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1 **Fluoxetine effects assessment on the life cycle of aquatic invertebrates**

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10

11 **Abstract**

12 Fluoxetine is a serotonin re-uptake inhibitor, generally used as an antidepressant. It is
13 suspected to provoke substantial effects in the aquatic environment. This study reports the effects
14 of fluoxetine on the life cycle of four invertebrate species, *Daphnia magna*, *Hyaella azteca* and
15 the snail *Potamopyrgus antipodarum* exposed to fluoxetine spiked-water and the midge
16 *Chironomus riparius* exposed to fluoxetine-spiked sediments. For *D. magna*, a multi-
17 generational study was performed with exposition of newborns from exposed organisms. Effects
18 of fluoxetine could be found at low measured concentrations (around $10 \mu\text{g L}^{-1}$), especially for
19 parthenogenetic reproduction of *D. magna* and *P. antipodarum*. For daphnids, newborns length
20 was impacted by fluoxetine and the second generation of exposed individuals showed much
21 more pronounced effects than the first one, with a NOEC of $8.9 \mu\text{g L}^{-1}$. For *P. antipodarum*,
22 significant decrease of reproduction was found for concentrations around $10 \mu\text{g L}^{-1}$. In contrast,
23 we found no effect on the reproduction of *H. azteca* but a significant effect on growth, which
24 resulted in a NOEC of $33 \mu\text{g L}^{-1}$, expressed in nominal concentration. No effect on *C. riparius*
25 could be found for measured concentrations up to 59.5 mg kg^{-1} . General mechanistic energy-
26 based models showed poor relevance for data analysis, which suggests that fluoxetine targets
27 specific mechanisms of reproduction.

28

29 *Keywords* : invertebrates, fluoxetine, pharmaceuticals, sublethal effects, multi-generational
30 test, mechanistic models.

31

32 1. Introduction

33

34 For the past few years, there has been a growing concern about ecotoxicological risk
35 of pharmaceuticals. Indeed, human medicines have been detected in many countries in
36 sewage treatment plant effluents, surface waters, seawaters, groundwater and some drinking
37 waters (Fent *et al.*, 2006). As pharmaceuticals are present at relatively low levels in the
38 environment, risk for acute toxic effects is unlikely, but chronic environmental toxic effects
39 cannot be excluded (Carlsson *et al.*, 2006). However, little is known about the chronic effects
40 of these substances. Moreover, environmental risk assessment based on acute data is
41 inappropriate (Ferrari *et al.*, 2004). For instance, carbamazepine and propranolol would be
42 inaccurately identified as having negligible risks in France and Germany. There is a real lack
43 of long term effects studies, in particular chronic data on the entire life cycle and
44 investigation of multigenerational effects (Fent *et al.*, 2006).

45 Fluoxetine is a serotonin re-uptake inhibitor. It is apparently the most acute toxic
46 human pharmaceuticals reported so far (Fent *et al.*, 2006), which makes necessary more
47 studies about risks of low levels of exposure. In terms of environmental concentrations,
48 Kolpin *et al.* (2002) estimated at $0.012 \mu\text{g L}^{-1}$ the median concentration of fluoxetine in U.S.
49 streams. In terms of chronic effects, the recently published data on the effects of fluoxetine to
50 invertebrates provide contradictory information. Flaherty and Dodson (2005) found an
51 enhancement of reproduction for *Daphnia magna* exposed to a concentration of $36 \mu\text{g L}^{-1}$. In
52 contrast, Brooks *et al.* (2003) found a reproduction decrease for *Ceriodaphnia dubia* with a
53 NOEC of $56 \mu\text{g L}^{-1}$ and a LOEC of $112 \mu\text{g L}^{-1}$. Henry *et al.* (2004) also found reproduction
54 decrease for the same species with a NOEC of $89 \mu\text{g L}^{-1}$ and a LOEC of $447 \mu\text{g L}^{-1}$. Brooks *et al.*
55 *al.* (2003) derived growth LOECs for *Chironomus tentans* and *Hyalella azteca* of respectively
56 1.3 and 5.6 mg kg^{-1} sediment, but Nentwig (2007) did not find any significant effect on

57 *Chironomus riparius* for concentrations up to 5.86 mg kg⁻¹ sediment. This author obtained an
58 extremely low LOEC of 0.47 µg L⁻¹ for the reproduction of the snail *Potamopyrgus*
59 *antipodarum*, comparable to concentrations measured in surface water.

60 Toxic effects of fluoxetine on invertebrates are consequently still worth being studied.
61 We selected four invertebrates, with the following criteria: first, we tested species for which at
62 least one test result is available in the literature to allow comparisons between our data and
63 data from other studies ; second, as past studies indicated that the sublethal effects of
64 fluoxetine are likely to be on reproduction, we tried to have different reproduction strategies
65 (sexual reproduction and parthenogenesis). We consequently used a large test battery
66 encompassing several phylogenetic groups. The species selected were then *Chironomus*
67 *riparius* for sediment borne exposure, the crustacean *Daphnia magna* and *Hyalella azteca*,
68 and the mollusc gastropod *Potamopyrgus antipodarum* concerning water borne exposure.
69 This selection for water borne exposure is relevant to cover a large range of invertebrate
70 sensitivity for organic compounds, as presented by Wogram and Liess (2001). Indeed, these
71 authors showed that, relative to organic compounds, amphipoda are significantly more
72 sensitive than daphnids and that gastropoda are significantly less sensitive than daphnids. We
73 plan here to investigate effects on all the components of the life cycle of our species. In
74 particular, effects on adults survival and reproduction as well as effects on juveniles survival
75 and growth have been assessed. Moreover, for one of the species (*Daphnia magna*), fitness of
76 the newborns produced during exposure and exposed themselves at the same concentration as
77 their mother was assessed, to account for subtle effects on reproduction (like malformation)
78 which would be undetectable with results expressed only in terms of numbers of newborns.
79 Finally, the growth and reproduction data were analyzed using energy-based models, DEBtox
80 (Kooijman and Bedaux, 1996). These models permit to estimate parameters not dependent on
81 time, which is valuable to assess effects for long term exposure, and they allow insides

82 relative to the physiological mode of action of the compound (Kooijman and Bedaux, 1996;
83 Péry *et al.*, 2003).

84

85 **2. Materials and Methods**

86

87 *2.1. Chemical substance*

88 Fluoxetine-HCL was used for all ecotoxicity tests (CAS 59333-67-4). It was
89 purchased from Interchim (Montluçon, France ; product number 09674, batch number
90 RD0001).

91 *2.2. Chironomus riparius tests*

92 Larvae were exposed to fluoxetine-spiked sediment. Sediment has been taken from
93 Port-Galland, a tributary of the river Ain (France). This sediment has **been monitored for**
94 **years by Cemagref**. Chemicals concentration are at low level and it has never been toxic to
95 sediment organisms. Sediments were 2 mm sieved and homogenised before use. Organic
96 carbon content is 3%, organic nitrogen content is 0.35%. Water was constituted of ¼ water
97 from a spring, situated under our laboratory, added to demineralised water, resulting in pH of
98 7.8 and conductivity of 450 $\mu\text{S cm}^{-1}$. Sediments were spiked with fluoxetine 10 days before
99 starting the tests. Fluoxetine (Interchim, Montluçon, France) was introduced in 0.5 L water at
100 the concentration corresponding to the chosen nominal exposure concentrations and mixed
101 with 1.5 kg wet sediment. Tested exposure concentrations were 1.2, 3.7, 11, 33, 100 and 1000
102 mg kg^{-1} d.w., the latest being the concentration for which a compound is considered as **safe if**
103 no significant effect occur at this exposure concentration (OECD, 2004). Sediments were
104 transferred in beakers three days before starting tests. There were ten beakers per
105 concentration. We put 0.1 L sediment and 0.4 L water in these beakers. The beakers were set
106 in a water bath at 21°C with a 16:8 h light:dark photoperiod. Conductivity, temperature, pH,

107 amount of dissolved oxygen were measured daily. We used an aeration system (air introduced
108 through a Pasteur pipette in each beaker) to maintain oxygen level.

109 The experiment was initiated with two-day-old larvae (end of first instar) from our
110 laboratory culture. We put ten larvae per beaker. Length at the beginning of the test, measured
111 on 20 organisms, was 1.7 +/- 0.1 mm. Organisms were fed daily with 0.6 mg per larva
112 Tetramin® fish food.

113 At day 7, survivor and growth were monitored by sampling five beakers per
114 concentration. Individuals were counted, killed using formaldehyde, then length was
115 measured using a binocular microscope. Emergence was monitored for the five remaining
116 beakers per concentration which had been covered to prevent organisms from escaping. The
117 females were then put into 1 L mating chambers, with 0.1 L water, with males from
118 laboratory culture in a ratio of three males per female as described by Péry *et al.* (2002).
119 After mating and oviposition, each egg mass was removed and put into a 5 mL tube with 2
120 mL H₂SO₄, 2N overnight and the number of eggs was counted.

121

122 2.3. *Hyalella azteca* tests

123 Amphipods were exposed to fluoxetine-spiked water. Experimental protocol was the
124 same as in Péry *et al.* (2005) : amphipods were exposed in beakers and an artificial nylon-
125 shelter was introduced at the bottom of each beaker. The beakers were set in a water bath at
126 21 °C with a 16:8 h light:dark photoperiod. Test water in the beakers was continuously
127 renewed (four renewals a day): for each concentration, there was a continuous pumping of
128 clean water (same as for *C. riparius*) and stock solution at a speed calculated to obtained the
129 required exposure concentration and then mixed in a bottle. Stock solution was protected from
130 light and renewed every three days. The nominal exposure concentrations were 3.7, 11, 33
131 and 100 µg L⁻¹. Specific conductivity, temperature, pH, dissolved oxygen were measured

132 daily. Organisms were fed daily with 0.16 mg Tetramin® per individual. Two experiments
133 were conducted. In the first one, young organisms (between 7 and 9 days old, mean length
134 1.69 +/- 0.17 mm) selected in our laboratory culture were exposed (ten per beaker) and length
135 was measured every 7 days to assess effects on growth. Length measurements were
136 performed on the dorsal side from the base of the first antenna to the end of the next to last
137 segment, using an image analysis method (Sigma Scan Pro 5.0, SPSS Inc., Chicago). In the
138 second one, we exposed five precopula (one male and one female, resulting in ten organisms
139 per beaker) taken from our laboratory culture per beaker during 28 days, and we monitored
140 reproduction every week. For each experiment, there was four replicates per concentration.

141

142 2.4. *Daphnia magna* tests

143 Organisms were exposed individually in 100 mL-bottles which contained 80 mL of
144 solution. There were ten replicates per concentration. Tested fluoxetine concentrations were 0,
145 3, 10, 30, 100 and 300 µg L⁻¹. Test duration was 21 d, temperature was maintained at 20 °C
146 by putting the bottles in temperature-controlled chambers, water (M4 medium, as
147 recommended by ISO 10706) was renewed every day. Daphnids length was measured at days
148 7, 14 and 21 using image analysis and reproduction (number of newborns) was monitored
149 every day. Length of the newborns was measured for the third brood. Food was algae
150 *Pseudokirchneriella subcapitata* from our laboratory culture. Each organism received
151 10⁷ algal cells per day the two first days, 2. 10⁷ algal cells per day the three following days, 3.
152 10⁷ algal cells per day the two following days and 4.10⁷ algal cells per day until the end of the
153 test. These feeding conditions are *ad libitum* conditions, as it has been chosen by previous
154 tests (unpublished results).

155 To assess effects on two generations, an experiment in the same conditions was
156 performed with newborns from the fifth brood. This experiment with the newborns started

157 exactly the day when the experiment with their mother ended. There was not enough
158 surviving newborns to start this new test for nominal concentration 300 µg L⁻¹.

159

160 2.5. *Potamopyrgus antipodarum* tests

161 Snails from the species *Potamopyrgus antipodarum* came from our laboratory culture.
162 The test beakers were filled with 0.5 L fluoxetine-spiked water (the same as for amphipods),
163 three days before the beginning of the tests. The beakers were set in a water bath at 21 °C
164 with a 16:8 h light:dark photoperiod. The exposure system was the same as for *H. azteca*. The
165 nominal exposure concentrations were 3.7, 11, 33 and 100 µg L⁻¹. Specific conductivity,
166 temperature, pH, dissolved oxygen were measured daily. Organisms were fed with 0.6 mg
167 Tetramin® fish food (Tetrawerke, Melle, Germany) per individual per day. We performed
168 two experiments, one to assess effects on growth, the other one to assess effects on
169 reproduction. Growth was monitored every week through shell length measurements using a
170 binocular. At the beginning of the growth test, each beaker contained ten organisms, which
171 had been selected in the culture according to their length (0.48 +/- 0.026 mm). **Reproduction**
172 **was monitored once a week, by counting and removing all newborns using a binocular.** The
173 test was initiated with individual adult length superior to 4 mm at the beginning of the test,
174 and also ten individuals per replicate. For each experiment, there were three replicates per
175 concentration. The experiments lasted six weeks.

176

177 2.6. *Analytical Procedures*

178 Spiked water was sampled for all exposure concentrations in the *D. magna* and *P.*
179 *antipodarum* tests at day 10 for daphnids and day 42 for snails. We could not have chemical
180 measurements for amphipod tests. To get enough volume to perform chemical measurements,

181 waters for all replicates of a given concentration were pooled. Spiked sediments were sampled
182 for all concentrations at the end of the toxicity test.

183 Water samples (10-250 mL), adjusted to pH 3 with sulphuric acid, were spiked with
184 the surrogate standard fluoxetine-d5 (Isotec, Miamisburg, USA). Samples were enriched at a
185 flow rate of 10-20 mL min⁻¹ (ca. 200 mbar) with OASIS HLB SPE cartridges (200 mg, 30
186 µm, Waters, Milfort, USA) and the SPE material was dried for 1 h under a nitrogen stream.
187 Fluoxetine was eluted using 4 x 2 mL of methanol/acetic acid (98/2, v/v). After blowing down
188 to 100 µL the samples extracts, they were reconstituted to 1 mL of the LC eluent A (see
189 below).

190 Sediment samples (1 g) were spiked with the surrogate standard fluoxetine-d5 and extracted
191 by pressurized liquid extraction (PLE) with MeOH/water/acetic acid (49:49:2) at 100 bar and
192 120 °C during two static cycles of five min. Afterwards, the extract was made up to 50 mL
193 and one aliquot of 0.1-1 mL diluted in 500 mL of groundwater. SPE clean-up was carried out
194 with OASIS HLB cartridges eluted with MeOH-MTBE (95:5).

195 The sample extracts were measured by LC tandem MS (Agilent 1100 with degasser,
196 quaternary pump and autosampler, Agilent Technologies, Waldbronn, Germany/API 4000
197 with ESI ionization, Applied Biosystems, Foster City, CA, USA) operating in the positive ion
198 mode using multiple reaction monitoring (MRM). Chromatographic separation took place at
199 room temperature by means of a Synergi Polar RP 80A column (150 x 3 mm, 4µm)
200 (Phenomenex®, Aschaffenburg, Germany). A mixture of 20 mM ammonia solution (pH 5.7
201 adjusted with acetic acid): acetonitrile (98:2) (A) and a mixture of A:acetonitrile (2:3) (B)
202 were used as mobile phases. Two MRM transitions were monitored for each substance for
203 identification and quantification of the analytes (fluoxetine: 310/44 and 310/148 amu;
204 fluoxetine-d5: 315/44 and 315/143 amu).

205 Calibration curves showed a good correlation in the range 5-2000 ng.mL⁻¹. Limits of
206 quantification for fluoxetine in sediment and water samples were 10 ng.g⁻¹ and 5 ng.L⁻¹,
207 respectively.

208

209 *2.7. Statistical analysis*

210 To analyse the data, we used standard methods (ANOVA, Dunnett-t tests) but also
211 DEBtox models (See a complete description in Kooijman and Bedaux, 1996 and in the OECD
212 guideline about statistics in ecotoxicology (OECD, 2006)). These models are based on the
213 DEB theory (Kooijman, 2000), which describes growth and reproduction as a function of
214 bioenergetics parameters like for instance costs of maintenance or food assimilation rate.
215 Effects on growth and reproduction are described as the consequences of effects on one of
216 these bioenergetics parameters. These effects are proportional to the difference between
217 accumulated compound concentration and a threshold concentration, called the NEC (No
218 Effect Concentration). The estimate of this threshold concentration, obtained through
219 maximum likelihood methods, does not depend on the duration of the test.

220

221 **3. Results**

222

223 *3.1. Chironomus riparius tests*

224 Temperature was constant (21±1 °C) so as pH (7.9 ±0.3). Conductivity was 490 ±/
225 35 µS cm⁻¹, and the percentage of dissolved oxygen was always above 90 %. Growth (length
226 of 12.1 mm at 7 days) and survival (72%) in the control were enough to validate the test.
227 Chemical measurement showed a recovery of 63 ± 4% for fluoxetine spiked on the
228 sediments. Traces of fluoxetine near detection limit (0.1 mg kg⁻¹) were found in the control.

229 There was no significant effect on *Chironomus riparius* growth, emergence and reproduction
230 for concentrations up to 59.5 mg kg⁻¹ (ANOVA, p>0.05). Final length for all these
231 concentrations were between 11.9 and 12.1 mm and total number of eggs per female were
232 between 426 and 456. For measured concentration 666 mg kg⁻¹, there was no emergence,
233 survival at day 7 was low (34%) and growth at day 7 was very significantly reduced (p<0.01,
234 Dunnet-t test), by 31%.

235

236 3.2. *Hyaella azteca* tests

237 Temperature was constant (20.9±0.4 °C) so as pH (7.55 ±0.2). Conductivity was
238 391 ± 17 µS cm⁻¹, and the percentage of dissolved oxygen was always above 90%.

239 No adult died for any of the concentrations during the test. There was no significant
240 effect of fluoxetine on reproduction (ANOVA, p>0.5), with mean number of newborns per
241 female from 12.8 to 15.9. For the young organisms, more than 87.5% amphipods survived in
242 all the concentrations. Effects on growth were significant for nominal concentration 100 µg L⁻¹
243 at days 14, 21 and 28 (p<0.01, Dunnet-t test), as presented by Figure 1. This resulted in a
244 LOEC of 100 µg L⁻¹ and a NOEC of 33 µg L⁻¹. We used DEBtox models, with the three
245 possible physiological modes of action for growth (effects on food assimilation, on growth
246 energy costs or on maintenance energetic costs), and with a Von Bertalanffy growth rate of
247 0.08 d⁻¹ (parameter required by the software, estimated with a least square method using
248 control data). Growth was very low the first week, so we used DEBtox only from day 7 to 28,
249 but taking into account that compound accumulation has started from the very first day. The
250 best fit was obtained for the mode of action “increase of energetic costs for growth”, the two
251 other modes of action leading to estimations significantly different from the data at day 28
252 obtained for nominal concentration 100 µg L⁻¹. To propose a rough explanation for that, we
253 should point that, in the DEBtox context, “increase of energetic costs for growth” is

254 characterized by effects on growth rate but no effect on ultimate length. By looking at Figure
255 1, it seems that all growth curves tend to reach the same ultimate length. The NEC estimated
256 by DEBtox was $19 \mu\text{g L}^{-1}$, but the software was unable to provide a confidence interval,
257 which means that all numbers between 0 and infinity were in this confidence interval.

258

259 3.3. *Daphnia magna* tests

260 Temperature was constant ($19.9 \pm 0.34 \text{ }^\circ\text{C}$) so as pH (7.9 ± 0.27) and conductivity
261 ($642 \pm 30 \mu\text{S cm}^{-1}$). Chemical measurements showed a recovery of fluoxetine in the
262 exposure system from 80 to 102%.

263 In the first test, a significant effect on growth was found at day 7 for concentrations 102
264 and $241 \mu\text{g L}^{-1}$. At days 14 and 21, this effect was only significant for exposure concentration
265 $241 \mu\text{g L}^{-1}$. Moreover, there was 40% mortality for this concentration at day 21 and a
266 significant decrease of reproduction by 32%. No effect on reproduction was found for the
267 other concentrations. The measurements of the newborns length for the third brood of the first
268 test showed significant effects of fluoxetine for exposure concentrations 31, 102 and $241 \mu\text{g}$
269 L^{-1} (Figure 2). This parameter is the most sensitive to fluoxetine, resulting in a NOEC of 8.9
270 $\mu\text{g L}^{-1}$ and a LOEC of $31 \mu\text{g L}^{-1}$. Concerning the second test, effects were much more
271 pronounced than for the first one, but with the same LOEC and NOEC. 70 % of the newborns
272 were found dead at 21d for exposure concentration $102 \mu\text{g L}^{-1}$. Moreover, reproduction was
273 significantly reduced for exposure concentration $31 \mu\text{g L}^{-1}$ (by 18%) and length was
274 significantly lower than the control for exposure concentrations 31 and $102 \mu\text{g L}^{-1}$.

275

276 3.4. *Potamopyrgus antipodarum* tests

277 Temperature was constant ($20.8 \pm 0.4 \text{ }^\circ\text{C}$) so as pH (7.6 ± 0.3). Conductivity was 400
278 $\pm 24 \mu\text{S cm}^{-1}$, and the percentage of dissolved oxygen was always above 90%. Chemical

279 measurements showed a bad recovery of fluoxetine in the exposure system (from 27 to 69%),
280 with measured exposure concentrations : 1, 4.2, 13 and 69 $\mu\text{g L}^{-1}$.
281 There was no significant effect of fluoxetine on growth for all weekly measurements
282 (ANOVA, $p>0.5$). As for reproduction, we observed a significant decrease at 69 $\mu\text{g L}^{-1}$
283 (Figure 3) but no significant effect at lower concentrations (Dunnett-t tests, $p<0.05$), resulting
284 in a NOEC of 13 $\mu\text{g L}^{-1}$ and a LOEC of 69 $\mu\text{g L}^{-1}$. We used DEBtox models to analyse data
285 on reproduction. We selected the physiological mode of action « increase of the energetic
286 costs of reproduction ». Indeed, the selection of effects on reproduction due to effects on
287 growth would have no sense here, because we exposed adults. We obtained a NEC of 5 $\mu\text{g L}^{-1}$
288 with 95% confidence interval 4.3-10.4 $\mu\text{g L}^{-1}$. All values in this confidence interval are lower
289 than the estimated NOEC.

290

291 4. Discussion

292

293 The chemical measures for the tests with daphnids and chironomids showed a correct
294 spiking with fluoxetine. In contrast, the chemical measurements for the tests snails showed an
295 irregular, sometimes low efficiency of water spiking (especially for low concentrations),
296 despite a continuous renewal of the solution. Recently Kwon and Armbrust (2006) have
297 demonstrated that fluoxetine is hydrolytically and photolytically stable in aqueous solutions
298 including natural waters. Fluoxetine may thus not be degraded in our system. Our
299 hypothesis is thus that fluoxetine, which is likely to sorb very quickly on the sediments, could
300 sorb very quickly on the fish food provided to the snails and the amphipods in our test or to
301 the plastic tubes of the renewing system. As the exposure system was the same for *H. azteca*
302 as for *P. antipodarum*, and as the tests for the snails were performed immediately after the
303 tests with amphipods, we could consider that the actual concentrations that have been

304 measured for snails exposure to fluoxetine are also valid for amphipods exposure to
305 fluoxetine. The NOEC of $33 \mu\text{g L}^{-1}$ expressed in nominal concentration for growth of
306 amphipods would be a NOEC of $13 \mu\text{g L}^{-1}$ expressed in measured concentration.

307 The classification of sensitivity for our species was quite different from our expectations based
308 on the works of Wogram and Liess (2001) on effects on organic compounds on invertebrates,
309 which classified amphipods, daphnids and snails in order of decreasing sensitivity, which
310 confirms the necessity to treat pharmaceuticals specifically among all organic compounds. In our
311 tests, the most sensitive species is *P. antipodarum*, with a NEC of $5 \mu\text{g L}^{-1}$ relative to
312 reproduction. Moreover, the effects of fluoxetine were on reproduction for daphnids and snails
313 (juveniles fitness and total amount of newborns respectively), whereas they were on growth for
314 *H. azteca*. Our choice of tested species was consequently relevant to capture a large range of
315 different types of responses.

316 For daphnids, the highest effects were found on the development of the embryos, with smaller
317 newborns resulting in significant effects on their future reproduction. Exposure to fluoxetine
318 has thus consequences on the fitness of the newborns, which energy-based models like
319 DEBtox are not able to account for. Indeed, they assume that the total amount of energy
320 invested per newborn is not concentration dependent, so that newborns should have the same
321 length and ability to resist to toxic exposure. This suggests a direct action of fluoxetine on the
322 development of newborns, which may not be the consequence of energy depletion in the adult
323 female. Consequently, no result from modeling was presented for daphnids toxicity tests, for
324 our models cannot account for fluoxetine mode of action. For *H. azteca*, the use of DEBtox
325 models was also irrelevant. DEBtox was unable to provide a confidence interval, which
326 means that all numbers between 0 and infinity were in this confidence interval. This suggest
327 that energy-based models like DEBtox are unable to account accurately for the observed

328 effects, and that, probably, the main target of fluoxetine in *H. azteca* is not the dynamics of
329 energy.

330 We can compare our results with other studies from the literature. Fluoxetine appeared to
331 have different effects on growth, fecundity and reproduction depending on species. The
332 freshwater snail *P. antipodarum* has shown to be the most sensitive invertebrate species for
333 reproduction as a NOEC of $3.2 \mu\text{g L}^{-1}$ (56 days) has been reported by Nentwig (2007). This is
334 very coherent with the NEC of $5 \mu\text{g L}^{-1}$ we found in this project. For *H. azteca*, fluoxetine
335 treatments inhibited growth with a NOEC of $33 \mu\text{g L}^{-1}$ expressed in nominal concentration.
336 Brooks *et al.* (2003) also showed an inhibition of growth due to fluoxetine exposure. We have
337 contradictory results compared to the data from Flaherty and Dodson (2005) who found an
338 enhancement of reproduction for *Daphnia magna* exposed to a concentration of $36 \mu\text{g L}^{-1}$
339 fluoxetine. In contrast, our results are coherent with Brooks *et al.* (2003) who found a
340 reproduction decrease for *Ceriodaphnia dubia* with a NOEC of $56 \mu\text{g L}^{-1}$ and a LOEC of 112
341 $\mu\text{g L}^{-1}$. Henry *et al.* (2004) also found reproduction decrease for the same species with a
342 NOEC of $89 \mu\text{g L}^{-1}$ and a LOEC of $447 \mu\text{g L}^{-1}$. Nentwig (2007) found a LOEC of 1.12 mg kg^{-1}
343 ¹ (measured value) when studied fluoxetine effects on *C. riparius* emergence which was
344 associated to a significant increase of number of eggs per clutch. However, he observed no
345 effect on growth for concentrations up to 5.86 mg kg^{-1} and he recommends confirming the
346 potential reduced emergence and increased clutch size observed after fluoxetine exposure. In
347 our study, we observed no effect for concentrations below 59 mg kg^{-1} . Fluoxetine is
348 consequently very unlikely to have effects in the field on *Chironomus riparius*.

349 To conclude, data sets on acute and chronic toxicity of the selected case study
350 pharmaceuticals have been derived in our study. Fluoxetine seems to interact with growth and
351 reproduction processes in invertebrates. Depending on the tested species, effects of fluoxetine
352 can be found at low exposure concentrations, around $10 \mu\text{g L}^{-1}$. The fact that the second

353 generation of daphnids was more sensitive than the first one highlights the need for
354 investigation of the effects of pharmaceuticals on at least two generations of invertebrates.
355 Energy-based models were developed and used to describe effects on growth and
356 reproduction, but were not relevant to estimates threshold effect for fluoxetine.

357

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363

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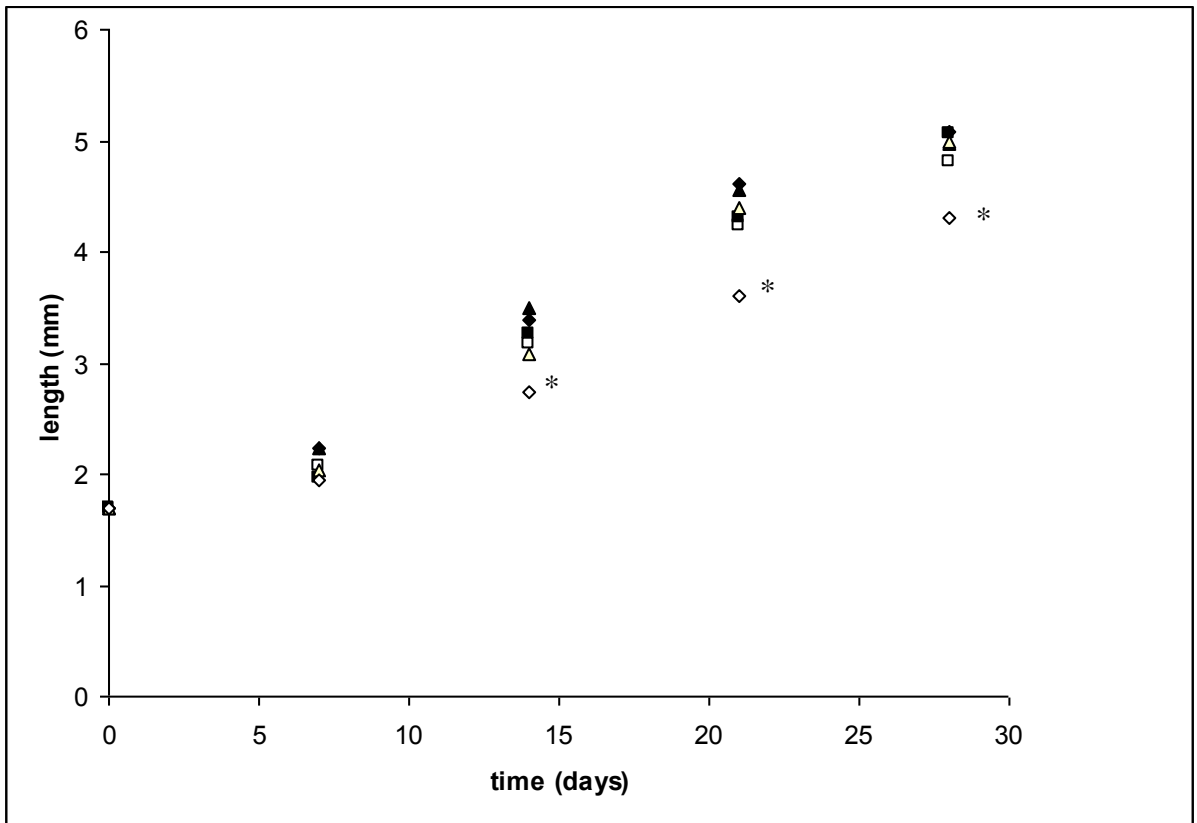
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413 Figure 1. Length of the young *H. azteca* as a function of time and nominal concentration
414 (control : black diamonds, 1.2 $\mu\text{g L}^{-1}$: black squares, 3.7 $\mu\text{g L}^{-1}$: black triangles, 11 $\mu\text{g L}^{-1}$:
415 white squares, 33 $\mu\text{g L}^{-1}$: white triangles, 100 $\mu\text{g L}^{-1}$: white diamonds). Asterisk accounts for
416 significant difference with the control ($p < 0.05$, Dunnett-t test).

417 Figure 2. Length of *Daphnia* newborns from the third brood as a function of fluoxetine
418 nominal concentration. Asterisk indicates significant difference from the control ($p < 0.05$,
419 Dunnett-t test).

420 Figure 3. Number of newborns per *P. antipodarum* adult (mean value and standard deviation) as
421 a function of fluoxetine nominal concentration. Asterisk indicates significant difference from the
422 control ($p < 0.05$, Dunnett-t test).

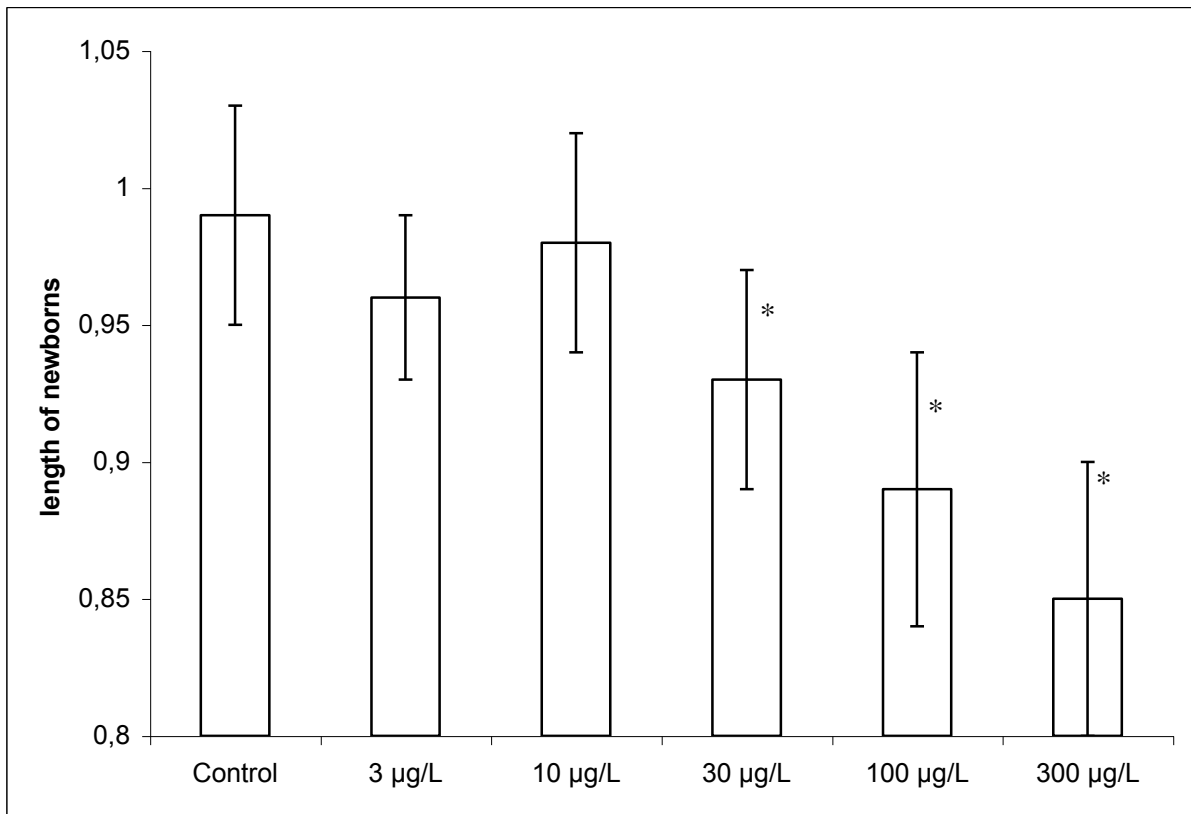
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425 Figure 1.

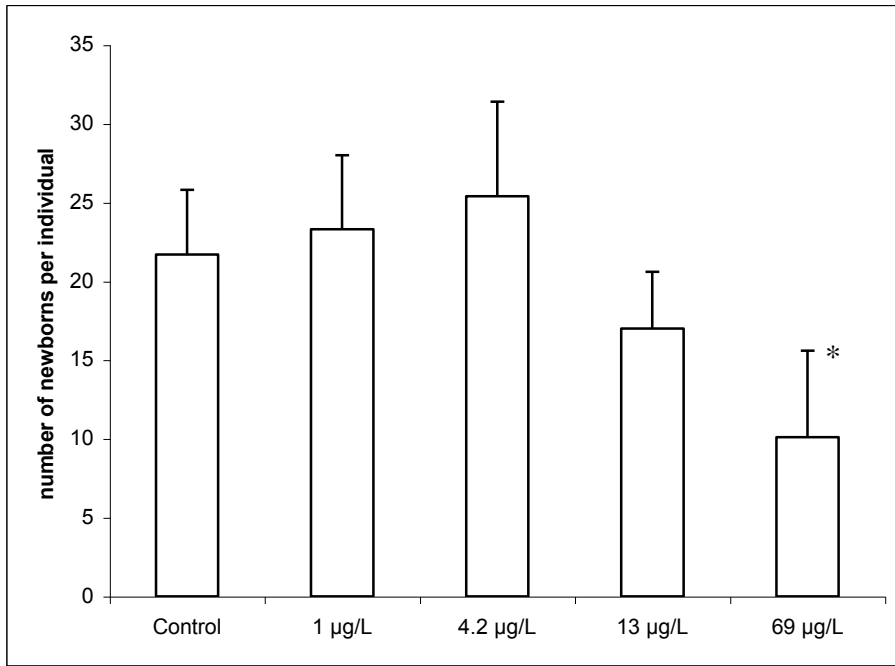
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427

428 Figure 2.

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430

431 Figure 3.

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