21 dpE, the
178 same time points when host phenotypes were also altered (Tables S2 and S3; Fig. 2).
179 Together, these results identify bacterial genera in the zebrafish intestine and tank
180 water that were altered by treatment with 1 μg/l TBT.
181

182 ***Supercolonization of the zebrafish intestine with Plesiomonas ZOR0011 reduces***
183 ***adiposity but does not affect body size***

184 We hypothesized that the effects of TBT on adiposity and body size may be
185 mediated in part by the observed alterations in the relative abundance of specific
186 bacterial genera. To test that possibility, we exposed CR zebrafish to specific bacterial

187 isolates for 14 days (Figure 1B; see Methods). We reasoned that this supercolonization
188 approach would allow us to test the effects of increasing abundance of specific
189 community members in an otherwise normal intestinal microbial community, presumably
190 resembling the specific changes in microbiota composition induced by TBT exposure.
191 We referenced LEfSe-identified intestinal biomarker taxa associated with TBT
192 exposure and alteration in adiposity and body size phenotypes (Fig. 2; Table S2)
193 against a collection of bacterial strains previously isolated from the zebrafish intestine
194 (64, 80). We found strains representing biomarker genera *Plesiomonas* (Class
195 Gammaproteobacteria, Order Enterobacterales; strain *Plesiomonas* ZOR0011), *Vibrio*
196 (Class Gammaproteobacteria, Order Vibrionales; strain *Vibrio* ZWU0020), and
197 *Chitinibacter* (Class Betaproteobacteria, Order Neisseriales; strains *Chitinibacter*
198 ZOR0017 and *Chitinibacter* ZOR0013), all of which grow aerobically and were originally
199 isolated at similar ages to that of our experimental cohort (64). In accord with our LEfSe
200 results, the relative abundance of *Chitinibacter* OTU in the intestines of fish exposed to
201 1 µg/l TBT was higher compared to DMSO controls at 14 and 21 dpE (P = 0.0045 and P
202 = 0.0224, respectively; Fig. 3A). Further, the relative abundances of *Vibrio* and
203 *Plesiomonas* OTUs in the intestines of fish exposed to 1 µg/l TBT were lower compared
204 to DMSO controls at 14 dpE (P = 0.0335 and P = 0.0205, respectively; Fig. 3C and E).
205 In addition, *Plesiomonas* OTU was also more abundant in the intestines of fish treated
206 with 0.1 µg/l TBT at 7 dpE (P = 0.0098; Fig. 3E). The relative abundances of these three
207 OTUs remained unaltered in the tank water at all time points and all TBT concentrations
208 (Fig. 3B, D, and F). These results show that TBT altered the relative abundance of all
209 three selected bacterial genera (*Plesiomonas*, *Vibrio*, and *Chitinibacter)* in the intestine

210 at 14 dpE, the time point of TBT exposure when the earliest host phenotype was first
211 observed (Fig. 2), and one of the only two timepoints in which TBT was a major
212 determinant of microbiota composition (Table S1). Based on the directionality of the
213 TBT-induced changes in the relative abundance of these OTUs by 14 dpE, we
214 hypothesized that an increase in *Chitinibacter* would promote adiposity and reduce body
215 size; while an increase in *Plesiomonas* or *Vibrio* would reduce adiposity and increase
216 body size.

217 Zebrafish entering the supercolonization experiment were size-matched to those
218 that had entered the *in vivo* TBT exposure experiment to ensure they were at similar
219 developmental stages. Mock supercolonization using only autoclaved RAS water was
220 used as a negative control. We sampled fish from individual tanks to evaluate the
221 impact of the bacterial treatments on body size, adiposity, as well as intestinal and
222 aquatic microbial communities (Fig. 1B). Prior to exposure, measurements of zebrafish
223 adiposity and body size did not reveal differences between experimental groups (Fig.
224 4A and B). However, after 14 days of supercolonization, animals treated with
225 *Plesiomonas* ZOR0011 had a significant reduction in adiposity (P = 0.0334; Fig. 4B and
226 D) without effects on standard length or other body size measurements (Fig. 4A and C;
227 Fig. S1). The other three bacterial strains did not significantly affect either of these traits.
228 The observation of the predicted adiposity phenotype after supercolonization with
229 *Plesiomonas* ZOR0011 supports the possibility that the reduction in the relative
230 abundance of *Plesiomonas* bacteria following TBT exposure may mediate, at least
231 partially, the resulting host phenotypes.

232 We next used 16S rRNA gene sequencing to verify if supercolonization
233 effectively increased the relative abundance of the introduced bacterial strains in the
234 water or zebrafish intestinal microbial communities. We examined the relative
235 abundance of *Chitinibacter*, *Plesiomonas*, and *Vibrio* OTUs in the aquatic communities
236 after 14 days of supercolonization with the four different intestinal bacterial strains.
237 None of those bacterial treatments significantly increased the relative abundance of
238 *Chitinibacter*, *Plesiomonas* or *Vibrio* OTUs in the tank water when compared to negative
239 controls (Fig. 4E). Nevertheless, results from the more sensitive LEfSe analysis
240 revealed that the OTUs assigned to each introduced bacterial strain were in fact
241 biomarker genera of their corresponding treatment group (Table S4). Therefore,
242 supercolonization effectively increased the relative abundance of introduced bacterial
243 genera in the aquatic environment, thereby potentially allowing them to also colonize
244 the zebrafish intestine. However, changes in the relative abundance of members of the
245 intestinal microbial communities of supercolonized animals were unique to the strain
246 *Plesiomonas* ZOR0011. The relative abundance of OTU *Plesiomonas* in the intestine
247 was increased upon supercolonization with *Plesiomonas* ZOR0011 (P = 0.0022; Fig.
248 4E), a result confirmed by LEfSe analysis (Table S5). For the other three strains tested,
249 the respective OTUs were not altered nor identified as biomarkers by LEfSe analysis.
250 These data support the hypothesis that the host adiposity phenotype caused by *in vivo*
251 exposure to 1 µg/l TBT may be mediated in part by the chemically-induced alteration of
252 the relative abundance of *Plesiomonas* in the zebrafish intestine.
253

254 ***Supercolonization with Plesiomonas ZOR0011 alters zebrafish intestinal***
255 ***microbiota***

256 We next asked if supercolonization with *Plesiomonas* ZOR0011 or any of the
257 other selected bacterial strains, altered the composition of the intestinal or aquatic
258 microbiota. As expected, composition of intestinal and aquatic microbiotas remained
259 significantly different regardless of the treatment (all $P \leq 0.008$; Fig. S2; Table S6).
260 Supercolonization with the different bacterial strains did not alter α-diversity of aquatic or
261 intestinal communities (total number of OTUs, Chao1, Shannon or Faith's phylogenetic
262 indices). However, the composition of intestinal microbial communities in zebrafish
263 supercolonized with *Plesiomonas* ZOR0011 or *Chitinibacter* ZOR0017 displayed
264 significant differences compared to negative controls ($P = 0.001$ and $P = 0.027$
265 respectively; Fig. S2, Table S6). Also, the intestinal microbial communities in zebrafish
266 supercolonized with *Plesiomonas* ZOR0011 and *Chitinibacter* ZOR0017 are distinct (P
267 $= 0.001$; Fig. S2, Tables S6). These results show that supercolonization with
268 *Plesiomonas* ZOR0011 and *Chitinibacter* ZOR0017 had distinct effects on intestinal
269 microbiota composition.
270

271 ***Monoassociation of zebrafish with Plesiomonas ZOR0011 inhibits adiposity and***
272 ***body size***

273 Supercolonization with *Plesiomonas* ZOR0011 reduced adiposity, but also
274 altered the composition of the established intestinal microbiota. We therefore sought to
275 test the sufficiency of *Plesiomonas* ZOR0011 to directly influence adiposity and body
276 size in the absence of other microbes using a gnotobiotic approach. Although methods

277 for gnotobiotic husbandry of zebrafish embryos and larvae have been established, they
278 were not optimized to support growth of zebrafish into later stages when adipose
279 tissues develop (68-70). We therefore developed new methods for long-term gnotobiotic
280 zebrafish husbandry (see Methods) to raise germ-free (GF) zebrafish to 14 dpf before
281 conducting a monoassociation with *Plesiomonas* ZOR0011 or *Chitinibacter* ZOR0013.
282 *Chitinibacter* ZOR0013 was selected as a control because it did not elicit a phenotype
283 nor altered the composition of the intestinal microbiota after supercolonization. As an
284 additional reference control, we tested the impact of TBT on zebrafish body size and
285 adiposity in the absence of any microbes by exposing GF zebrafish to 1 μg/l TBT or
286 DMSO. We subjected GF zebrafish to these treatments for 21 days to match the length
287 of adiposity phenotype during the TBT exposure (Fig. 4B and D). We detected no
288 differences in mortality (all $P \geq 0.1986$ at 7 dpE/dpC; and $P \geq 0.5213$ at 21 dpE/dpC) or
289 the density (all $P \geq 0.8869$ at 7 dpE/dpC; and $P \geq 0.9474$ at 21 dpE/dpC) of the flasks
290 between the chemical or bacterial conditions tested (Methods). Whereas there were no
291 phenotypical alterations in fishes after 7 days of treatment, we observed a reduction in
292 adiposity of gnotobiotic zebrafish 21 days post-colonization (dpC) with *Plesiomonas*
293 ZOR0011 ($P = 0.0399$; Fig. 5A, B, and D), resembling the effect of supercolonization of
294 CR zebrafish with this bacterial strain (Fig. 4B and D). In contrast to its
295 supercolonization phenotype, monoassociation with *Plesiomonas* ZOR0011 also
296 reduced body size of gnotobiotic zebrafish ($P = 0.0256$; Fig. 5A and C). Finally, neither
297 monoassociation with *Chitinibacter* ZOR0013 or exposure to 1 μg/l TBT in the GF
298 background elicited body size or adiposity phenotypes after 21 days (Fig. 5). Together,
299 these results show that *Plesiomonas* ZOR0011 is sufficient to directly reduce adiposity

300 and body size, and suggest that obesogenic effects of TBT on zebrafish may require the
301 presence of microbiota.

302 ***TBT affects specific members of the intestinal microbiota directly in the absence***
303 ***of the zebrafish host***

304 The studies above did not resolve whether TBT affects *Plesiomonas* and other
305 intestinal bacteria *in vivo* directly, or indirectly through host-dependent processes. To
306 investigate the direct effect of TBT on zebrafish intestinal bacteria, we harvested
307 intestinal microbiota from CR zebrafish and cultured them *in vitro* in non-selective rich
308 media containing different concentrations of TBT similar to those tested in the *in vivo*
309 chemical exposure (0.01, 0.1, and 1 µg/l TBT) and two additional higher concentrations
310 (10 and 1000 µg/l TBT) anticipating the presence of highly-resistant bacteria observed
311 in some aquatic environments (42). Zebrafish used as microbiota donors were size-
312 matched to zebrafish from control tanks at the endpoint of the *in vivo* TBT exposure
313 (see Methods). We incubated the communities *in vitro* for 24 hours at room temperature
314 under aerobic and anaerobic conditions and used 16S rRNA gene sequencing to
315 interrogate their composition. Of a total of 272 OTUs detected across the different
316 treatments and the input community, only 62 were observed both in the input
317 community and at least one condition after *in vitro exposure to TBT*. Nevertheless, most
318 of the OTUs detected in the *in vitro* cultures (85/90) were also observed in the intestinal
319 microbiota of zebrafish at 21 dpE during the *in vivo* chemical exposure in at least one of
320 the chemical conditions. Therefore, although OTUs from the *in vitro* experiments
321 represent only the culturable fraction of an *in vivo* community, they provide a relatively

322 broad representation of OTUs found in the intestinal microbiota of zebrafish from the
323 same size that underwent TBT exposure *in vivo* (Table S7).
324 As in the *in vivo* chemical exposures, diversity analysis showed that TBT was not
325 a major determinant of the composition of the culturable zebrafish intestinal microbiota
326 at any of the concentrations tested *in vitro* (all $P \geq 0.05$, Table rename). In addition, α-
327 diversity was not altered by the exposure to TBT (total number of OTUs, Chao1,
328 Shannon or Faith's phylogenetic indices). LEfSe analysis identified 37 biomarker taxa of
329 *in vitro* TBT exposure at the genus level, in at least one of the pairwise comparisons
330 between the DMSO and the different TBT concentrations (Table S8). Of the 37
331 biomarker genera, 11 of them were also identified as biomarkers of the *in vivo* exposure
332 to 1 µg/l TBT at time points when zebrafish phenotypes were detected (14 and 21 dpE,
333 Table S8). These 11 biomarkers included genera *Vibrio and Plesiomonas*. *Plesiomonas*
334 showed an increased relative abundance (2.7-fold) only at the highest TBT
335 concentration of 1000 µg/l under aerobic conditions. In contrast, the lower TBT
336 concentrations used in the *in vivo* chemical exposure experiment (0.01, 0.1, and 1 µg/l
337 TBT) did not affect the relative abundance of *Plesiomonas* in microbiota cultured *in vitro*
338 (Tables S7 and S8). These results suggest that *Plesiomonas* sensitivity to TBT may be
339 determined in part by the host.
340

341 **DISCUSSION**

342 TBT is considered a prototypical obesogen (27-31). In zebrafish and other
343 teleosts, TBT promotes the accumulation of lipids in different tissues and organs (32,
344 81-88). Exposure of post-embryonic zebrafish to TBT impaired adipose tissue

345 mobilization in response to food restriction (33, 34), but its impact on adipose tissue
346 accumulation in fishes was unknown. Our results establish that a 21-day TBT exposure
347 promotes adiposity in zebrafish, indicating its obesogenic activity on teleost fishes. We
348 also found that the TBT-induced adiposity phenotype was accompanied by reduced
349 body size. This is in accord with reports that TBT compounds reduced body length in
350 zebrafish (89), other teleost species (90, 91) and in *Xenopus tropicalis* (92), as well as
351 body weight in other vertebrates (93-95), although these studies did not examine co-
352 occurring lipid or adipose phenotypes. In humans, stunted growth and other forms of
353 malnutrition coexist with obesity (96) and earlier manifestations of undernutrition may
354 predispose to obesity (97). We observed that the impacts of TBT on SL (by 14 dpE)
355 preceded overt impacts on overall body size and adiposity (by 21 dpE), but it remains
356 unclear if these adiposity and body size phenotypes are mechanistically linked. It is
357 possible that TBT-induced impairments in fat mobilization from adipose tissues in
358 zebrafish (34) reduce the energy available for growth. Identification of the direct and
359 indirect mechanisms by which TBT affects the relationship between adiposity and body
360 size is therefore warranted.

361 There is a growing appreciation that xenobiotics may exert their impacts on
362 animal health by altering the composition of their microbiotas (15, 16, 59-61, 98-100).
363 While TBT has well-documented direct actions on adipocyte differentiation and other
364 aspects of host physiology (27, 28, 35-40), it was only recently shown to alter the
365 composition of gut microbiota in rodents (59-61). In attempt to test if TBT-modified gut
366 microbiota is sufficient to affect host physiology, Zhan and coworkers transplanted TBT-
367 modified fecal microbiota into recipient mice pre-treated with antibiotics to deplete their

368 resident microbiota, and recapitulated adiposity and metabolic phenotypes observed in
369 donors (60). However, this experimental approach does not control for potential
370 confounding effects of antibiotic-resistant microbes, the direct effects of antibiotics on
371 the recipient host (101), or the possibility of bioaccumulation of TBT in fecal microbiota.
372 We found that TBT altered the relative abundance of a subset of intestinal bacterial
373 genera in CR zebrafish. Whereas the alterations in intestinal microbiota composition
374 was relatively modest, this information allowed us to identify TBT-affected bacterial
375 genera for further sufficiency tests. It also remains possible that, beyond these
376 compositional changes, TBT affects the functional potential of the microbiota as has
377 been demonstrated with other xenobiotics (102-104).

378 We developed multiple experimental approaches to test if microbes affected by TBT
379 exposure *in vivo* were themselves sufficient to evoke the phenotypes observed in their
380 hosts. Supercolonization is a strategy that introduces microbial strains into animals
381 already colonized with a microbiota, and has been effective for defining the impact of
382 the introduced microbes on the host and the intestinal ecosystem (105-107). By
383 supercolonizing CR zebrafish with available individual bacterial strains representing
384 TBT-affected genera, we found that *Plesiomonas* ZOR0011 was the only strain,
385 amongst those tested, sufficient to reduce adiposity in zebrafish. One advantage of the
386 supercolonization approach is that it inherently accommodates the potential for
387 microbial interactions. Indeed, *Plesiomonas* ZOR0011 supercolonization altered the
388 microbiota composition, making it unclear whether the impact of *Plesiomonas* ZOR0011
389 supercolonization on host adiposity was direct or indirect via alterations in the

390 abundance of other microbiota members. For these reasons we used a complementary
391 gnotobiotic approach to study the isolated effect of *Plesiomonas* ZOR0011.

392 Although methods for gnotobiotic zebrafish husbandry have been in use for over
393 17 years (62, 67, 108), almost all studies have focused on early free-feeding larval
394 stages. This postponed the need to develop nutrition and housing systems for raising
395 zebrafish into later life stages. The husbandry methods that we developed for this study
396 permit growth of zebrafish to standard lengths at which most adipose tissue depots
397 develop (70). We anticipate these methods will also be useful for testing the role of
398 microbial exposures on other aspects of post-embryonic physiology in the zebrafish.
399 Using these new methods, we showed that the exposure of GF zebrafish to a TBT
400 concentration that was phenotypical in CR zebrafish did not affect body size or
401 adiposity. This suggests that GF zebrafish may be resistant to TBT-induced
402 phenotypes, but additional studies are needed to determine if this is limited to specific
403 developmental stages or TBT dosages. Nevertheless, our combined approaches
404 showed that *Plesiomonas* ZOR0011 was sufficient to inhibit adiposity in zebrafish, both
405 in the absence and the presence of a conventional microbiota.

406 A major finding of this study is the discovery that a bacterial strain isolated from
407 the zebrafish intestine, *Plesiomonas* ZOR0011, has leptogenic effects on zebrafish
408 hosts. *Plesiomonas* bacteria are common members of the intestinal microbiota in
409 laboratory-reared zebrafish at all life stages and across multiple aquaculture facilities,
410 and have also been observed in adult zebrafish recently captured from their natural
411 habitat (63, 64). *Plesiomonas* abundance in the zebrafish intestine has also been
412 shown to be sensitive to other xenobiotics besides TBT (109-113), but this study is the

413 first to explore its direct physiologic effects on this host. Although the relative
414 abundance of *Plesiomonas* in the intestine was relatively low in our study, it had a
415 significant effect on host physiology and on microbiota composition. Other studies have
416 also underscored how members of the microbiota of relatively low abundance can have
417 significant effects on host-microbe commensalism (66, 114). The mechanisms by which
418 *Plesiomonas* ZOR0011 exerts its leptogenic effects on zebrafish and affects microbiota
419 composition remain unknown. The genus *Plesiomonas,* thought to consist of a single
420 species *P. shigelloides,* is common in aquatic ecosystems and inhabits diverse aquatic
421 vertebrates and invertebrates (115). *Plesiomonas* has also been detected in the
422 intestinal microbiota in healthy humans and some mammals, yet also frequently
423 associated with gastroenteritis in humans (115). We did not observe any overt
424 pathology or mortality in zebrafish supercolonized or monoassociated with *Plesiomonas*
425 ZOR0011, thus its sufficiency to reduce adiposity and body size may be consistent with
426 a role on digestive physiology or energy balance. Moreover, our recent finding that the
427 relative abundance of *Plesiomonas* members was markedly reduced by starvation in
428 adult zebrafish, suggests potential roles in host nutritional physiology (116). Further
429 studies are needed to discern the mechanisms by which *Plesiomonas* ZOR0011 exerts
430 its leptogenic effect.

431 Whereas the focus of this study was to assess impacts of TBT on gut microbiota
432 and its consequences *in vivo*, we also investigated the direct effect of TBT on zebrafish
433 microbiota *in vitro* in the absence of the host. Our results demonstrated that the impact
434 of xenobiotics like TBT on members of the zebrafish intestinal microbiota can differ
435 between *in vivo* and *in vitro* scenarios. Many genera including *Plesiomonas* were not

436 affected by phenotypical levels of TBT *in vivo* (0.1 and 1 μg/l TBT), and their relative
437 abundance *in vitro* was only significantly affected by much higher concentrations (1000
438 μg/l TBT). We were unable to test the effect of TBT exposure to ≥ 10 μg/l on intestinal
439 microbiota *in vivo* as this concentration caused >92% mortality as early as 1 dpE (see
440 Methods). The global increase in synthetic chemicals like TBT is recent in the shared
441 evolutionary history of vertebrate animals and their commensal bacteria with whom they
442 continue to co-evolve (117-119). The effect of chemical novelty on intestinal bacteria
443 like *Plesiomonas* is presumably an anthropogenic impact on organisms across the tree
444 of life, as individuals or as holobionts. Zebrafish, a well-established animal model for
445 human disease, can also serve as a model for wild and farmed fishes, vulnerable to
446 xenobiotic exposure and central to food security (120-122). Further understanding of the
447 specific effect of xenobiotics on intestinal microbiota can contribute to both planetary
448 sustainability and the improvement of human and animal health.

449

450

451 **MATERIALS AND METHODS**

452 *Conventional zebrafish husbandry and obtention of experimental cohorts*

453 All zebrafish experiments were approved by the Institutional Animal Care and
454 Use Committees of Duke University Medical Center under protocol A115-16-05 in
455 accordance with the Public Health Service Policy on the Human Care and Use of
456 Laboratory Animals under the United States of America National Institutes of Health
457 Office of Laboratory Animal Welfare (OLAW). Adult zebrafish (wild-type Ekkwill strain)
458 were raised under conventional conditions on a Pentair recirculating aquaculture system

459 (RAS) and set up for natural breeding to obtain the embryos constituting the different
460 experimental cohorts. Four adult fish (1:1 male to female ratio) from five families were
461 set in mating boxes for group spawning. The resulting clutches of embryos were
462 collected synchronously, transferred to Petri dishes containing RAS water, and
463 maintained at a density of ~70 fish per dish and incubated at 28 °C. Abnormal embryos
464 were removed during the first 24 hours post-fertilization (hpf). At ~36 hpf, embryos were
465 pooled and randomized into groups of 62 embryos which were transferred to clean 3-
466 liter tanks (30 tanks total). Each 3-liter tank, filled with 500 ml of RAS water, was kept
467 static until 5 dpf. At 5 dpf, the 3-liter tanks were connected to the RAS and maintained
468 according to our standard husbandry protocols (123) with one modification to feeding
469 before their transfer to the modular flow through aquaculture system (mFTS, see below)
470 where the experimental cohorts were housed during the experiments. The modification
471 was that, instead of receiving an exclusive powdered diet of Zeigler AP100 <50-micron
472 larval diet (Pentair, LD50-AQ) twice daily from 5 dpf until 14 dpf, larvae were
473 supplemented twice daily with 500 µl of *Artemia* (Brine Shrimp Direct, BSEACASE) that
474 was collected, rinsed and resuspended in an equal volume of RAS water.
475 The experimental cohort for the *in vivo* chemical exposure to TBT was
476 transferred to the mFTS on 18 dpf. On the day of transfer to the mFTS, zebrafish were
477 collected from the RAS in sterile 25 mm-deep Petri dishes, where they were
478 anesthetized using 0.2 g/L buffered Tricaine to measure their SL (77) for size-matching
479 individuals between 7 and 11 mm. The SL of the experimental cohort prior to TBT
480 exposure (0 days post-exposure, dpE) was of 8.39 ± 0.82 mm (mean ± SD), which was
481 measured at 22 dpf. A total of 20 pools of 30 individuals constituted the experimental

482 cohort during the *in vivo* chemical exposure study. For the supercolonization
483 experiments, the cohort was obtained in the same manner with the exception that
484 transfer to the mFTS occurred on 24 dpf. Zebrafish entering the supercolonization
485 experiment were size matched to those that had entered the TBT exposure to avoid
486 developmental differences between these two experiments. The SL of the experimental
487 cohort prior to bacterial supercolonization (0 days post-supercolonization, dpSC) was of
488 8.77 ± 0.84 (mean ± SD), which was measured at 27 dpf. 15 pools of 30 individuals
489 constituted the experimental cohort during the supercolonization study.
490
491 *Modular flow through system (mFTS) construction and operation*
492 Because TBT is likely degraded by filtration through activated carbon (124) and
493 UV exposure (125) which are used to maintain water quality on the RAS, we designed,
494 built and installed a modular flow-through system (mFTS) in one of the racks at our
495 RAS. The mFTS housed the experimental cohorts for both the *in vivo* TBT exposure
496 and the supercolonization experiments.
497 Each shelf in our facility fits five 10-liter polycarbonate tanks connected to the
498 RAS by five manifolds that deliver pre-conditioned RAS water into the experimental
499 tanks. The mFTS used the standard polycarbonate tanks from the RAS system which
500 we modified by drilling and installing polycarbonate food-grade beverage dispenser
501 faucets serving as spigots for draining the tanks during water exchanges (2712212,
502 Carlisle). Inside the tanks, the spigots were covered with fiberglass mesh (33105,
503 Lowe's) serving as baffles to prevent fish from escaping during water exchanges. The
504 spigots from the five tanks in each shelf were connected through fitted vinyl hoses to a

505 3/4-inch PVC pipe mounted with a slight angle of inclination with respect to the fish tank
506 shelf. This angle prevented backflow during the water exchanges. One PVC pipe was
507 used to collect the outflow of 5 tanks from a shelf. Four PVC-pipes were finally
508 connected, through a 90-degree 3/4-inch elbow, to a fitted 1-inch vinyl hose and
509 secured with an adjustable clamp (57188, Lowe's). The 1-inch vinyl hose ran
510 perpendicular to the fish tank shelves into a drain, to collect the outflow of the mFTS
511 system. Thus, the mFTS thus allowed us to drain several fish tanks simultaneously to
512 perform water exchange in a relatively automated and uniform manner. The outflow of
513 each fish tank was controlled by the location of the spigot along the height of the tank.
514 We installed the spigot to regulate an outflow of 5.5 L, with 2.5 L retained below the
515 spigot after draining. After draining, the tanks in the mFTS were refilled with the
516 conditioned water from the RAS system via the RAS manifold. The modified tanks in the
517 mFTS were initially filled with 7.5 L of conditioned RAS. A total of 1.6 volumes of water
518 were exchanged daily to maintain an adequate water quality.
519 The mFTS held twenty 10-liter modified polycarbonate tanks during the *in vivo*
520 chemical exposure to TBT, and fifteen during the supercolonization experiments. Water
521 quality parameters were assessed in the mFTS during the two studies. Conductivity was
522 measured in the conditioned RAS water before water exchange. pH, nitrate and nitrite,
523 and toxic ammonia were measured in the mFTS and in the conditioned water using test
524 kits according to the manufacturer's instructions (147008, 2745425, and 224100
525 respectively, HACH). Water quality parameters were measured on every tank in the
526 mFTS on the day before adding the experimental cohort to the tanks, and on every time
527 point sampled during the studies. The pH and conductivity in the conditioned water

528 used for the water exchange was recorded daily. The nitrite levels in the conditioned
529 water were 0 ppm, nitrate between 2 and 10 ppm, and toxic ammonia was 0 mg/l.
530 During the *in vivo* chemical exposure to TBT, the pH in the tanks ranged between 7 and
531 7.6, nitrite between 0 and 0.15 ppm, nitrate between 2 and 10 ppm, and toxic ammonia
532 between 0 and 0.00521 mg/l. The water pH and nitrate in the experimental tanks during
533 the chemical exposure was comparable to that of the conditions water, while the nitrite
534 and the toxic ammonia were higher. During the supercolonization experiments, water
535 pH in the experimental tanks ranged between 7.37 and 7.41, nitrite was 0, nitrate
536 ranged between 2 and 10 ppm, and toxic ammonia between 0 and 0.00106 mg/l. When
537 compared to the conditioned water, the pH was lower, the nitrite and nitrate were
538 comparable, and the toxic ammonia was slightly higher. Finally, the conductivity in the
539 RAS water used during the water exchanges during the *in vivo* exposure to TBT ranged
540 between 712 and 845; and during the supercolonization experiments, between 773 and
541 801. Overall, water quality parameters in the tanks were within the optimal ranges for
542 the zebrafish (126). The only substantial environmental difference between the mFTS
543 and the RAS was the temperature. In the RAS, the water temperature is maintained at
544 28.5 °C by an in-line heating element. In the mFTS, tanks were filled with conditioned
545 RAS water but then equilibrated to air temperature over time. Water temperature in the
546 tanks after the water exchange was recorded daily in the two studies. During the *in vivo*
547 exposure TBT, the average water temperature recorded in the afternoon was 22.3 °C;
548 and in the supercolonization experiments, 22.9°C. Notably, the water temperature
549 equilibrated with the room temperature during the evening, resulting in a lower water
550 temperature in the morning. During the *in vivo* exposure to TBT, the average water

551 temperature recorded in the morning was 19.1°C, and in the supercolonization
552 experiments 20.1°C. Because the mFTS was located inside our main zebrafish facility,
553 we were not able to change the room temperature or install space heaters.
554 During their housing in the mFTS, zebrafish received an exclusively live feed
555 diet. *Artemia* (Brine Shrimp Direct, BSEACASE) was offered twice a day. *Artemia* was
556 collected, rinsed and resuspended in RAS water. The volume and the concentration of
557 *Artemia* (volume collected from hatching cone/volume of RAS water used for
558 resuspension after rinsing) were adjusted to prevent the accumulation of uneaten food
559 at the bottom of the tanks and recorded daily. Each tank in the mFTS received the same
560 amount of food, and the order of feeding was alternated daily to prevent any feeding
561 bias that could affect growth. Uneaten food and debris accumulated at the bottom of the
562 tank were removed as required using a 10 ml sterile serological pipette during the water
563 exchange.
564
565 *Repeated cross-sectional study of in vivo TBT exposure*
566 Four stocks of 1 mg/ml, 0.1 mg/ml, 0.01 mg/ml, and 0.001 mg/ml of TBT (Sigma,
567 T50202) in DMSO (Fisher, D136-L) were prepared. All stocks were maintained in the
568 dark at 4° C until use. Small volumes of these stocks were aliquoted in sterile
569 borosilicate glass vials to avoid repeated freeze-thaw cycles. DMSO stocks were
570 aliquoted in the same manner.
571 Tanks in the mFTS were initially dosed with 75 µl of the corresponding stocks of
572 TBT and DMSO stocks to achieve final concentrations of TBT in water of 10 µg/l, 1 µg/l,
573 0.1 µg/l, and 0.01 µg/l, respectively; as well as DMSO vehicle control. By aliquoting the

574 same volume of stocks, we also maintained an equal volume of DMSO in all treatments.
575 Stocks were added before refilling tanks after the water exchange to facilitate mixing.
576 We assumed that TBT was not degraded over a 24-hour period, so we added a volume
577 of stock daily to adjust the concentration of TBT in the tanks for the total loss of
578 chemical during the daily water exchange.

579 Each TBT concentration and the control had four tank replicates and 30 fish per
580 tank (Fig. 1A). The TBT concentration of 10 µg/l resulted in a mortality rates between
581 92.31 and 100% after one day of exposure so this treatment was excluded from the
582 study and the schematic in Fig. 1A. The tank replicates for each treatment were
583 randomly distributed in our zebrafish facility racks to avoid feeding and water exchange
584 biases (not represented in Fig. 1A). Four time points were sampled during this repeated
585 cross-sectional study: one before starting the exposure to TBT (0 days post-exposure,
586 dpE); as well as 7, 14, and 21 dpE (Fig. 1A). The fish were not offered food on the day
587 of sampling and had received their last meal and chemical treatments 14 hours before
588 sampling. 200 ml of water from each tank were collected in sterile conical vials: 150 ml
589 were used for the aquatic microbial community analysis (see below), and the remainder
590 50 ml we used for measuring the water quality parameters (pH, nitrate, nitrite, and toxic
591 ammonia) and for counting colony forming units (CFU). Five individual fish per tank (20
592 fish per treatment) were sampled at each time point indicated in Fig. 1A. Body size (SL,
593 HAA, and body area), adipose tissue accumulation, and composition of the intestinal
594 microbial communities from each fish were also analyzed (see below, Fig. 1A).

595

596 *Zebrafish body size and adipose tissue analysis*

597 Nile Red (Sigma, N1142) staining was performed as described in (33, 70, 78)
598 except that the Nile red solution was prepared with filtered and autoclaved RAS water
599 (FASW). Zebrafish were euthanized in 1.34g/L buffered Tricaine and laid on 1.5%
600 methylcellulose in FASW on the right flank for imaging. We used a Leica M205
601 fluorescence stereomicroscope with a GFP bandpass filter to image Nile Red
602 fluorescence in adipose tissues and corresponding brightfield images of each animal.
603 All images per time point were acquired using the same exposure settings. Image
604 analysis was conducted in FIJI/ImageJ 1.5p (127). SL, HAA, and total body area were
605 measured to evaluate body size as described in (70, 77, 78).
606
607 *Intestinal and aquatic sample collection*
608 After imaging, euthanized zebrafish were transferred to a Petri dish coated with
609 sterile 3% agarose, onto which fish were immobilized by the tail using an insect pin
610 dipped in 70% ethanol. After decapitation with an autoclaved razorblade, the whole
611 gastrointestinal (GI) tract was removed using forceps. Individual whole intestines were
612 separated from the other organs of the GI and transferred to sterile screw cap tube
613 containing 100 µl of sterile 0.1-mm-diameter zirconia beads (BioSpec Products,
614 NC0268065) and 400 µl of sterile lysis buffer (20 mM Tris-HCl pH 8.0, 2 mM EDTA pH
615 8.0, 1% Triton X-100; modified from (72)). Tank water from the upper water column was
616 filtered as described in (72). Filters were then extracted using flame-sterilized forceps
617 and cut in half using a sterile scalpel. One half of the paper disc was rolled and inserted
618 into a sterile screw cap tube containing 200 µl of sterile 0.1-mm-diameter zirconia
619 beads, to which 200 µl of sterile lysis were immediately added. Intestinal and water

samples were flash frozen in a dry ice-ethanol bath and stored at −80°C for subsequent 16S rRNA gene sequencing.

Supercolonization with zebrafish intestinal bacterial strains

In the supercolonization experiments described here, we used four bacterial strains isolated from the zebrafish intestine: *Chitinibacter* ZOR0017, *Chitinibacter* ZOR0013, *Plesiomonas* ZOR0011; and *Vibrio* ZWU0020 (64, 80). Strains ZOR0017, ZOR0013, and ZOR0011 were generously provided by Karen Guillemin (University of Oregon). Single colonies grown on tryptic soy agar (TSA) plates were picked and grown overnight in tryptic soy broth (TSB). 750 µl of the overnight cultures were collected to extract DNA and confirm the identity of the strain by sequencing the 16S rRNA gene using the universal 8F and 1492R primers. An aliquot of this overnight verified culture was diluted and plated on TSA for growth of well-spread single colonies that were used to inoculate fresh daily overnight cultures used for preparing the bacterial suspension administered daily during the supercolonization experiments. The daily overnight cultures of 10 ml of tryptic soy broth (TSB) were incubated aerobically at 30 ^{o}C for 14 to 16 h in rotary mixer at 100 rpm. 300 µl of the daily overnight culture were collected for 16S rRNA sequencing-based verification of the bacterial strains.

A starter culture of 10 ml of fresh TSB was inoculated with the overnight culture at a starting OD_{600} of 0.1 and incubated for 6 to 8 h at 30°C in a roller drum at 100 rpm. The starter culture was used to inoculate 200 ml of fresh sterile TSB in an culture flaks (i.e. supercolonization culture). Because of differences in the growth rate of the different strains, and to obtain supercolonization cultures of approximately the same density at

643 the same time, the supercolonization culture for *Chitinibacter* ZOR0017 and
644 *Chitinibacter* ZOR0013 was inoculated at a starting OD_{600} of 0.05, whereas
645 *Plesiomonas* ZOR0011 and *Vibrio* ZWU0020 were inoculated at a starting OD_{600} of
646 0.025. The supercolonization culture was incubated at 30°C in a shaker at 100 rpm for
647 14 hours.

648 To prepare the bacterial suspension, we calculated the volume of the
649 supercolonization culture required to prepare 200 ml of bacterial suspension in fresh
650 TSB at an OD_{600} of 1. The diluted bacterial suspension was transferred to an autoclaved
651 Nalgene centrifuge bottle (B1033, Sigma), and spun in a Sorvall RC5B centrifuge at
652 5000rpm at a set temperature range of 10 – 25°C. The pellet was washed twice with
653 100 ml of autoclaved RAS water, and resuspended in 60 ml of autoclaved RAS water
654 (i.e., bacterial suspension for daily supercolonization). 750 µl of each bacterial
655 suspension were sampled daily to count CFU on TSA and 16S rRNA sequencing-based
656 verification of the bacteria strains. The CFU/ml in the tanks after supercolonization
657 (estimated from the CFU/ml in the bacterial suspension) was measured daily. The
658 average CFU/ml was not significantly different amongst the bacterial strains over the 14
659 days of supercolonization (One-way ANOVA and Tukey's multiple comparisons test; P =
660 0.7770). The bacterial suspension was divided into 20 ml aliquots used to supercolonize
661 each tank. Before adding the bacterial suspension, the tanks were drained to the 2.5 L
662 volume. To promote supercolonization of the intestine with this inoculum, zebrafish were
663 offered *Artemia* after adding the bacterial suspension. Zebrafish, bacteria, and *Artemia*
664 were incubated for 2.5 h, before continuing with the water exchange as described
665 above. Control tanks received a mock inoculation of 20 ml of autoclaved RAS.

666 Each bacterial treatment and the control had three tank replicates (15
667 experimental tanks total with ~30 fish per tank) randomized in order. We performed
668 daily supercolonization for 14 days, which was a period equivalent to the earliest time
669 point at which we observed body size and adiposity phenotypes during the *in vivo*
670 chemical exposure (Fig. 2). Five fish per tank were sampled at (0 dpSC) for intestinal
671 microbiota and body size analysis. Fifteen fish were sampled at the 14 dpSC (as there
672 was only one time point in this experiment) for body size and adipose tissue analysis.
673 Five of these fifteen fish were also sampled for intestinal microbiota analysis (Fig. 1B).
674 Tank water was collected from each tank for aquatic microbiota analysis at 0 and 14
675 dpSC as previously described for aquatic microbiota and water quality analysis.
676
677 *Gnotobiotic zebrafish husbandry and obtention of experimental cohorts*
678 The maintenance of the flasks containing GF zebrafish, diet administered and
679 monoassociation procedures were adapted from (108, 128) to maintain GF animals up
680 to 35 dpf. This substantial improvement of gnotobiotic husbandry was necessary for our
681 experiment because adipose tissue starts to develop when zebrafish are SL of ~4.2 mm
682 which in conventional husbandry is detectable around 12 dpf (69, 70). Moreover, we
683 wanted to mimic the phenotypical *in vivo* chemical exposure to 1 µg/l TBT (both body
684 size and adiposity at 21 dpE; Fig.2) and supercolonization with *Plesiomonas* ZOR0011
685 (at 14 dpSC; Fig.4) under conventional husbandry conditions.
686 Four conventionally reared adult fish (1:1 male to female ratio) from two different
687 families were set in mating boxes for group spawning. Zebrafish embryos were obtained
688 by natural mating. The collection of the embryos and the derivation procedure to obtain

689 GF zebrafish was conducted as described in (108). All procedures after GF derivation
690 were conducted using sterile technique, sterile materials wiped with ethanol and UV-
691 irradiated, inside a laminar flow cabinet. Sterility tests throughout this experiment were
692 conducted as described (108). Zebrafish were maintained in cell culture flasks with
693 sterile gnotobiotic zebrafish medium (GZM) in a 28.5°C incubator with a 14:10 hour
694 light: dark cycle. Flasks were filled with 50 ml of GZM from 0 to 21 dpf, and this volume
695 was increased to 100 ml GZM from 23 to 35 dpf to accommodate the growth of the
696 animals. 60% volume GZM exchange was conducted daily to maintain water quality
697 levels. pH, nitrate, nitrite, and toxic ammonia were measured in all flasks as previously
698 described. The pH in the flasks ranged between 6.6 and 7, nitrite between 0 and 0.15
699 ppm, nitrate between 0 and 10 ppm, and toxic ammonia between 0 and 0.0042 mg/l.
700 The pH in the flasks was lower than in the conditioned water in our RAS facility and the
701 *in vivo* chemical exposure, but comparable to the pH during the supercolonization
702 experiments. The nitrite in the flasks was higher than in the conditioned water in our
703 RAS zebrafish facility and the supercolonization experiments, but comparable to the *in*
704 *vivo* chemical exposure. The nitrate in the flasks was comparable to the conditioned
705 water in our RAS zebrafish facility, the *in vivo* chemical exposure and the
706 supercolonization experiments. The toxic ammonia in the flasks was higher than in our
707 RAS zebrafish facility, but comparable to the *in vivo* chemical exposure and the
708 supercolonization experiments. Overall, water quality parameters in the flasks were
709 within the optimal ranges for zebrafish (126).
710 Flasks of GF zebrafish were raised for 13 days post-fertilization before randomly
711 assigning flask to the chemical and microbial treatments (see below). These flasks also

712 underwent a thorough fish health check and verification of GF status. On 14 dpf, one
713 fish per flask was sampled to measure the SL. The average SL of the cohort was 4.21 ±
714 0.22 mm (n=29), similar to the SL expected at this age under conventional husbandry.
715 Exposure of GF zebrafish to TBT and their monoassociation with *Plesiomonas*
716 ZOR0011 or *Chitinibacter* ZOR0013 were initiated on 15 dpf. Flasks of GF zebrafish (5
717 flasks per treatment except for *Chitinibacter* ZOR0013 which had only four, 19 flasks
718 total) were randomly assigned to chemical and bacterial treatments. Two time points
719 were sampled: 7 and 21 days post-exposure (dpE) or monoassociation (days post-
720 colonization, dpC). Flasks were sampled terminally at both time points: two flasks per
721 treatment at 7 dpE, and 3 samples per treatment at 21 dpE (except for the *Chitinibacter*
722 ZOR0013 treatment from which only two flasks) were sampled at each time point. The
723 fish were not offered food on the day of sampling and had received their last meal and
724 treatments 14 hours before sampling. Body size and adipose tissue area from each fish
725 in the flask were analyzed as previously described. There were no significant
726 differences in the rearing density of the flasks assigned to the chemical/bacterial
727 treatments that could result in differences in body size (Kruskal-Wallis, Dunn's multiple
728 comparison test of flasks from each chemical/bacterial treatment against the germ-free
729 DMSO control; all $P \geq 0.8869$ at 7 dpE/dpC; and $P \geq 0.9474$ at 21 dpE/dpC). Similarly,
730 there were no differences in mortality in flasks sampled at 7 or 14 dpE/dpC (Kruskal-
731 Wallis, Dunn's multiple comparison test of flasks from each chemical/bacterial treatment
732 against the germ-free DMSO; all $P \geq 0.1986$ at 7 dpE/dpC; and $P \geq 0.5213$ at 21
733 dpE/dpC).
734

735 *Culture and provision of germ-free Artemia-Tetrahymena diet*

736 One of the main challenges of long-term gnotobiotic husbandry is the transition in
737 zebrafish larvae from reliance on yolk to free-feeding before they are capable of hunting
738 live *Artemia*. Also, we wanted to reproduce as closely as possible the diet zebrafish
739 received in the mFTS during the *in vivo* chemical exposure, and the supercolonization
740 experiments. However, previous pilot experiments showed that an *Artemia*-only diet
741 was not compatible with survival past 14 dpf (data not shown). Thus, we designed a GF
742 live diet of *Tetrahymena thermophila* strain CU428.2 (TSC_SD00178, Tetrahymena
743 Stock Center) and San Francisco strain *Artemia* (Brine Shrimp Direct). GF *Artemia* was
744 offered daily starting 5 dpf, and supplemented with GF *T. thermophila* on 5, 8, and 11
745 dpf. GF *Artemia* and *T. thermophila* cultures were prepared as described below.

746 *T. thermophila* stocks were maintained in 5 ml of Modified Neff's Media (MNM; 3
747 mg $FeCl_3.6H_20$, 10 g proteose peptone, 2.5 g yeast extract, and 5 g glucose) with 5 µl
748 Penicillin-Streptomycin Stabilized solution (Sigma-Aldrich, P4458) and 24 µl of
749 Amphotericin B solution (Sigma-Aldrich, A2942) at room temperature. These stocks
750 were renewed every two weeks by transferring 100 µl of stock to fresh media. 200 µl of
751 MNM stock were used to inoculate 14.8 ml of Proteose Peptone Yeast Agar (PPYE; 2,5
752 g proteose peptone; 2.5 g yeast extract) media with 10 µl of Penicillin-Streptomycin
753 Stabilized solution (Sigma-Aldrich, P4458). PPYE cultures were maintained at 28.5° C
754 for one week before its use for inoculation of milk yeast extract media (MYE; 10g/l yeast
755 extract, 10 g/l non-fat powdered milk). MYE is a rich media that is used for growing *T.*
756 *thermophila* for feeding zebrafish (129). 4 ml of a one-week old PPYE culture were used
757 to inoculate 40 ml of sterile MYE and was incubated at 28.5° C overnight in a 50 ml

758 conical vial. For food preparation, the conical vial was centrifuged at 5000 rcf for 3
759 minutes, 25 ml of the supernatant were decanted, and the conical vial was refilled to the
760 50 ml mark with sterile GZM to rinse the pellet. The pellet was rinsed two more times
761 with sterile GZM by decanting 35 ml of the supernatant, before its final resuspension in
762 40 ml of sterile GZM. This preparation had an average of ~30 000 cells/ml. 4 ml of the
763 suspension of *T. thermophila* were used to feed each fish flask. Sterility tests were
764 conducted on the *T. thermophila* MNM and PPYE cultures, as well as on prepared food
765 suspensions as described in (108).

766 To prepare GF Artemia, ~250 µl of Artemia cysts were rehydrated in 10 ml of
767 sterile saltwater (12.5g/l NaCl, 0.83g/l NaHCO3) for at least 2 hours with gentle shaking
768 in a nutator. The cysts were left to settle, and the saltwater was exchanged for 10 ml of
769 household bleach (8.25% sodium hypochlorite) for decapsulation. The Artemia-bleach
770 suspension was mixed on a nutator for 3 minutes, and then left to settle at the bottom of
771 the conical tube for 5 more minutes. After decapsulation, the bleach and floating cysts
772 were removed. The cysts were rinsed by adding saltwater, mixed by gentle rolling for
773 1.5 minutes, before letting the cysts settle for 30 seconds. The procedure was repeated
774 4 times, before resuspending the cysts in 10 ml of sterile saltwater, and incubation for at
775 least 24 h at room temperature with gentle shaking under a warm light for hatching. For
776 food preparation, the actively swimming *Artemia* were collected using a sterile
777 serological pipette, collected on a 100 µm cell strainer (431752, Corning) and rinsed
778 with 10 ml of sterile GZM. *Artemia* was transferred to a conical vial and resuspended in
779 GZM. The volume and the concentration (i.e. volume of collected from hatching
780 vial/volume of GZM for resuspension) of *Artemia* was adjusted to prevent the

781 accumulation of food debris in the flasks. Volume and concentration were recorded
782 daily. Each flask of fish received the same amount of food, and the order of feeding was
783 alternated daily to prevent to prevent any feeding bias that could affect growth. Uneaten
784 food and debris were removed daily during the GZM exchange.
785
786 *Germ-free zebrafish exposure to TBT*
787 Stocks of 1 mg/ml of TBT (Sigma, T50202) in DMSO (Fisher, D136-L) and of
788 DMSO vehicle were prepared and aliquoted using sterile technique. Although the
789 presence of bacteria in these chemicals was unlikely, the sterility of the stocks was
790 tested by plating 100 µl of the stock solutions on TSA and incubated for 1 week at
791 28.5°C under aerobic conditions before starting the experiment. The plates were
792 inspected daily, and no microbial growth was observed.
793 Flasks containing GF zebrafish were initially dosed with 5 µl of the TBT stock and
794 DMSO to achieve a final concentration of 1 µg/l TBT as well as an equal total volume of
795 DMSO vehicle in the control flasks. Stocks were added before fresh GZM during the
796 media exchange to facilitate mixing. We assumed that TBT was not degraded over a
797 24-hour period, so we added a volume of stock daily to adjust the concentration of TBT
798 in the flask for the total loss of chemical during the daily GZM exchange.
799
800 *Monoassociation with Plesiomonas ZOR0011 and Chitinibacter ZOR0013*
801 Monoassociation is the colonization of GF zebrafish with a single microbial strain.
802 A starter culture of each bacterial strain was prepared by inoculating 10 ml of sterile
803 TSB with a single colony, followed by overnight aerobic incubation in a roller drum at

804 100 rpm at 30° C. 300 µl of the starter culture were collected to confirm the identity of
805 the strain by sequencing as described for the supercolonization experiments. The
806 starter culture was used to inoculate 40 ml of sterile TSB in a sterile 50 ml conical via
807 (i.e. monoassociation culture). To obtain monoassociation cultures of approximately the
808 same density at the same time, *Chitinibacter* ZOR0013 was inoculated at a starting
809 OD_{600} of 0.05 and *Plesiomonas* ZOR0011 at 0.025 as *Plesiomonas* ZOR0011 grew
810 faster in TSB than *Chitinibacter* ZOR00R13. The monoassociation culture was
811 incubated at 30° C in a shaker at 100 rpm for 14 hours. To prepare the bacterial
812 suspension for the monoassociation, we calculated the volume of the culture needed to
813 prepare 40 ml of a bacterial suspension at an OD_{600} of 0.25. We chose the OD_{600} and
814 dilutions to ensure that the zebrafish fish received amounts of bacteria in the
815 monoassociation comparable to those in the supercolonization experiments. The diluted
816 bacterial suspension was spun at 5000 rpm for 10 minutes. The supernatant was
817 decanted, and the pellet was washed twice with 40 ml of sterile GZM. The washed
818 bacterial pellet was finally resuspended in 40 ml of autoclaved GZM, and this
819 suspension was used for the monoassociation. 750 µl of each bacterial suspension
820 were sampled to count CFU on TSA plates and to confirm the identity of the strain by
821 16S rRNA gene sequencing as described above. The CFU/ml in the flask after
822 inoculation were of 1.01E+08 CFU/ml for *Chitinibacter* ZOR0013 and 1.20E+07 CFU/ml
823 for *Plesiomonas* ZOR0011 (estimated from the CFU/ml of the bacterial suspension).
824 Nevertheless, the average CFU/ml counts from GZM from individual flasks sampled at 7
825 dpC and 21 dpC were not significantly different (t-test, P = 0.7541 and P = 0652
826 respectively) between *Chitinibacter* ZOR0013 and *Plesiomonas* ZOR0011, suggesting

827 that this initial difference was not maintained and likely did not affect the results of our
828 experiments. In addition, whereas the estimated CFU/ml in the fish flasks after
829 monoassociation was lower than the average estimated CFU/ml in the tanks after the
830 supercolonization experiments, these values were within the minimum to maximum
831 CFU/ml range in tanks after supercolonization measured during the 14 days of
832 supercolonization (data not shown).

833 GF flasks assigned to the bacterial treatments received 6 ml *Chitinibacter*
834 ZOR0013 or *Plesiomonas* ZOR0011 suspension. In addition, the monoassociated flasks
835 were initially dosed with 5 µl of DMSO since the GF DMSO control was shared with the
836 chemical exposure to TBT in the GF background. DMSO was added before fresh GZM
837 during the media exchange to facilitate mixing, and a volume was added daily to adjust
838 for the loss chemical during the daily GZM exchange. Bacteria were added only once
839 during the experiment at 15 dpf.

840

841 *In vitro exposure of zebrafish intestinal microbial communities to TBT*

842 Four stocks of 1000 µg/ml, 10 µg/ml, 1 µg/ml, 0.1 µg/ml, and 0.01 µg/ml of TBT
843 (Sigma, T50202) in DMSO (Fisher, D136-L) were prepared and aliquoted using sterile
844 technique. All stocks were maintained in the dark at 4°C until use. Borosilicate tubes
845 containing 15 ml of sterile tryptic soy broth (TSB) were used for the aerobic *in vitro*
846 exposure; and borosilicate tubes containing 14.25 ml of sterile TSB and 0.75 µl of 10 g/l
847 filter sterilized L-cysteine were used for the anaerobic *in vitro* chemical exposure. 15 µl
848 of the stocks were added to the growth media to achieve final TBT concentrations of 0

849 µg/l (DMSO vehicle control), 0.01 µg/l, 0.1 µg/l, 1 µg/l, and 10 µg/l, and 1000 µg/l TBT.
850 Each TBT concentration was prepared in triplicate.

851 An experimental cohort of zebrafish was obtained and conventionally reared. On
852 day 25 post-fertilization, zebrafish were size matched between 7 mm and 9 mm. Two
853 pools of 30 size-matched individuals were transferred to two clean and autoclaved fish
854 tanks and maintained in the RAS for 4 weeks before sampling to match the SL at 21
855 dpE *in vivo* chemical exposure to TBT. Two fish per tank were collected as donors (n =
856 4; average SL = 16.75 ± 1.5 mm). To reproduce the sampling of whole intestines during
857 the *in vivo* chemical exposure, zebrafish were incubated in 1 mM epinephrine in FASW
858 before euthanasia with 1.34 g/L filter sterilized tricaine. Whole gastrointestinal (GI) tracts
859 were dissected as previously described, and cut open using sterile technique before
860 transfer to a sterile screw cap tube containing 200 µl of sterile 1-mm-diameter zirconia
861 beads (NC9847287) and 500 µl of FASW. Four GI tracts were pooled in a single
862 beaded screw cap tube. Intestinal samples were vortexed for one minute twice to obtain
863 an intestinal slurry used to inoculate TSB media with TBT and DMSO prepared as
864 described above (10 µl of slurry in 5 ml of media). The remainder of the slurry
865 constituted the input intestinal microbial community and was transferred to a sterile
866 Eppendorf tube. The beads were rinsed with 500 µl of FASW, and after vortexing, this
867 rinsing volume was pooled with the slurry. The collected material was centrifuged,
868 before centrifugation at 7500 rcf for 15 minutes. After removing the supernatant, the
869 pellet was flash frozen in a dry ice-ethanol bath and stored at −80°C until DNA isolation
870 for 16S rRNA gene sequencing.

871 TSB cultures dosed with TBT or DMSO were incubated aerobically and
872 anaerobically for 24 hours without agitation at room temperature. Anaerobic incubation
873 was performed in a Coy anaerobic chamber. 750 µl of each culture tube was collected
874 in a sterile 1.5 ml Eppendorf tube and spun at 7500 rcf. The pellets were flash frozen in
875 a dry ice-ethanol bath and stored at −80°C until DNA isolation for 16S rRNA gene
876 sequencing for microbial community analysis.

877

878 *DNA isolation*

879 The Duke Microbiome Shared Resource extracted bacterial DNA from all
880 samples using Qiagen MO BIO PowerSoil DNA Kit that allows for the isolation of
881 samples in a 96 well plate format using the Retsch MM400 plate shaker. Quantity of
882 samples were assessed using the PerkinElmer Victor plate reader with the
883 ThermoFisher Scientific Qubit dsDNA HS assay kit.

884

885 *Sequencing of 16S rRNA gene amplicons*

886 Bacterial community composition in isolated DNA samples was characterized by
887 amplification of the V4 variable region of the 16S rRNA gene by polymerase chain
888 reaction using the forward primer 515 and reverse primer 806 16S rRNA following the
889 Earth Microbiome Project protocol (http://www.earthmicrobiome.org/) by the Duke
890 Microbiome Shared Resource. These primers (515F and 806R) carry unique barcodes
891 allowing for multiplexing and co-sequencing of hundreds of samples at a time.
892 Equimolar 16S rRNA gene PCR products were quantified and pooled for sequencing.

893 Sequencing was performed by the Duke Microbiome Center using an Illumina MiSeq
894 instrument configured for 150 bp paired-end sequencing runs.
895
896 *Bioinformatic analysis*
897 Data analysis conducted in QIIME v. 1.9.1 (131). Paired reads were joined with
898 join_paired_ends.py, using standard parameters, and joined reads were subsequently
899 demultiplexed with split_libraries_fastq.py, with the phred quality threshold set to Q30.
900 Demultiplexed amplicons were filtered for chimeric sequences with
901 identify_chimeric_seqs.py, using the usearch61 (132) detection method, and
902 substituting usearch v. 6.1 with vsearch v. 2.7.1 (133). Only amplicons predicted as
903 chimeric by both denovo and reference-based detection were removed. After
904 completion of base quality and chimera filtering, totals of 21,069,394 (*in vivo* TBT
905 exposure study), 1,283,416 (supercolonization study) and 5,801,110 (*in vitro* TBT
906 exposure study) amplicons were used in downstream analyses. Amplicons were
907 clustered into operational taxonomic units (OTUs), using QIIME's closed reference OTU
908 picking strategy, implemented in pick_closed_reference_otus.py. The script was
909 configured to cluster amplicons with uclust (132) and assign taxonomy against the 97%
910 clustered 16S reference sequence set of SILVA, v. 1.23 (134). A subsequent basic
911 statistical diversity analysis was performed, using QIIME's core diversity analysis
912 workflow script (core_diversity_analyses.py), calculating alpha- and beta-diversity, and
913 relative taxa abundances in sample groups. Relative taxa abundances were further
914 analyzed with LEfSe (Linear discriminant analysis effect size, (79) to identify differential
915 biomarkers in sample groups.

916 *Data availability*

917 16S rRNA sequence data are available at the NCBI Short Read Archive under
918 accessions PRJNA692784 (*in vivo* TBT exposure study), PRJNA693006
919 (supercolonization study), PRJNA693015 (*in vitro* TBT exposure study).

920

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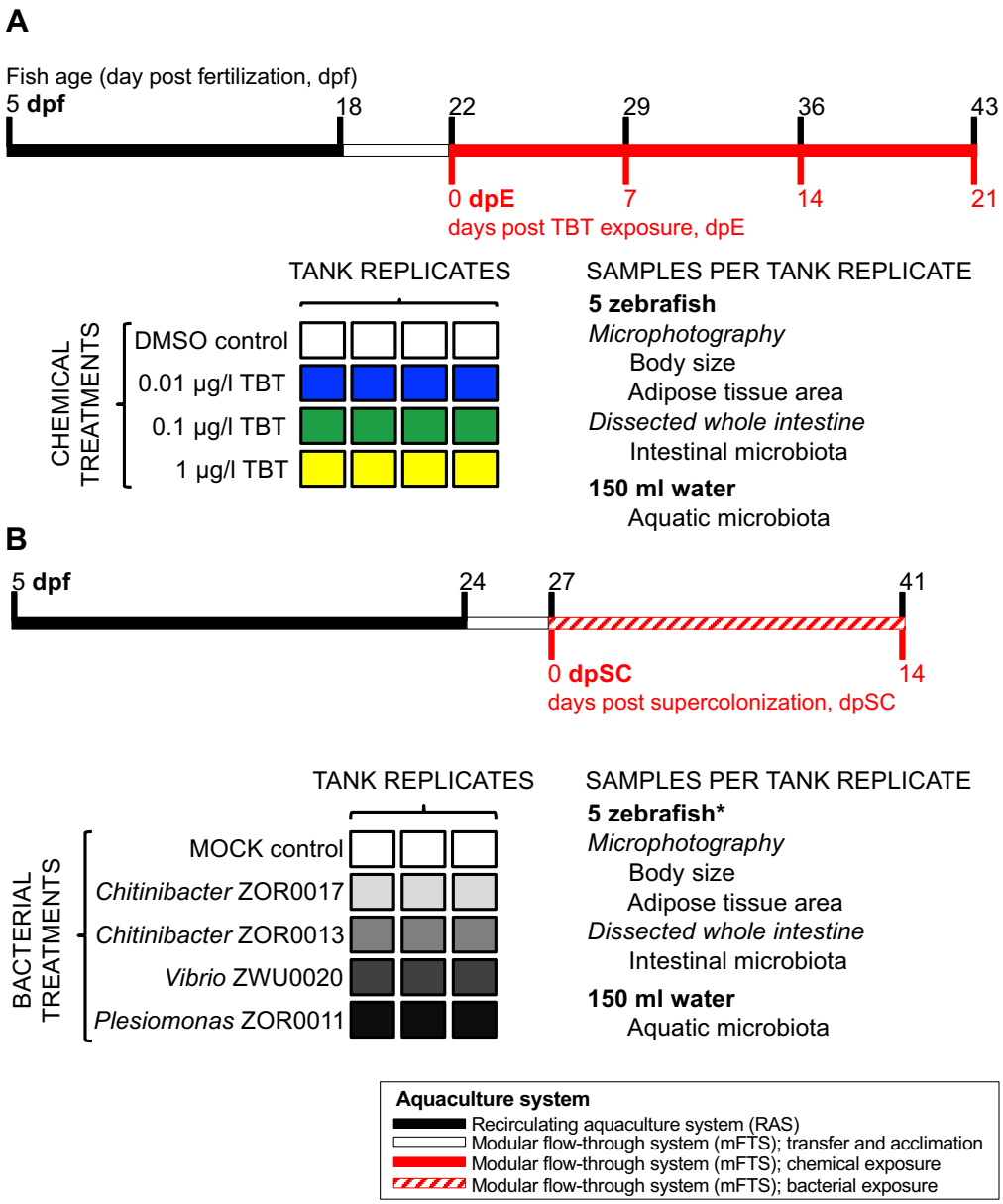
1358 **FIGURES**



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1360 **Figure 1: Schematic representation of *in vivo* experiments using conventionally-**
1361 **reared zebrafish.** (A) Experimental design for exposure to tributyltin chloride (TBT). We
1362 prepared four tank replicates per chemical treatment. The concentrations of TBT in tank
1363 water are indicated in the schematic. DMSO was used as vehicle control. (B)
1364 Experimental design for supercolonization with individual bacterial strains. We prepared

1365 three tank replicates per bacterial treatment, including the four indicated bacterial
1366 strains and a mock supercolonization negative control of water from the recirculating
1367 aquaculture system (RAS). For both experiments, the timeline represents the fish age in
1368 days post-fertilization (black ticks, dpf) in relation to the days of chemical exposure (red
1369 ticks, dpE) or supercolonization (red ticks, dpSC). The different bar colors represent the
1370 aquaculture systems used for housing the zebrafish and are listed in the box below
1371 panels A and B. We sampled 5 fish per tank to measure body size and adiposity, prior
1372 to dissecting their whole intestine for 16S rRNA gene sequencing analysis of the
1373 intestinal microbial communities. We also sampled 150 ml of tank water for 16S rRNA
1374 gene sequencing analysis of the aquatic microbial communities. (*) The same sampling
1375 strategy was used in all timepoints of both experiments, except at 14 dpSC when we
1376 sampled all the remainder fish as this experiment had only two timepoints.

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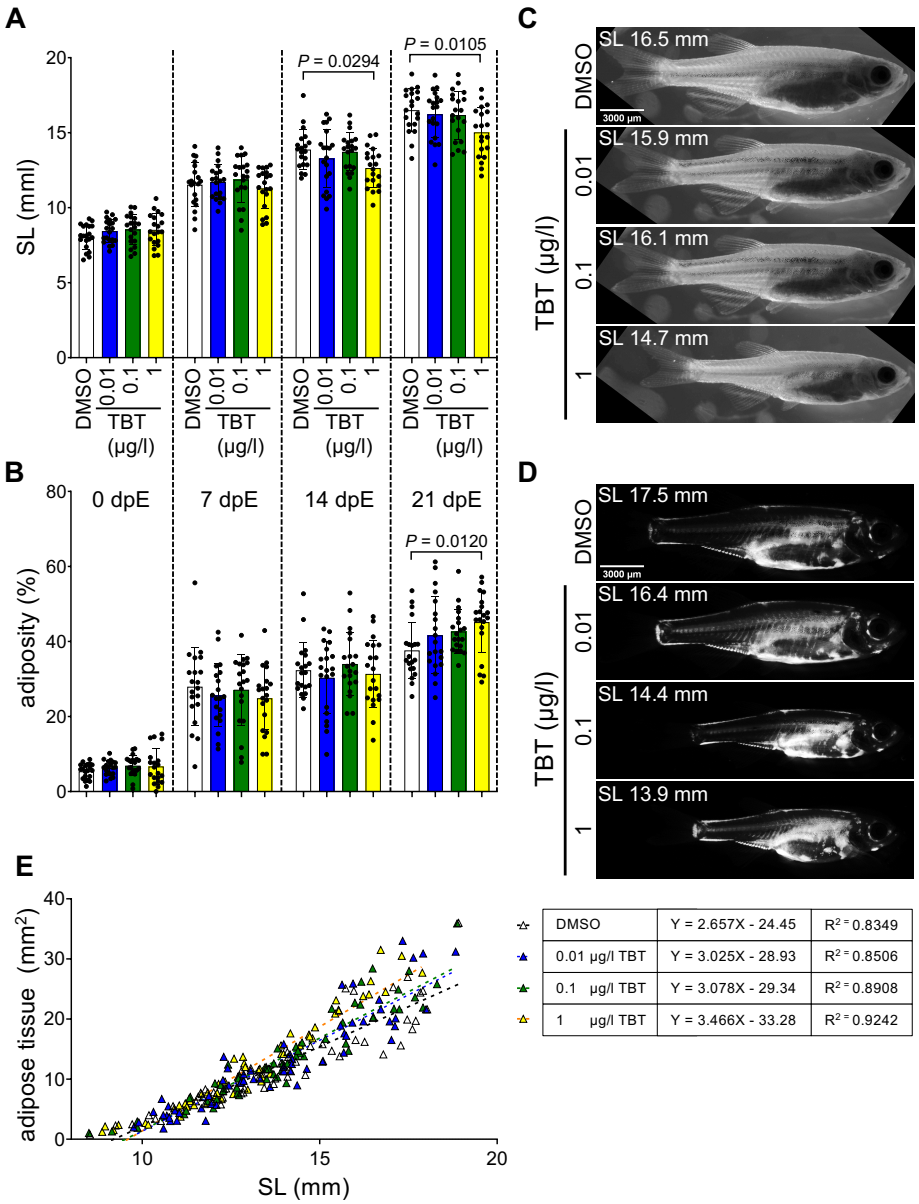

Figure 2: Exposure to 1 µg/l TBT increases adiposity and inhibits body size *in vivo* after 21 days. (A) Standard length (SL, the distance between the snout and the caudal peduncle) measurements. (B) Adiposity measurements (the total two-dimensional area of adipose tissues per body area excluding fins). Bars represent the mean and error bars the standard deviation. Means were compared to the DMSO control using one-way ANOVA and post-hoc Dunnett's multiple comparison tests. When

1385 significant, the multiplicity adjusted *P*-value is reported for the comparison. (C)
1386 Brightfield images of whole fish at 21 dpE. Selected fish are representative of the mean
1387 SL per chemical treatment. (D) Nile red fluorescence images reveal adipose tissues in
1388 whole fish at 21 dpE. Selected fish are representative of the mean adiposity per
1389 treatment. Their corresponding SL is reported. (E) Linear regression analysis between
1390 SL (x-axis) and total adipose tissue area (y-axis). All samples across all timepoints (7
1391 dpE, 14 dpE, and 21 dpE) and treatments are included. Deviation from linearity per
1392 chemical treatment was tested and found not significant (all $P \geq 0.0853$). Equations and
1393 goodness of fit values per chemical treatment are reported on the panel. The best fit
1394 values of the slopes per treatment were compared to the DMSO control using one-way
1395 ANOVA and post-hoc Dunnett's multiple comparison tests. The only significant adjusted
1396 *P*-value corresponded to the comparison between the DMSO and 1 µg/l TBT treatments
1397 ($P < 0.0005$).

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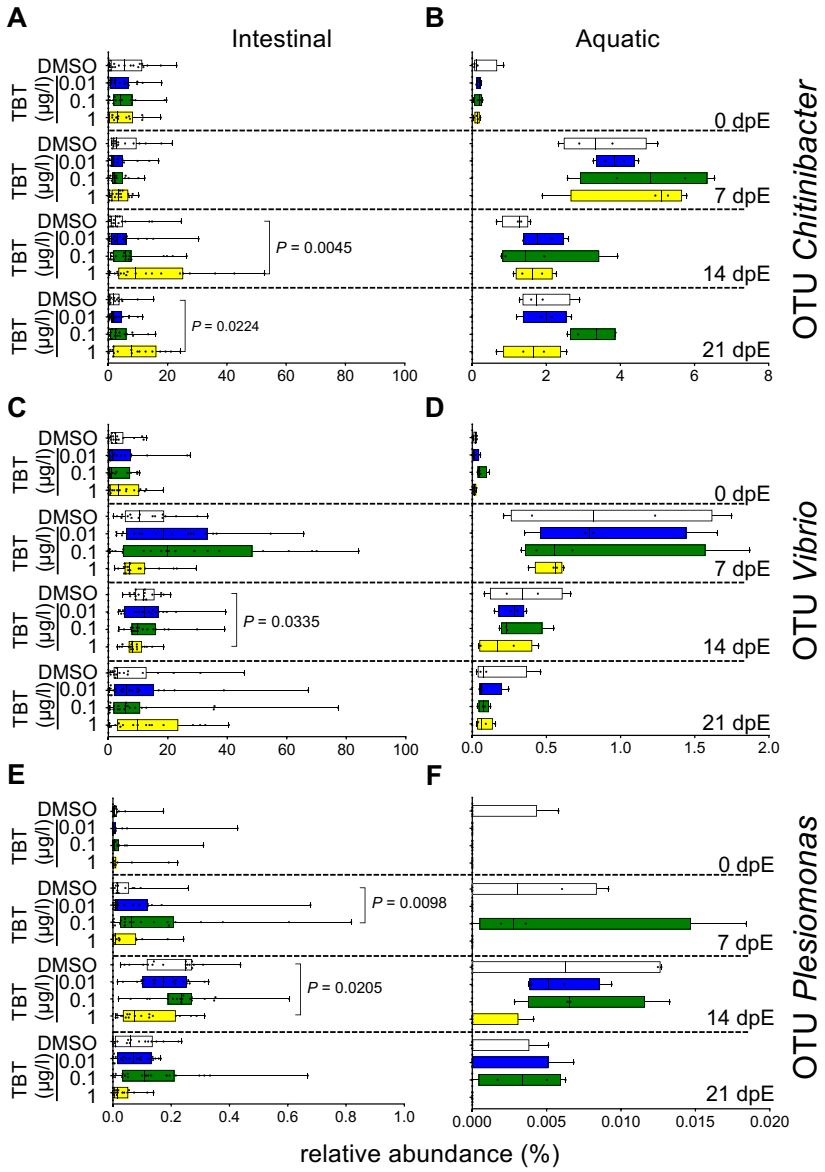

Figure 3: TBT exposure alters the relative abundance of specific bacterial taxa in the zebrafish intestine but not in the water. Relative abundance of selected members of intestinal microbial communities during in vivo exposure to the TBT concentrations and at the time points indicated. Selected OTUs from the intestinal (A, C, E) and aquatic (B, D, F) microbial communities assigned to genera *Chitinibacter* (A, B), *Vibrio* (C, D), and *Plesiomonas* (E, F). Box and whisker plots represent the interquartile

1406 range, with the line in the box defining the median. Whiskers represent the minimum
1407 and maximum value. The mean of each bacterial treatment was compared to the control
1408 using the Mann-Whitney test per time point and sample types. When significant, the *P*-
1409 values are reported for the comparisons.

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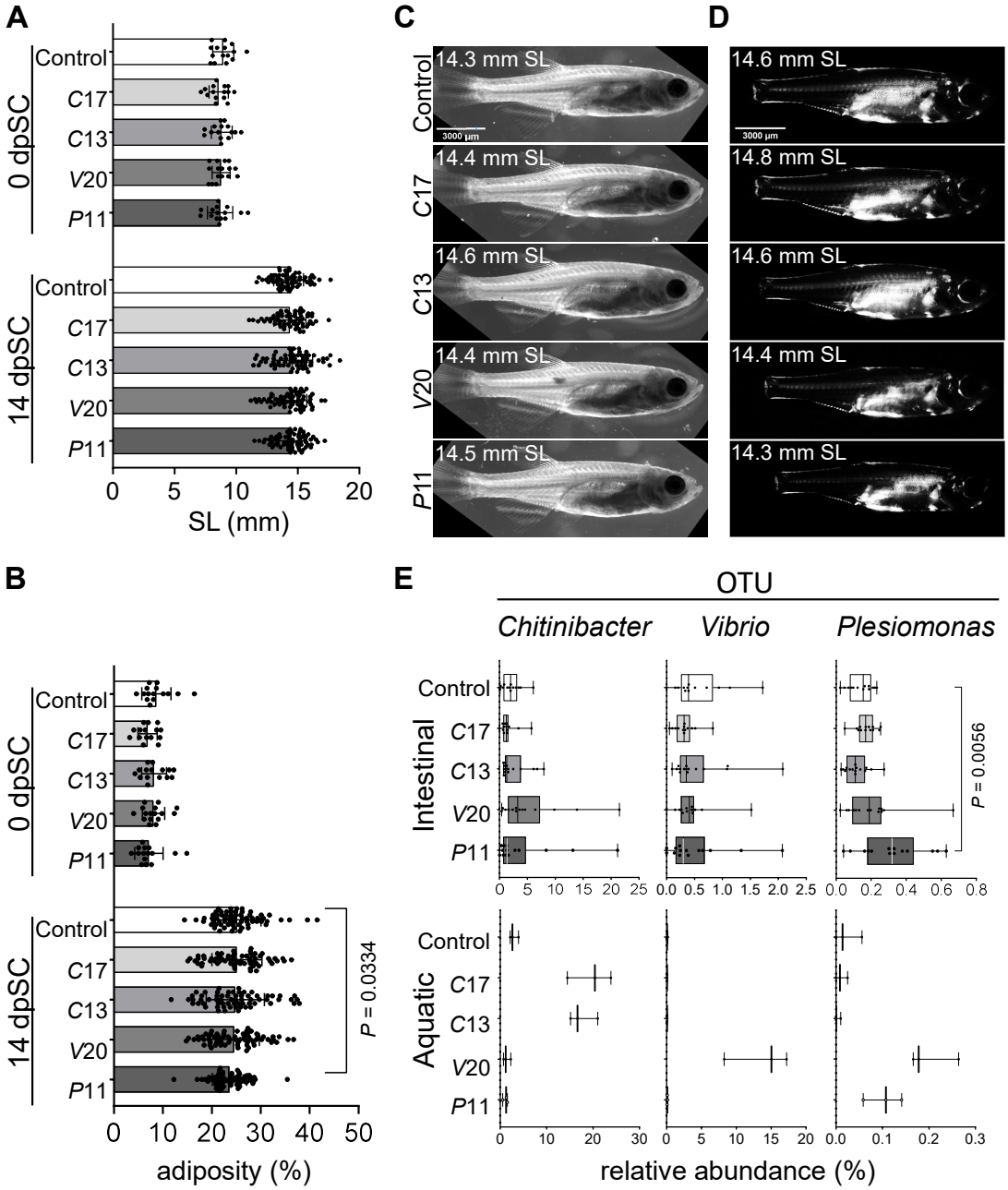

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1413 **Figure 4: Supercolonization with *Plesiomonas* ZOR0011 is sufficient to inhibit**

1414 **adiposity.** Zebrafish were supercolonized daily for 14 days with one of four bacterial

1415 strains: *Chitinibacter* ZOR0017, C17; *Chitinibacter* ZOR0013, C13; *Vibrio* ZWU0020,

1416 V20; or *Plesiomonas* ZOR0011, P11. (A) Standard length (SL) and (B) adiposity

1417 measurements before initiating the bacterial treatment (0 days) and after 14 days of
1418 daily supercolonization (dpSC). Bars represent the mean and error bars the standard
1419 deviation. Means were compared to the negative control using unpaired t-test. When
1420 significant, the *P*-value is reported for the comparison. (C) Brightfield images of whole
1421 fish representative of the mean SL per treatment at 14 dpSC. (D) Nile red fluorescence
1422 images reveal adipose tissues in whole fish representative of the mean adiposity per
1423 treatment at 14 dpSC. Their corresponding SL is reported. (E) Relative abundances of
1424 OTUs assigned to genera *Plesiomonas*, *Chitinibacter*, and *Vibrio* in aquatic and
1425 intestinal microbial communities at 14 dpSC. Box and whisker plots represent the
1426 interquartile range, with the line in the box defining the median. Whiskers represent the
1427 minimum and maximum value. The mean of each bacterial treatment was compared to
1428 the control using the Mann-Whitney test. When significant, the *P*-values are reported for
1429 the comparisons.

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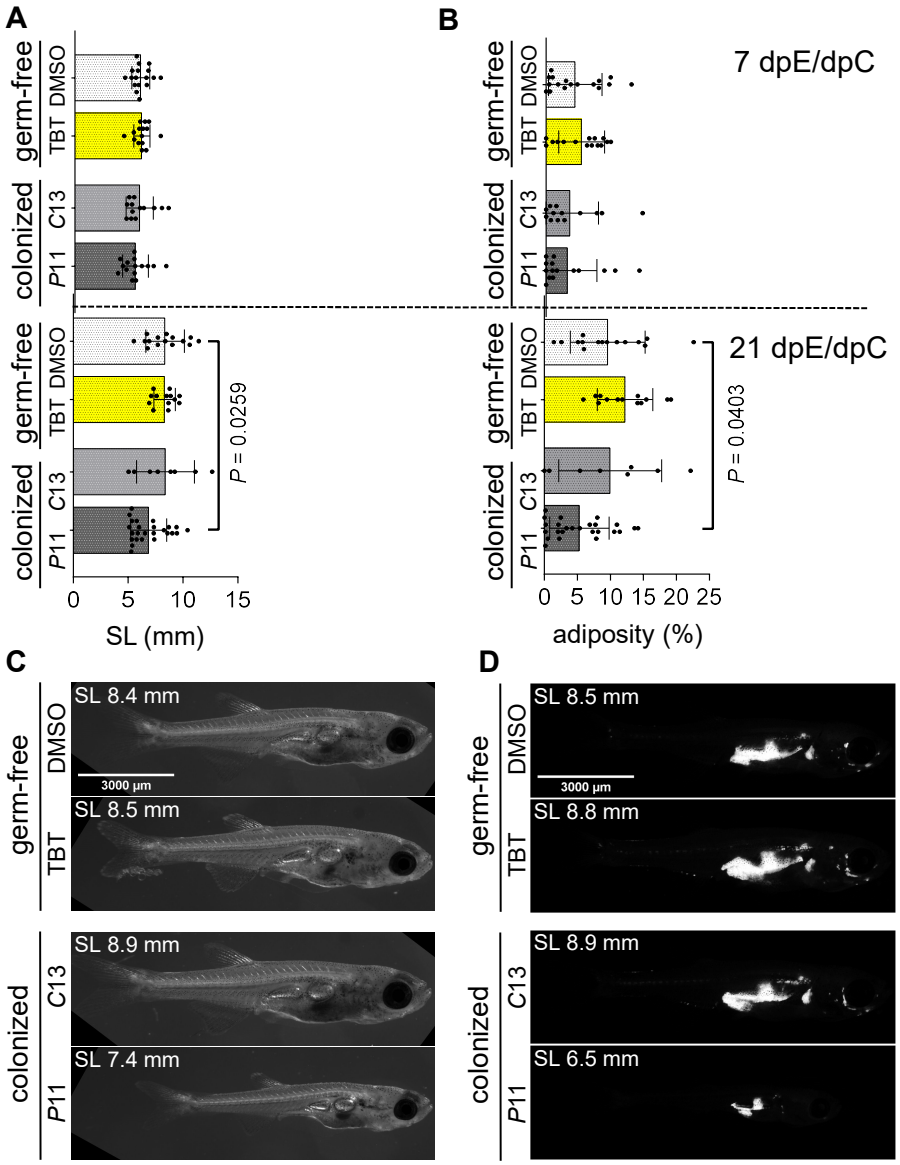

Figure 5: Monoassociation reveals that *Plesiomonas* ZOR0011 is sufficient to reduce body size and adiposity in zebrafish without an established microbiota. (A) Standard length and (B) adiposity after exposure of germ-free zebrafish to TBT; or monoassociation (colonized) with *Chitinibacter* ZOR0013 (*C*13) and *Plesiomonas* ZOR0011 (*P*11). Measurements were taken 7 and 14 days post-exposure (dpE) or post-colonization (dpC). Bars represent the mean and error bars the standard deviation. Means of each treatment were compared to the DMSO control using one-way ANOVA

1439 and the post-hoc Dunnett's multiple comparison test. When significant, the multiplicity
1440 adjusted *P*-value is reported for the comparison. (C) Brightfield images of whole fish
1441 representative of the mean SL per treatment measured after 14 days. (D) Nile red
1442 fluorescence images reveals adipose tissues in whole fish representative of the mean
1443 adiposity per treatment measured after 14 days. Their corresponding SL are reported.

1444