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1 **Chemical Pollution Levels in a River Explain Site-Specific Sensitivities to Micropollutants**
2 **within a Genetically Homogeneous Population of Freshwater Amphipods**

3

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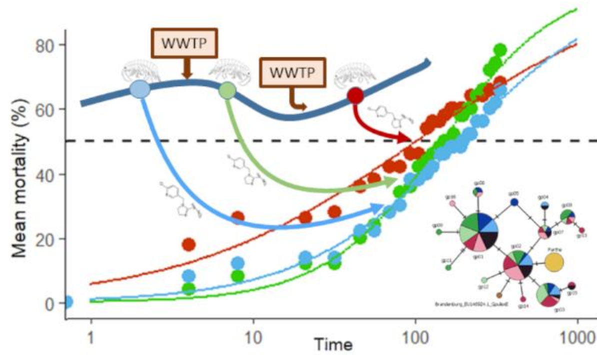


Abstract

Anthropogenic micropollutants alter chemical and ecological conditions of freshwater ecosystems and impact aquatic species that live along the pollution gradient of a river. Species sensitivity to micropollutants depends on the site-specific exposure, however, it remains unclear to what degree this sensitivity relates to species' genetic structure. Here, we explored the relationship between toxic sensitivity and genetic structure of the amphipod species *Gammarus pulex* (Linnaeus, 1758) along an organic micropollutant gradient in the Holtemme River in central Germany. We determined the river's site-specific micropollutant patterns and analyzed the genetic structure of *G. pulex* using nuclear and mitochondrial genetic markers. Furthermore, we examined the exposure sensitivities and bioaccumulation of the commonly detected insecticide imidacloprid in *G. pulex* from different sites. Our results show that throughout the Holtemme River, *G. pulex* forms a well-connected and homogenous population with no observable pollution–related differences in genetic structure. However, *G. pulex* from polluted sites responded more sensitively to imidacloprid; survival times for half of the amphipods were up to 54% shorter, the percentage of immobile individuals increased up to 65%, and the modeled imidacloprid depuration rate was lower in comparison to amphipods from non-polluted sites. Altogether, these results suggest that

39 the level of sensitivity of *G. pulex* amphipods to micropollutants in the river depends on the degree
40 of pollution: amphipods may thrive in food-rich but polluted habitats, yet their sensitivity is
41 increased when chronically exposed to organic micropollutants.

42 Keywords: *Gammarus pulex*, anthropogenic pollution, imidacloprid, LC-HRMS, population
43 genetics, microsatellites, selection

44 Synopsis: *Gammarus pulex* amphipods from river sections with higher levels of organic pollution
45 show increased sensitivity to the pesticide imidacloprid; the amphipods' sensitivity depends
46 largely on the toxic pressure that they are exposed to in their habitat.

47 1. Introduction

48 Chemical water pollution, river regulation, and invasive species affect river ecosystem functioning
49 and indigenous aquatic species.[1–3] In particular organic micropollutants, bioactive compounds
50 such as pesticides[4] and pharmaceuticals[5] that are only partially eliminated by wastewater treatment
51 plants (WWTP), are important, but often neglected stressors in rivers.[6] These pollutants have been
52 shown to significantly contribute to a deteriorated chemical and ecological river status.[7,8]
53 Specifically, the type and degree of pollution was demonstrated to influence the aquatic species
54 composition.[9,10] Some species, such as the amphipod *Gammarus pulex* (Linnaeus, 1758), can
55 nonetheless occur along pollution gradients in both pristine and polluted habitats of a river.

56 In rivers with different levels of pollution, the toxic sensitivity of *G. pulex* differs depending on
57 the degree of pollution in the respective habitat.[11–14] Differences in sensitivities to chemicals of up
58 to three fold were detected among amphipods from polluted and unpolluted sites.[11,12,15] Such
59 discrepancies in sensitivities may arise due to different mechanisms; sensitivity of amphipods at

polluted sites can decrease due to genetic and physiological adjustment to pollution (i.e., adaptation and acclimation, respectively) or can increase due to impairment from chronic chemical exposure.[16]

Adaptation to pollution can occur as a result of co-acting mutagenic and selective effects of toxic pollutants in exposed populations.[17] Mutations increase the rates of new alleles in such populations, while the selective pressure of micropollutants, such as pesticides, increases the frequency of resistant alleles due to higher survival and reproduction rates of the individuals with these alleles.[18–20] Adaptation due to a mutation in a pyrethroid receptor resulting in reduced sensitivity to the pyrethroid insecticide was shown among genetic lineages of an amphipod, *Hyallea azteca* (Saussure, 1858), living in polluted habitats.[21] Environmental pollution can also cause changes in genetic diversity.[18,22,23] In naturally exposed populations of *Daphnia magna* Straus, 1820 that showed reduced sensitivity to the pesticide carbaryl, reduced allelic richness and observed heterozygocity were detected by neutral genetic markers.[24] In addition, different sensitivities were shown for different cryptic genetic lineages of *Gammarus* amphipods.[25] Some of these lineages occur sympatrically in a river,[26] yet it is unclear to which degree their sensitivities to toxins depend on site-specific pollution and lineage-related genetic differences.

Acclimation, a physiological, behavioral, or morphological response of amphipods to different pollution levels,[27,28] can similarly to adaptation result in a reduced sensitivity against toxicants. Acclimation can occur within populations under stressful conditions if individuals are able to physiologically adjust to directional selection and still reproduce.[16] Acclimation is for example illustrated by a study, in which the parental generation (F0) of *Gammarus fossarum* Koch, 1836

amphipods that was acclimated to toxic conditions showed lower sensitivity to cadmium than the F2 generation that was continuously kept in cadmium-free conditions.[29]

In addition to the above-mentioned mechanisms, external factors can also modify sensitivity of amphipods to micropollutants. Thus, sensitivity increased due to a rise of temperature in rivers,[11] food shortage,[30] and when exposure to micropollutants occurred in a certain sequence. The latter in particular, was found to increase sensitivity in *G. pulex* to chemical exposure under repeated exposures to two pesticides in a specific order.[31] An explanation for this may be provided by a study finding a carry-over due to slow toxicodynamic recovery from diazinon exposure and an increased mortality under subsequent exposure to propiconazole compared to the sequential exposure in the reversed order.[32]

Despite abundant information on toxic effects of organic micropollutants on *G. pulex,* it remains unclear how the pollution gradient in a river affects the genetic structure of *G. pulex* and how the genetic structure relates to the species' sensitivity to toxicant exposure. We therefore investigated two competing hypotheses: 1) the sensitivity of *G. pulex* to organic micropollutants in polluted river sections is reduced due to the site-specific genetic or physiological adjustment to exposure, i.e., adaptation and acclimation, respectively, and 2) micropollutants in the river increase the sensitivity of *G. pulex* from polluted sites.

We performed a study at the Holtemme River, serving as a landscape model for studies of the effects of anthropogenic pollution on riverine ecosystem functioning.[18,33–35] We analyzed the widespread Palearctic amphipod species *G. pulex*, which occurs in rivers with different degrees of pollution.[36–38] It is common in the Holtemme River, where two distinct populations were described in the past.[18] To test our hypotheses, we 1) determined the degree of organic micropollution

103 pressure on *G. pulex* along the river using a toxic unit scale and 2) compared it to the genetic
104 structure of *G. pulex* in the river. In laboratory exposures, we 3) determined the sensitivities to
105 toxic chemicals of *G. pulex* sampled along the pollution gradient employing the common
106 insecticide imidacloprid, and 4) measured imidacloprid tissue levels in exposed amphipods from
107 different sites to determine if differences in sensitivity can be related to imidacloprid uptake and
108 depuration rates.

109 **2. Materials and Methods**

110 2.1. Sample Collection

111 Samples were taken at eight locations (H1–H8) along a 47 km stretch of the Holtemme River
112 (mean annual discharge: 1.34 $m^3 s^{-1}$)[39] in Saxony-Anhalt (Germany) (Fig. S1). The river comprises
113 a micropollutant gradient; the water from the spring in the Harz National Park starts off as a pristine
114 mountainous headwater that becomes increasingly polluted by WWTP effluents and runoffs from
115 agricultural land and urban areas of the towns of Wernigerode and Halberstadt.[18,33] Reference
116 samples were collected near the spring of the Parthe River (Saxony, Germany).

117 At each site, up to 100 *G. pulex* amphipods were collected with a Surber sampler (0.5 mm mesh
118 size) from at least five spots across the entire river width. For DNA analysis, amphipods were
119 stored in absolute ethanol. Amphipods for chemical analysis were rinsed with distilled water and
120 frozen at -20°C until analysis. Concurrently, a water grab sample consisting of 1 mL river water
121 was collected at each site from 10 cm water depth with a sterile pipette and frozen at -20°C until
122 analysis. For detailed information on sampling locations refer to Tab. S1 in the Supporting
123 Information (SI).

124

125 ## 2.2. Chemical Analysis

126 Pooled *Gammarus pulex* individuals (900 mg) from each site were extracted with the QuEChERS
127 (Quick, Easy, Cheap, Effective, Rugged and Safe) method according to Inostroza et al. (2016b).[40]
128 An extract from each site was analyzed by liquid chromatography-high resolution mass-
129 spectrometry (LC/HRMS, Thermo Ultimate 3000 HPLC system coupled to a Thermo QExactive
130 Plus quadrupole-orbitrap instrument). Water samples were analyzed using the same instrument.
131 Details on sample preparation and instrument settings with a target screening method are included
132 in section S1 in the SI. Subsequently, the levels of organic compounds of anthropogenic origin,
133 comprising pesticides, pharmaceuticals, household and industrial chemicals with a wide range of
134 hydrophobicity known to occur in the Holtemme River[33,40] were determined (Tab. S2).

135

136 ## 2.3. Micropollutant Toxic Effect Estimation

137 The toxic capacities of the analyzed pollutants in *G. pulex* tissue were estimated based on the
138 respective toxic units (TUs). According to the finding that chemical levels of several orders of
139 magnitude below EC_{50} values affect freshwater macroinvertebrate communities, log TU values
140 equal or higher than -3 were taken to indicate pollutant levels causing adverse effects as suggested
141 by Schäfer et al. (2012).[41] From the measured tissue concentrations the freely dissolved fraction
142 (C^f) of each compound *i* was estimated according to equilibrium partitioning theory:

143 $$C_i^{fd} = \frac{C_i^{tG}}{f_{LIPID} K_{OW}}$$

144

145 where C^{tG} is the total measured concentration [ng/g of wet tissue] in *G. pulex,* f_{LIPID} the lipid
146 fraction value (1.34% of the total body mass; Ashauer et al., 2010),[42] and K_{ow} is the n-octanol-
147 water partition coefficient. The freely-dissolved concentrations of neonicotionoids calculated by
148 this equation with K_{ow} values predicted by JChem deviated by more than two orders of magnitudes
149 from the measured tissue concentrations (based on Fig. 4 and literature data).[42,43] Therefore,
150 instead of using K_{ow}, we calculated the partitioning ratio as the ratio between the tissue and water
151 equilibrium concentrations measured in the uptake experiments for imidacloprid (Fig. 4) and with
152 data from another publication (thiacloprid).[37] Reference standard toxicity data (LC_{50}) were
153 retrieved from the EPA ecotoxicology database (https://www.epa.gov/chemical-
154 research/ecotoxicology-database). If LC_{50} data were not available for *G. pulex,* data for *Daphnia*
155 *magna* were used. The TUs for each compound with available LC_{50} (Tab. S5) were summed up in
156 order to predict an additive effect of all compounds at each site:[44]

157 $$log\sum TU = log\sum\left(\frac{C_i^{fd}}{LC_{50,i}}\right)$$

158

159 2.4. DNA extraction, Sequencing and Genotyping

160 Genomic DNA was extracted from 140 *G. pulex* individuals from differently polluted sites (H1,
161 H3–H8) using the Qiagen DNeasy Blood & Tissue kit. To avoid contamination by endoparasites,
162 common in the gut of freshwater amphipods, only appendages (pereopods) were used. After DNA
163 quality check using gel electrophoresis and a nanodrop spectrophotometer, a fragment of the
164 mitochondrial COI gene was amplified for twenty samples per site. For details on PCR conditions
165 and primer selection refer to the section S2 and Tab. S7.

166 For microsatellite analysis, 17 markers[45–47] (Tab. S8) were amplified from 80 DNA samples
167 mainly belonging to polluted and non-polluted sites analyzed in the exposure experiments (H1,
168 H3, H4, H6). The amplification was done according to the protocol described in Švara et al.
169 (2019)[47] and Schuelke (2000).[48] Allele sizes were determined using an ABI Prism 3130XL Genetic
170 Analyzer.
171
172 2.5. Genetic Variation Analysis
173 The genetic variation of *G. pulex* from the Holtemme River was investigated with two methods,
174 comprising protein-coding mitochondrial COI sequence analysis and analysis of non-coding
175 microsatellite nuclear loci. With the two methods, cryptic diversity at the species (COI) and
176 population (microsatellites) levels can be examined. The sequenced COI fragments were
177 assembled and aligned with sequences of *G. pulex* from other European rivers acquired from the
178 National Center for Biotechnology Information (NCBI) and compared for their phylogenetic
179 relation and genetic distances by the maximum likelihood analysis in MEGA7.[49] Genetic
180 differentiation was analyzed by pairwise fixation index (F_{st}) comparison in Arlequin 3.5.[50] For
181 microsatellite loci, diversity parameters and diversification between amphipods from different
182 locations were estimated in Fstat 2.9.3.2[51] and Arlequin 3.5. The population genetic structure in
183 the river was determined in Structure 2.3.4[52] and the effective population sizes were estimated in
184 NeEstimator 2.0.2.[53] Analyses and visualization of the genetic data are described in detail in
185 section S3 in the SI.
186

187 2.6. Imidacloprid Toxicity Experiment

188 *Gammarus pulex* from three sampling locations (H1, H4, H6) were exposed to imidacloprid (≥98%
189 Purity, CAS-No. 138261-41-3, Sigma-Aldrich) at 130 µg/L (0.025% DMSO) and 270 µg/L
190 (0.05% DMSO), along with medium and solvent controls (0.05% DMSO) for 14 d. Exposures
191 were set up in 1 L glass beakers in a volume of 500 mL Aachner Daphnien Medium (ADaM)[54] as
192 an exposure medium. For further details on the experimental set-up refer to section S4 in the SI.

193 During the experiment, the beakers were checked for dead/immobile amphipods (lethal/sub-lethal
194 effect) at least every twelve hours. Amphipods were classified as dead when no movement of
195 extremities was observed and as immobile when repeated contacts with a glass rod did not
196 stimulate movement although pleopod motion indicated that amphipods were alive. As a measure
197 of sensitivity, the time until mortality reached 50% (LT_{50}) in each treatment was quantified with
198 the non-linear Hill model[55] (see S5) and compared using the 95% confidence intervals. For
199 comparison of immobility data from different treatments and samplings sites, the Kruskal-Wallis
200 rank sum test was applied as normal distribution of data was not assumed. Data analysis was done
201 in GraphPad Prism version 5.01 and in R.[56]

202

203 2.7 Imidacloprid Uptake and Depuration Kinetics

204 To determine the kinetics of imidacloprid bioaccumulation and depuration in *G. pulex* tissue, *G.*
205 *pulex* from the locations H2 (non-polluted) and H6 (polluted) were exposed to imidacloprid as
206 described in sections 2.6 and S4. Exposures were performed at 25 µg/L (≙ $1/10^{th}$ of LC_{50}) for seven
207 days (uptake period) and subsequently in uncontaminated ADaM for four days (depuration period).
208 Control amphipods were kept in ADaM with 0.05% DMSO for seven days and afterwards in
209 uncontaminated ADaM for four days. Amphipods were sampled at 17 time points. Four to six

210 amphipods with a total tissue mass of 150 mg were pooled and immediately frozen at -20°C. After
211 QuEChERS[40] extractions, imidacloprid concentrations in the tissue were measured using
212 LC/HRMS (see section S1).

213 Uptake data were fitted with the one phase association model, using the least squares method.
214 Initial internal concentration C_0 was set to zero with the accumulation rate constant K, time t, and
215 maximal saturation estimated with the model. Depuration data were fitted with the one phase decay
216 model, using the least squares fitting method. To compare the accumulation and depuration
217 efficiency, the models were compared using an extra sum-of-squares F-test. Modelling was
218 performed with GraphPad Prism version 5.01.

219

220 **3. Results**

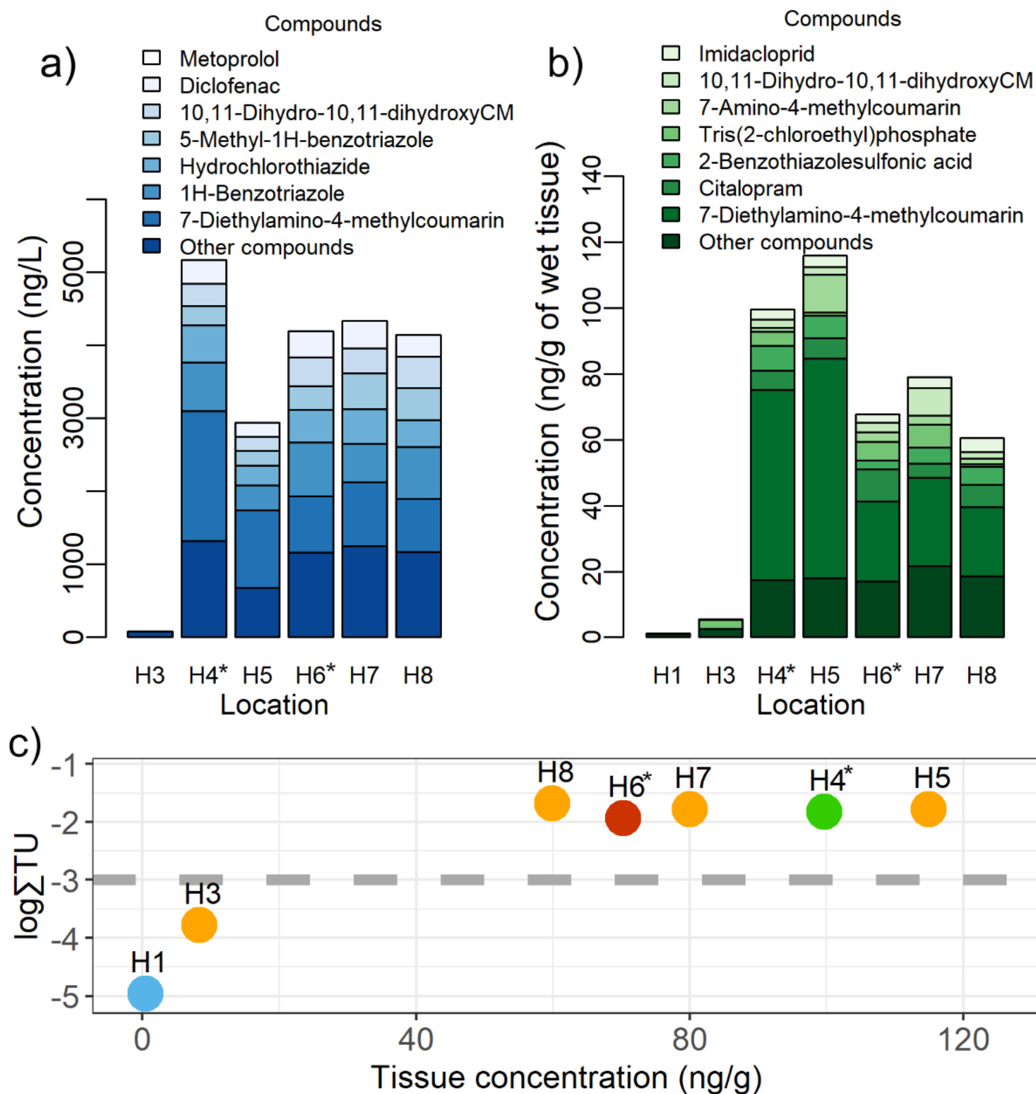
221 3.1. Organic Micropollutants in the Holtemme River

222 3.1.1. Micropollutants in Water Samples

223 The number and amount of identified micropollutants was strongly related to the presence of
224 WWTP effluent (Fig. 1a, Tab. S4). Out of 60 screened organic compounds, four were found in the
225 water samples from site H3 upstream of WWTP1 and 32 in water samples from sites H4–H8,
226 downstream of WWTP1. The concentrations of the analyzed compounds were, in comparison to
227 the upstream site, higher downstream of WWTP1 (Fig. 1a). From the analyzed compounds, 7-
228 diethylamino-4-methylcoumarin showed the highest concentrations, between 873–1785 ng/L, at
229 sites H4–H8. The effluent of WWTP1 is the source of this fluorescent dye.[57] The corrosion
230 inhibitors 1H-benzotriazole and 5-methyl-1H-benzotriazole and the diuretic hydrochlorothiazide

231 showed relatively high concentrations in the samples from locations downstream of WWTP1 (H4–

232 H8) with 350–734 ng/L, 204–486 ng/L, and 268–511 ng/L, respectively.



233

234 Figure 1. Organic micropollutant levels in the Holtemme River. (a) Detected concentrations of the

235 seven most prevalent compounds in each water sample from the Holtemme River. (b) Detected

236 concentrations of the seven most prevalent compounds in the *G. pulex* tissue extracts. (c) Sum of

237 toxic units (TUs) for each sampling site based on the calculated TUs for all compounds detected

238 in *G. pulex* tissue samples. The colors of the circles representing sites H1, H4, and H6 correspond

239 to the colors in figures 3 and 4. The dashed line at 10^{-3} TUs marks the threshold for expected
240 adverse effects; at TUs > 10^{-3} adverse effects are expected to occur. Asterisks denote the locations
241 directly downstream of WWTPs.

242

243 ### 3.1.2. Micropollutants in *Gammarus pulex* Tissue Samples

244 The WWTP effluents significantly contributed to the amount and abundance of micropollutants in
245 the *G. pulex* tissue samples, as in total 10 compounds were detected in *G. pulex* samples from
246 upstream (sites H1, H3) and 28 from downstream of WWTP1 (sites H4–H8) (Tab. S3). The
247 micropollutant concentrations detected in tissue samples collected downstream were up to 200
248 times higher than in the samples collected from site H1 (Fig. 1b). Among the detected compounds
249 in the tissue extracts the industrial compound 7-diethylamino-4-methylcoumarin at 21–67 ng/g
250 wet tissue in samples from downstream of WWTPs, was most abundant. It was followed by the
251 transformation product 7-amino-4-methylcoumarin, the antidepressant citalopram at 4.2–9.6 ng/g
252 and the rubber additive transformation product 2-benzothiazolesulfonic acid at 2-8–7.7 all at sites
253 H4–H8.

254 With their high toxic potential for *G. pulex,* identified insecticides were of special interest. The
255 neonicotinoid insecticide imidacloprid was detected in the amphipod tissue samples from the sites
256 downstream of WWTPs (2.4–4.3 ng/g at sites H4–H8) (Fig. 1b). The second detected
257 neonicotinoid, thiacloprid, was found also upstream of WWTP1 (0.21–0.35 ng/g at sites H1 and
258 H3), but the concentrations were higher downstream of WWTP1, reaching 1.2 ng/g at the site H8
259 (0.64–1.2 ng/g at sites H4–H8). Fipronil was detected downstream of WWTP2 at sites H6 and H7

260 (0.64 and 0.12 ng/g, respectively). Pesticide tissue concentrations were the highest in the samples
261 from H8, the last location before the confluence with the Bode River.

262

263 3.1.3. Toxic Unit Values

264 The amounts of the detected compounds at each site are reflected by TUs. For 14 compounds
265 detected in *G. pulex* tissue, toxicity data were available in the EPA ecotoxicology database (Tab.
266 S5). The sum of TUs in samples from all locations downstream of WWTP1 exceeded 10^{-2}, while
267 at locations H1 and H3 TUs were below 10^{-3} (Fig. 1c, Tab. S6). In the samples from sites H4–H8,
268 cumulated TUs amounted to > 10^{-3} with imidacloprid as the major contributor to these TUs (> 10^{-2}
269 TUs). Additionally, the corrosion inhibitors 1H-benzotriazol and 5-methyl-1H-benzotriazole, the
270 neonicotinoid insecticide thiacloprid, and the pharmaceuticals verapamil, metoprolol, and
271 propranolol, each with up to 10^{-4} TUs, contributed substantially to the sum of TUs.

272

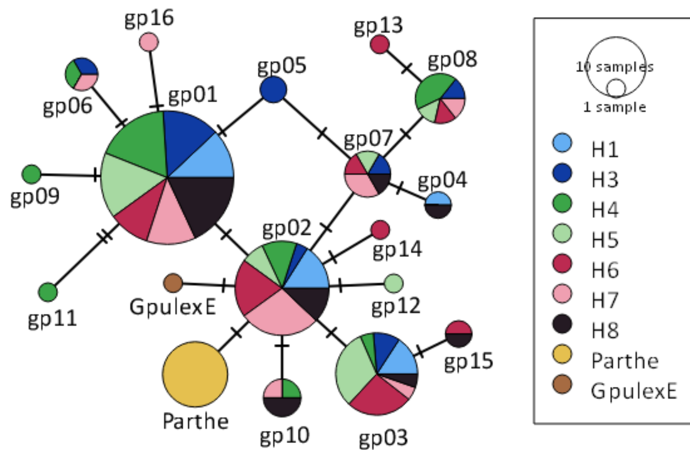
273 3.2. Population Genetic Analysis

274 3.2.1. COI Sequences Comparison

275 Comparisons of 658 base pair COI sequences of 127 *G. pulex* amphipods from seven locations in
276 the Holtemme River and of twelve *G. pulex* amphipods from the reference river Parthe revealed a
277 significant variation across sequences. Fifteen variable nucleotide sites were identified in the
278 sequences of amphipods from the Holtemme River and an additional variable site in the amphipods
279 from the reference group. The sequences from the Holtemme River comprised 16 distinct
280 haplotypes, of which nine were represented by more than a single specimen. The three most

281 common haplotypes gpcoi1, gpcoi2, and gpcoi3, were found among 39.4%, 19.7%, and 16.5% of
282 the amphipods, respectively. Sequences were most diverse at location H6 with eight and least
283 diverse at location H1 with four different haplotypes. Site-specific haplotypes were identified at
284 sites H4 and H6, while no site-specific haplotypes were found at H1 and H8.

285 The population genetics structure of *G. pulex* from the Holtemme River based on the COI analysis
286 was not pollution-related. All of the most common haplotypes are present in the samples from
287 polluted as well as non-polluted locations, with only a few location-specific haplotypes (Fig. 2).
288 *Gammarus pulex* from the Parthe River belonged to one distinct haplotype characterized by a
289 single different base, and a reference sequence for *G. pulex* E from the Brandenburg region by the
290 difference of two bases. The fixation index for COI sequences across all nucleotides within the
291 Holtemme River was 0.012, suggesting low genetic structuring. Pairwise F_{st} values were mostly
292 lower than 0.05 and not significant (Tab. S10). Two significant values between locations H3:H6
293 and H4:H6 were detected with fixation indices 0.10 and 0.07, respectively, explaining the low
294 diversification. On the phylogenetic tree (Fig. S2), a cluster of samples belonging to *G. pulex*
295 lineage E *sensu* Grabner et al.[47,58] from the Holtemme River, Parthe River, and from the
296 Brandenburg region (G_pulex_E) can be recognized consistently, without supported structure
297 within this cluster. Phylogenetic comparison also showed small genetic distances of less than 0.003
298 among all *G. pulex* samples from the Holtemme River (Tab. S9). The distances to the samples
299 from the Parthe River and Brandenburg reference sequences, which are also spatially closest to
300 the Holtemme River, were all below 0.003. Genetic distances to other *G. pulex* lineage C and D
301 were 29 and 40 times higher, respectively.



302

303 Figure 2. Minimum spanning network of the analyzed COI sequences of *Gammarus pulex*
304 belonging to the clade E from seven sampling locations at the Holtemme River (H1 and H3–H8)
305 and two reference locations Parthe and Brandenburg (GpulexE) in different colors. Each pie chart
306 represents a different haplotype. Their sizes represent the number of samples detected for each
307 haplotype. Hatch marks between the pie charts represent a single nucleotide change.

308

309 3.2.2. Microsatellite Analysis

310 Similar to COI sequence analysis, large microsatellite variability with no pollution-related
311 structure was detected among the Holtemme River samples. In total, 75 alleles were found with
312 allele variability of 54–59 alleles across 17 microsatellite loci in amphipods from each of the four
313 analyzed locations (Tab. 1). From one to nine alleles per microsatellite locus were found in total
314 (Tab. S8) with nine alleles detected for loci gp10 and gp28, eight for gp37, and only a single allele
315 for locus Gapu-9 as all pairs of loci were unlinked. A higher number of private alleles was observed
316 in amphipods at sites with higher allelic richness, with no significant differences in expected and
317 observed heterozygosities across all loci. Null alleles were detected for four loci, namely g8, g9,

318 gp11, gp37, at frequency rates of 0.06, 0.02, 0.08, and 0.36, respectively. The highest effective
319 population size (∞) was detected at site H6 and the lowest (87.3) at site H1. No structural
320 divergence within the sampled amphipods was detected as the likelihood values estimated in
321 Structure Harvester suggest a single population based on the K value (Fig. S3). Pairwise F_{st}
322 comparison of different locations did not confirm significant COI structuring results, but showed
323 a weak (F_{st} = 0.017), yet significant difference between H1 and H3 (Fig. S11). A slightly increased
324 inbreeding rate was detected at H6 (Tab. 1).

325

326 Table 1. Microsatellite diversity indices including the total number of detected alleles (N), allelic
327 richness per all loci (AR), detected number of private alleles per all loci (N_{pa}), observed (H_o) and
328 expected (H_e) heterozygosity, inbreeding coefficient (F_{is}), and effective population size (N_e).

Location	N	AR	N_{pa}	H_o	H_e	F_{is}	N_e
H1	59	2.83	0.26	0.38	0.41	0.03 (-0.05–0.10)	87.3 (27.4–∞)
H3	54	2.58	0.19	0.40	0.42	0.00 (-0.09–0.10)	∞ (55.3–∞)
H4	57	2.65	0.23	0.40	0.43	0.03 (-0.01–0.14)	∞ (45.2–∞)
H6	59	2.86	0.37	0.42	0.49	0.08 (-0.03–0.18)	∞ (149.7–∞)

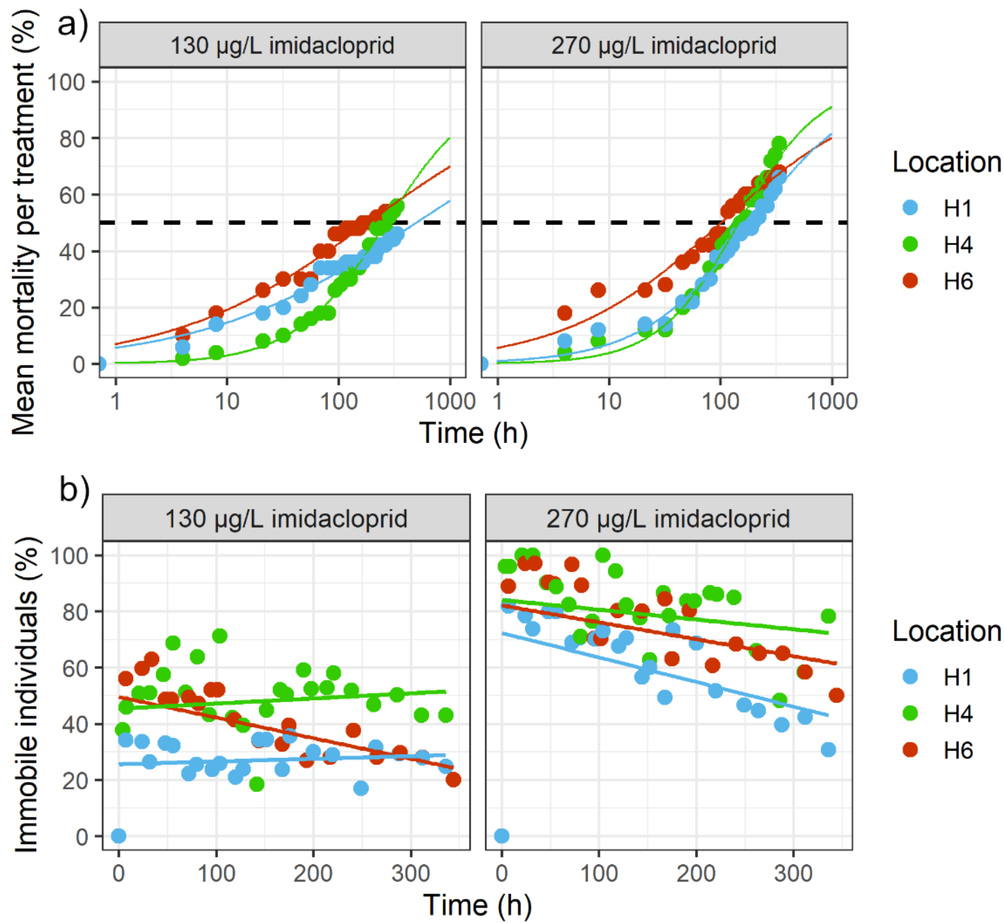
329

330 3.3. Imidacloprid Toxicity Experiments

331 The laboratory exposure experiments with different imidacloprid concentrations indicated site-
332 specific differences in sensitivities across *G. pulex* from the Holtemme River. The initial
333 mortalities occurred simultaneously at 4 h in amphipods from sites H1, H4, and H6 in both the 130
334 µg/L and 270 µg/L treatments. The mortality rates at the end of the experiment reached 46% (H1)
335 and 56% (H4, H6) in the 130 µg/L imidacloprid treatment and 66% (H1), 78% (H4), and 68%
336 (H6) in the 270 µg/L imidacloprid treatment. In the treatment with 130 µg/L imidacloprid, LT_{50}
337 values were reached at 184 (164.5–205.8) h (H6), 269.1 (234.9–308.2) h (H4), and 501.7 (304.1–

338 824.8) h (H1), while LT_{50} values in the 270 μg/L imidacloprid treatment were reached earlier, i.e.,
339 after 102.2 (92.2–113.3) h (H6), 146.9 (130.3–165.5) h (H4) and 187.3 (169.4–207.1) h (H1) (Fig.
340 3a). The confidence intervals of LT_{50} values did not overlap between H1–H6 and H4–H6 in the
341 low concentration treatments and between H1–H4, H1–H6, and H4–H6 in the high concentration
342 treatments. The LT_{50} differences between polluted and non-polluted sites were at 41.4 and 85.1 h
343 (22%–45%) in the high concentration treatments and at more than 232 h (54%) in the low
344 concentration treatments. In controls/solvent controls, mortalities first occurred after 82 h/92 h
345 (H1), 56 h/32 h (H4) and 68 h/82 h (H6). They reached 9%/8% (H1) and 12%/14% (H4, H6) by
346 the end of the experiment (Fig. S4).

347 For immobility rates, indicating sub-lethal effects of imidacloprid that amphipods can recover
348 from,[59] significant differences were observed in *G. pulex* from polluted (H4, H6) and non-polluted
349 locations (H1) (Tab. S12). In contrast to controls, in which all amphipods were mobile throughout
350 the experiment (Fig. S4), increased immobility was observed in all treatments at the first
351 observation time point (4 h) (Fig. 3b). On average, 35–60% and 77–96% of amphipods were
352 immobile in 130 μg/L and in 270 μg/L imidacloprid treatments, respectively. Twice as many
353 amphipods were immobile in treatments from polluted locations (H4, H6) compared to the non-
354 polluted site H1. By the end of the experiment the percentages of immobile amphipods decreased
355 to 43% in H4 and to 20% in H1 and H6 in the lower concentration treatments and to 77% in H4,
356 48% in H6, and 32% in H1 in the higher concentration treatments.



357

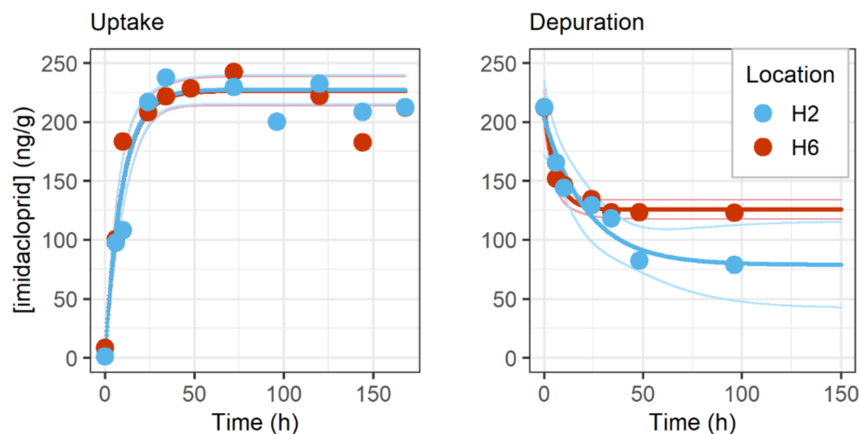
358 Figure 3. Toxic effects of imidacloprid on *Gammarus pulex* from different sampling locations. (a)
359 Mean mortalities of amphipods from locations H1, H4 and H6 in 130 µg/L and 270 µg/L
360 imidacloprid treatments in exposures over 14 d (336 h). Each dot marks the number of dead
361 amphipods per beaker in %. 50 individuals (10 individuals in each of 5 replicates) correspond to
362 100%. Regressions were calculated with the Hill equation (S5). The dotted line marks 50%
363 mortality. (b) Percentages of immobile amphipods in 130 µg/L and 270 µg/L imidacloprid
364 treatments over 14 d (336 h) of exposure. Lines were fitted to the data for each sampling location
365 using linear regression.

366

367 3.3. Uptake and Depuration

368 Upon exposure to imidacloprid, the tissue concentrations of imidacloprid in *G. pulex* from polluted
369 (H6) and non-polluted (H2) locations indicated similar uptake kinetics. After 48 h of exposure, the
370 mean tissue concentration in amphipods from sites H2 and H6 reached equilibrium at 225 ng/g
371 and 228 ng/g wet weight tissue, respectively (Fig. 4). Afterwards, tissue concentrations varied
372 between 200.5 ng/g and 261.9 ng/g, and between 182.6 ng/g and 258.7 ng/g in amphipods from
373 H2 and H6, respectively. The imidacloprid uptake rates of amphipods from different locations
374 were similar (0.125 and 0.091 in *G. pulex* from H2 and H6, respectively; $p = 0.605$).

375 Parameter estimates from the depuration models for *G. pulex* from polluted and non-polluted
376 locations differed significantly ($p = 0.016$), with depuration rate constants of 0.166 (H2) and 0.046
377 (H6). Imidacloprid tissue concentrations reached equilibrium in the amphipods from location H6
378 already after 34 h at 126 ng/g; thereafter, no further changes in tissue concentrations were seen
379 (Fig. 4). In contrast, imidacloprid tissue concentrations in amphipods from H2 did not reach
380 equilibrium by the end of the experiment with imidacloprid tissue concentrations at 79 ng/g. The
381 amphipods from the controls showed constant concentrations from the start of the experiment,
382 with 8.2 ng/g and 0 ng/g imidacloprid detected in the samples from H6 and H2, respectively.



383

384 Figure 4. Uptake and depuration kinetics of imidacloprid in tissue of amphipods sampled at
385 locations with low (H2) and high (H6) levels of organic pollutants. Regressions were modeled
386 with a one phase association (uptake) and a one phase decay (depuration) model. The lighter blue
387 and red lines denote confidence intervals of the models (95%).

388

389 ## 4. Discussion

390 We addressed the question whether sensitivities of *G. pulex* to pollution stress in a river with
391 different levels of pollution differ due to acclimation or adaptation or due to an impaired
392 organisms' condition as a consequence of chronic exposure to toxicants. Our data indicate that
393 differences in sensitivities of *G. pulex* to imidacloprid exposure along the Holtemme River rather
394 originate from local exposure to toxic anthropogenic micropollutants than from adaptive
395 adjustment at differently polluted sites, as the *G. pulex* population in the Holtemme River was
396 found to be genetically homogenous.

397 ### 4.1. Toxic Potential of Anthropogenic Micropollutants in the Holtemme River

398 Detected organic micropollutant concentration levels in the Holtemme River samples are in a range
399 similar to the levels reported for various European rivers.[18,33,40,60,61] Micropollutant analysis from
400 up- and downstream of WWTP1 indicated that this WWTP is a significant source of pesticides,
401 pharmaceuticals, and other organic micropollutants. Of the detected compounds, insecticides with
402 their comparatively larger TU values show a particularly high adverse potential for *G. pulex*.
403 Tissue concentrations of imidacloprid in amphipods sampled downstream of WWTP1 were above
404 4 ng/g (Tab. S3). Based on equilibrium partitioning, this concentration corresponds to a water
405 concentration of 0.4 ng/mL. This is in the range of imidacloprid concentrations measured in other

406 European rivers[62,63] that were found to affect the feeding behavior of *G. pulex* (0.81 ng/mL).[64]
407 Thus, imidacloprid in the Holtemme River water, in the presence of other adverse factors,[30] may
408 be a major contributor to sub-lethal effects (i.e., reduced feeding rates). *Gammarus pulex*
409 individuals sampled downstream of WWTP1 were therefore predisposed by exposure to
410 neonicotinoids and potential sub-lethal effects, which may already exert a selective pressure at
411 these sites in the Holtemme river.[65]

412 Notably, toxicity data for only a few compounds were available for *G. pulex* and therefore toxicity
413 data for *D. magna* were used. Although toxicities to *G. pulex* and *D. magna* correlate for most
414 compounds,[66] toxicity estimations for further compounds for this species would be extremely
415 valuable for more precise assessments of the impacts of chemicals in the environment of this
416 species. Likewise, we want to emphasize the importance of examining the micropollutant levels
417 in the tissue of riverine organisms in addition to water grab samples, as certain toxic compounds,
418 such as imidacloprid, were found in tissue only but not in water samples. Thus, comprehensive
419 information on the present micropollutants can only be obtained by looking at both matrixes, as it
420 enables a more precise toxicity assessment.[67]

421 4.2. River Pollution Patterns and *Gammarus pulex* Population Structure Are Not Linked

422 Although there is evidence for the presence of a selective pressure in the river, our genetic data on
423 population diversity and structure indicate the absence of genetic differentiation of *G. pulex*
424 populations in relation to pollution. This is consistent with preceding studies on *G. pulex*
425 population structure, which suggest that amphipods from one river mostly belong to one
426 genetically homogeneous population within a clade, but at a regional scale, i.e. between different
427 rivers, a complex population structure with distinct populations often exists.[26,47,68] Surprisingly, in

428 contrast to our observations and the aforementioned studies, two populations and increased rates
429 of private alleles for *G. pulex* in the Holtemme river due to anthropogenic pollution of the river
430 were demonstrated in a previous study.[18] As pollution conditions in the river were comparable
431 between the two studies, pollution seems not to be the cause for the observed differences. Different
432 sets of microsatellites used in the two studies are a likely explanation for differing results. For this
433 study we selected a robust microsatellite set and avoided primers with many stuttering peaks used
434 in the previous study (e.g. Gam 2, Gam 4), as suggested by Weiss and Leese[68] (see also Švara et
435 al., 2019).

436 The homogenous genetic structure of *G. pulex* in the Holtemme River is shaped by different
437 factors. Firstly, migration from the upstream sites with low pollution pressure to sites with higher
438 pollution pressure[18] most likely prevents major shift of allele frequencies in the polluted river
439 section. Although slightly inbred, amphipods living downstream of the WWTP effluents did not
440 show drastic reduction of the effective population size and allelic richness, the two parameters are
441 often observed in populations under selection due to toxic exposure.[17] Secondly, in comparison to
442 a low *G. pulex* abundance and effective population size found at upstream sites H1 and H2, high
443 abundances in the polluted river sections and large effective population sizes directly after WWTPs
444 can be maintained due to abundant food supply (fungi, biofilms) resulting from the input of
445 anthropogenic nitrate and organic carbon, that enter the river through WWTP effluents and
446 agricultural field drainage.[34,35] Additionally, the number of private alleles does not show any
447 dramatic increase downstream of the WWTPs in our study. Thus, slightly increased allelic richness
448 values downstream of WWTPs are probably due to a larger allele pool in lower reaches because
449 of migration to the river. Within rivers comparable to the Holtemme River, connectivity,

450 migration, and historic colonization have been argued to often determine population genetic
451 composition of *Gammarus* amphipods, rather than pollution.[68]

452 4.3. *In situ* Exposure to Anthropogenic Pollution Results in an Increased Sensitivity of *G. pulex*

453 As *G. pulex* from the Holtemme River form a single population, differences in molecular targets
454 originating from adaptation in amphipods from the different sites are an unlikely reason for
455 differential sensitivities of amphipods from different sites against exposure to imidacloprid. The
456 highest detected difference in survival time between amphipods from two sites in the Holtemme
457 River was 54%, which is partially in line with the findings of Weston et al. (2013) who found
458 differences in sensitivities of amphipods within the same clade and location to be smaller than one
459 fold.[21] Larger sensitivity differences between genetically divergent populations are associated with
460 respective mutations or shifted allele frequencies, which could also be expected in *G. pulex,* but
461 only on a regional scale, where several populations or even cryptic species are present.[25,26,47]

462 Given the genetic homogeneity of *G. pulex* across the Holtemme River, we can assume that the
463 physiological states of amphipods were different between upstream and downstream sampled
464 individuals. The amphipods used for the experiments here were lab-acclimated for seven days,
465 which is a period commonly used in comparable studies (1–7 d).[11,21,25] It proves sufficient to
466 harmonize *in situ* physiological state differences of the amphipods from the different sites due to
467 factors such as temperature (refer to Tab. S1), food availability, and competition. Yet, this time
468 period may not have been sufficient for recovery of amphipods from toxic micropollutants
469 accumulated in the tissue, as many compounds persist in *G. pulex* tissue for weeks (e.g.
470 imidacloprid).[42] In the elimination experiment, imidacloprid tissue levels decreased by about 50%
471 within two to three days, however, tissue levels then remained stable and did not show any

472 significant decrease until the end of the experiment (Fig. 4). It is conceivable that imidacloprid,
473 together with other micropollutants (e.g. thiacloprid) taken up by the amphipods at sites H4 and
474 H6, enhance such chronic toxic burden, that could in the exposure experiment be reflected in
475 higher immobility or mortality rates in the initial phase of the exposure to imidacloprid. Thus, the
476 reduced capacity to eliminate imidacloprid accumulated in the tissue by amphipods from polluted
477 sites, in addition to the effects of sequential exposure[31] and differences in damage recovery of
478 closely related amphipods,[69] may explain the finding of higher sensitivity of amphipods from
479 polluted sites against imidacloprid exposure.

480 4.4. Ecological Implications

481 Our data show that within a genetically homogeneous *G. pulex* population site-specific differences
482 in sensitivities to anthropogenic micropollutant exposure can occur. These sensitivities are related
483 to the site-specific pollution conditions. Sensitivity of amphipods to micropollutants is enhanced
484 when amphipods are chronically exposed to toxic compounds in their natural habitat, as these
485 compounds accumulate in the tissue. However, although more vulnerable from exposure to
486 anthropogenic micropollutants, *G. pulex* exposed to toxic micropollutants benefit from the high
487 abundance of food in the polluted but nutrient-rich habitats in the Holtemme River. Together with
488 higher food abundance, other factors, such as habitat availability, higher temperatures, and
489 favorable oxygen and pH conditions,[70,71] can contribute to higher growth rates[72] and increased
490 abundance of *G. pulex* in these reaches compared to the more oligotrophic upstream habitats. In
491 addition to favorable environmental parameters, large effective population size and high
492 abundance of *G. pulex* can be facilitated by migration of genetically diverse *G. pulex* from non-
493 polluted parts of the river. By contrast, predisposition of *G. pulex* in polluted river sections through

494 exposure to micropollutants may lead to temporal phases of increased sensitivity due to seasonal
495 pollution peaks. Such peak events may result in severe consequences for a *G. pulex* population in
496 a stream, such as large fluctuations of population size[73] and a reduced trophic transfer. After all,
497 *G. pulex* has a key role as a shredder of organic debris and as food source for fish.[74] Therefore, we
498 would like to emphasize the importance of information on the population genetic composition of
499 the studied organisms in toxicological studies with organisms originating from habitats with
500 different levels of pollution. As our study shows, toxic organic micropollutants did not select for
501 a *G. pulex* genotype adapted to thrive in polluted habitats in the river, but lead to higher sensitivity
502 against compound exposure in amphipods.
503
504 **Supporting Information**
505 LC/HRMS sample preparation and analysis; PCR conditions, sequencing and genotyping
506 information; sequence and microsatellite data analyses; exposure experiments conditions; non-
507 linear Hill model; haplotypes of COI sequences; map of the sampling sites; likelihood values from
508 Structure Harvester analysis; maximum likelihood phylogenetic tree; graphs with control data;
509 mortality data with standard errors; list of sampling sites; list of analyzed compounds; compounds
510 detected in *G. pulex* tissue samples; compounds detected in water samples; standard toxicity test
511 median acute effect concentration data; toxic units for compounds found in the *G. pulex*; primers
512 used for COI sequencing; primers used for microsatellite amplification; pairwise genetic distances
513 between COI sequences; pairwise F_{st} values for COI sequences; pairwise F_{st} values for
514 microsatellite data; Kruskal-Wallis one-way test of mobility data.
515
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