Fluoxetine effects assessment on the life cycle of aquatic invertebrates

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

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10

Abstract

11

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*, *Hyalella 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 µg 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 µg L^{-1}. For *P. antipodarum*,

22   significant decrease of reproduction was found for concentrations around 10 µg 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 µg 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

1. Introduction

32
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 propanolol 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 $0.012 \ \mu g \ L^{-1}$ 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 $36 \ \mu g \ L^{-1}$. In

52   contrast, Brooks *et al.* (2003) found a reproduction decrease for *Ceriodaphnia dubia* with a

53   NOEC of $56 \ \mu g \ L^{-1}$ and a LOEC of $112 \ \mu g \ L^{-1}$. Henry *et al.* (2004) also found reproduction

54   decrease for the same species with a NOEC of $89 \ \mu g \ L^{-1}$ and a LOEC of $447 \ \mu g \ L^{-1}$. Brooks *et*

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

3

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

4

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 µS 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 H_2SO_4, 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   10^7 algal cells per day the two first days, 2. 10^7 algal cells per day the three following days, 3.

152   10^7 algal cells per day the two following days and 4.10^7 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 ºC 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.* Hyalella 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^{-}

243 ^{1} 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 µg 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 +/- 0.34 °C) so as pH (7.9 +/- 0.27) and conductivity

261   (642 +/- 30 µs 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 µg L^{-1}. At days 14 and 21, this effect was only significant for exposure concentration

265   241 µg 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 µg

269   L^{-1} (Figure 2). This parameter is the most sensitive to fluoxetine, resulting in a NOEC of 8.9

270   µg L^{-1} and a LOEC of 31 µg 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 µg L^{-1}. Moreover, reproduction was

273   significantly reduced for exposure concentration 31 µg L^{-1} (by 18%) and length was

274   significantly lower than the control for exposure concentrations 31 and 102 µg L^{-1}.

275

276   *3.4.* Potamopyrgus antipodarum *tests*

277   Temperature was constant (20.8+/-0.4 °C) so as pH (7.6 +/-0.3). Conductivity was 400

278   +/- 24 µS 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 µg 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 µg 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 µg L^{-1} and a LOEC of 69 µg 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 µg L^{-1}

288   with 95% confidence interval 4.3-10.4 µg 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 hydrolitycally and photolytically stable in aqueous solutions

298   including natural waters. Fluoxetine may thus not be degradated 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 µg L^{-1} expressed in nominal concentration for growth of

306 amphipods would be a NOEC of 13 µg 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 µg 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 µg L^{-1} (56 days) has been reported by Nentwig (2007). This is

334   very coherent with the NEC of 5 µg L^{-1} we found in this project. For *H. azteca*, fluoxetine

335   treatments inhibited growth with a NOEC of 33 µg 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 µg 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 µg L^{-1} and a LOEC of 112

341   µg L^{-1}. Henry *et al.* (2004) also found reproduction decrease for the same species with a

342   NOEC of 89 µg L^{-1} and a LOEC of 447 µg L^{-1}. Nentwig (2007) found a LOEC of 1.12 mg kg^{-1}

343   (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 µg 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

363

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365

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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 µg L^{-1}: black squares, 3.7 µg L^{-1} : black triangles, 11 µg L^{-1} :

415   white squares, 33 µg L^{-1} : white triangles, 100 µg L^{-1} : white diamonds). Asterisk accounts for

416   significant difference with the control ($p < 0.05$, Dunnet-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

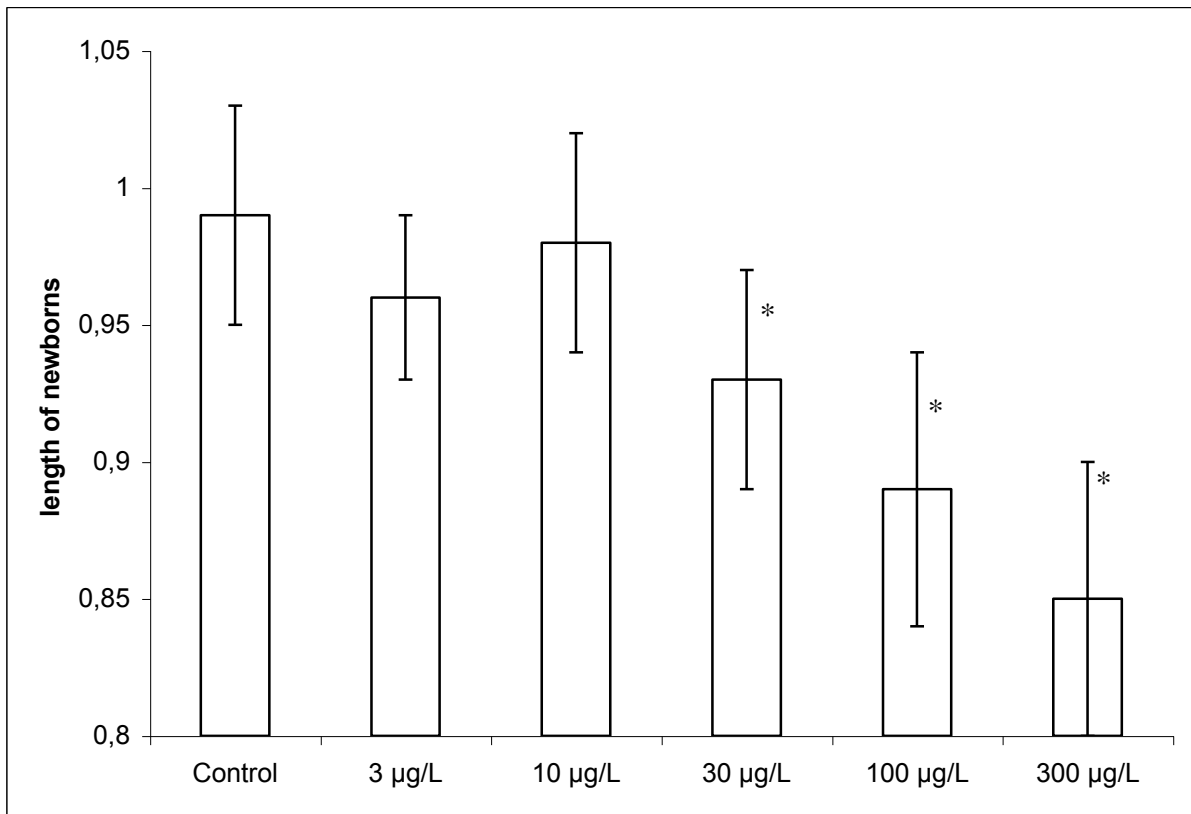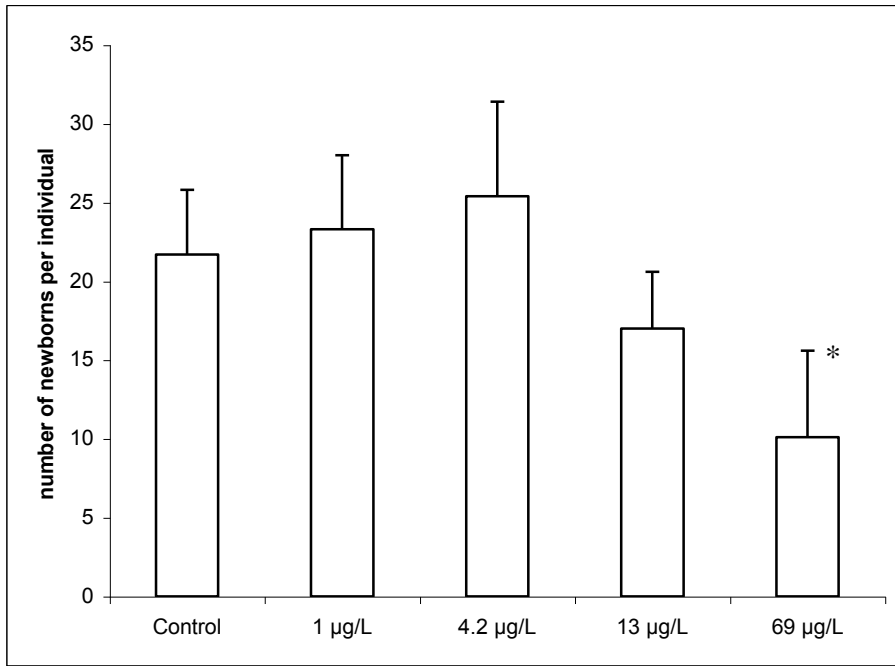422   control ($p < 0.05$, Dunnett-t test).

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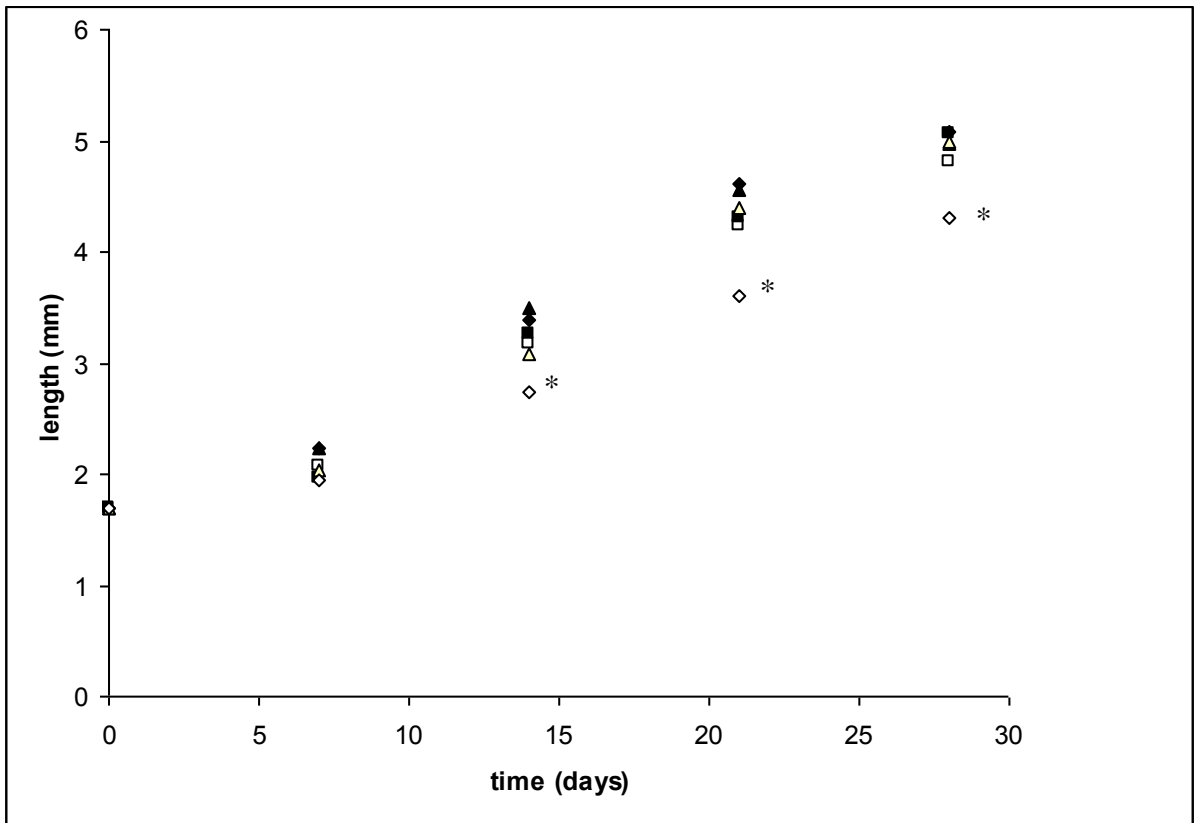
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425   Figure 1.
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