1 # Transgenerational toxicity of flumequine over four generations of *Daphnia magna.*

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6

7 ## Abstract

8 In this study, the effects of both continuous and alternate exposure to 2 mg L^{-1} of flumequine (FLU)
9 on survival, growth and reproduction of *Daphnia magna* were evaluated over four generations.
10 Mortality was the most evident effect, with an average mortality rate of 23±14 % across generations.
11 Individuals destined to succumb were identifiable well in advance through their discolouration and
12 lack of development, and limited or zero reproductive capacity. Inhibition of reproduction in
13 surviving mothers varied across the four generations (14.3±17 %) without an apparent correlation
14 with the duration of exposure over generations. Significant reproductive inhibition was observed in
15 the generation that followed three non-exposed generations (the fourth generation), pointing to a
16 transgenerational toxicity of FLU. In another experiment, i*n vitro* exposure of 72 *D. magna* embryos
17 to 2 mg L^{-1} FLU caused 14% mortality (versus 7% in the control). Among the 62 individuals that
18 hatched alive, six showed birth defects and only one was able to survive the next few days. The other,
19 apparently healthy newborns were randomly assigned to two groups and submitted to a reproduction
20 test, either in the absence or in the presence of 2 mg L^{-1} FLU. A high mortality rate and/or strongly
21 significantly inhibited reproduction were detected in both groups. As with previously run analogous
22 tests with enrofloxacin, the multigenerational and embryonic tests showed a clear disruption to this
23 crustacean population which would not be evidenced by the standard official acute and chronic tests.
24 This indicates the necessity of taking a different and more comprehensive approach to the evaluation
25 of substances having an inherent ability to interact with genetic material.

26

27 **Keywords:** *Daphnia magna*, flumequine, transgenerational toxicity, embryonic toxicity, heritable
28 damage.

29

30 **Introduction**

31 Fluoroquinolones are a group of synthetic antibacterial agents widely used in human and veterinary
32 medicine (Janecko *et al.*, 2016; Marchant, 2018). Flumequine (FLU) is a fluoroquinolone mainly
33 active against Gram-negative bacteria. It is largely used in farm animals to prevent and/or treat
34 respiratory and intestinal diseases such as pasteurellosis, colibacillosis and salmonellosis whilst, in
35 aquaculture, it is licensed for the treatment of furunculosis and vibriosis in fish. Veterinary medicinal
36 products (VMPs) containing FLU are available in the EU market in the form of soluble powder or
37 premix to be added to water or feed, respectively. In these forms, FLU is administered to large
38 collections of animals, usually for 5 days, as a so-called prophylactic or metaphylactic treatment. In
39 fact, much of the use of antibiotics that claims to be 'therapeutic' is intended to control disease in
40 intensive farms and often involves mass medication (Compassion in World Farming, 2011). Such a
41 treatment approach leads to a considerable environmental load of active pharmaceutical ingredients
42 and/or active metabolites, which are often criticized for their possible impact on wider bacterial
43 populations and non-target organisms, and may contribute to the increase in antibacterial resistance
44 that we see at present (Cabello, 2006; Ungemach *et* al., 2006; Bond and Jewel, 2014). FLU is highly
45 persistent in the aquatic environment (Hektoen *et al.*, 1995) and has recently been detected both in
46 freshwater and in sediment (Dinh *et al.*, 2017). It has also been found as a contaminant in organisms
47 of the first trophic level, such as the bryophita *Fontinalis antypiretica*, at concentrations up to 400 ng
48 g^{-1} (Delépée *et al.*, 2004). In soil, concentrations of up to 6.9 µg kg^{-1} have been reported (Tamtam *et*
49 *al.*, 2011). Indeed, the tendency to accumulate in soil and sediment is a general characteristic of
50 fluoroquinolones (Sukul and Spiteller, 2007), and very high concentration were detected for some of
51 these compounds in the environment surrounding poorly-controlled pharmaceutical manufacture
52 (Larsson *et al.*, 2007) and aquaculture facilities (Janecko *et al.*, 2016).
53 In a previous experiment (Dalla Bona *et al., 2016*), exposure to enrofloxacin (EFX), another
54 veterinary fluoroquinolone, which may exert genotoxic (Thomé *et al.*, 2012) or epigenetic (Csoka
55 and Szyf, 2009) effects in eukaryotes, led to an increasing toxicity over *Daphnia magna* generations.
56 Toxic effects (mortality and reproduction inhibition) were detected even in daphnids no longer
57 exposed to EFX for two consecutive generations. Hence, the transmission of genetic alterations or
58 regulations to *Daphnia* offspring was hypothesized. Given that there are indications of FLU
59 genotoxicity, not only in bacteria (Zounkovà *et al.*, 2011; Ma *et al.*, 2012) but also in animals (Kashida
60 *et al.*, 2002), we performed an analogous study with this drug, following a continuous and alternate
61 exposure scheme across four generations. To facilitate comparison a concentration of FLU, identical
62 to the one previously used for EFX (2 mg L^{-1}), was assayed. Moreover, as already explored with EFX
63 (Dalla Bona *et al.*, 2016), FLU was assayed *in vitro* on *Daphnia* embryos and then a reproduction test

64 was run, either in pure medium or in 2 mg L^{-1} FLU, using the neonates obtained. This was in order
65 to verify whether the effects observed during the multigenerational test, in groups returned to pure
66 medium, could be explained by their inevitable perinatal antibacterial exposure.
67 The classic *D. magna* reproduction test (OECD, 2012) has long been established as the gold standard
68 evaluation of the impact of xenobiotics on *D. magna* reproduction. Given the known interaction of
69 many xenobiotics with genetic material, other authors have questioned whether this test should be
70 extended in order to evaluate potential transgenerational effects of toxicants. The necessity of an
71 extension of the classic reproduction test was recently underlined (Castro et al., 2018). This study
72 takes a different and more comprehensive approach to the classic test, with the aim of evaluating in
73 more depth the toxicity of an antibacterial (FLU) which has an inherent ability to interact with genetic
74 material. Given the rigorous design of the experiment - which allowed for a range of exposure and
75 non-exposure to be assessed across four generations of daphnids - and its interesting findings, this
76 study provides a sound basis for further work to explore the transgenerational effects of
77 fluoroquinolones.

78 **Materials and methods**

79

80 *Test chemical*

81 FLU was purchased from Sigma-Aldrich (Milano, Italy) (CAS number 42835-25-6). Purity was
82 ≥98%. A stock solution of 100 mg L^{-1} in Rocchetta© low mineralized water (dry residue 174.1 mg
83 L^{-1}) was prepared at the beginning of each test, and stored in the dark at 4°C. Solubilization in
84 Rocchetta© was achieved by gentle stirring overnight at 37°C. The pH was measured using a
85 BASIC20 pH-meter (CRISON, Carpi, Italy).

86

87 *Culture conditions*

88 Resting eggs of *D. magna* were originally provided by ECOTOX (Milano, Italy). A single clone
89 culture was selected based on its adequate sensitivity to potassium dichromate (ISO, 1996).The
90 sensitivity was then checked periodically (every 4 months). The excellent health status of the culture
91 was evidenced over time by the low mortality rate (≤2% per week), the high reproduction rate (about
92 10 neonates per day per individual), and the absence of *ephippia* and/or males. The organisms were
93 maintained in Rocchetta© (see above) at 20±1 °C, with a 16-h light (100 lux): 8-h dark photoperiod.
94 They were fed three times per week with *Scenedesmus dimorphus* ($8x10^5$ cells mL^{-1}). Details of the
95 algal culturing method have already been reported (De Liguoro *et al*., 2012).

96

97 *Four-generation reproduction test*

98 A four generation reproduction test was conducted, adapted from the OECD Test Guideline 211
99 (OECD, 2012), with only a single concentration of FLU assayed, due to the complexity of the
100 experimental design (see below). The concentration (2 mg L^{-1}) was chosen on the basis of previous
101 results from Zounková et al. (2011) indicating an EC_{50} of 1.2 (0.44–3.1) mg L^{-1} for *D. magna*
102 reproduction inhibition. It was judged appropriate as it allowed direct comparison with results from
103 the previous test with EFX in which the same concentration had been tested. Each test group was
104 composed of 10 third-brood neonates <24 h old (OECD, 2012) allocated individually to a 150 mL
105 beaker containing 50 mL either of the test solution or of pure Rocchetta© medium, and incubated for
106 21 days under the same conditions (light, temperature) used for culturing. After exposing the group
107 (E0) of the first generation, 20 specimens from the collected off-spring (third-brood) were randomly
108 assigned to two groups (n=10). The first group (EE1) was exposed again to FLU while the second
109 one (EN1) was returned to pure medium. Given that this exposure scheme was to be followed across
110 four generations, a total of 15 (1+2+4+8) test groups plus 4 control groups were scheduled. The design
111 ensured that various possible patterns of exposure and non-exposure could be evaluated. During each
112 test, solutions were renewed every other day, the neonates removed and counted, and the feed (S.
113 dimorphus, $8x10^5$ cells mL^{-1}) supplied. Old (48-h) solutions, pertaining to each test group, were then
114 pooled and monitored for pH, conductivity and dissolved oxygen using YSI85 Multiparameter
115 Instrument (YSI Incorporated, Yellow Springs, OH, USA).
116
117 *Test on D. magna embryos*
118 Gravid daphnids were collected from cultures and examined microscopically for the level of embryo
119 development in the brood chamber. About thirty specimens, carrying embryos in early development
120 (stage1) (LeBlanc *et al.*, 2000), were selected for the experiment. The embryos were extracted by
121 immobilizing the head of the adult with a dissecting probe while a second probe was used to gently
122 free the embryos by separating the carapace (LeBlanc *et al.*, 2000). The collected embryos were
123 randomly taken and individually transferred to each well of 24-well Suspension Culture Plate
124 (CELLSTAR, Greiner bio-one) containing either 2 mL of test solution (*n*=72) or 2 mL of Rocchetta©
125 (*n*=72). They were incubated for 3 days under the conditions (light and temperature) normally used
126 for *D. magna* culture (see above).
127 After incubation, the number of hatched embryos was recorded and 10 neonates obtained from
128 exposed embryos (FLU 2 mg L^{-1}) were randomly selected and assigned to group 1 (prenatal exposure)
129 and 10 neonates randomly selected from controls assigned to group 2 (controls) respectively. Another
130 10 neonates obtained from exposed embryos (FLU 2 mg L^{-1}) were randomly selected and assigned to
131 group 3 (continuous exposure).

132 Daphnids of each group were allocated to individual 150 mL beakers containing 50 mL either of pure
133 Rocchetta© (Group 1 and 2) or FLU 2 mg/L (Group 3) and incubated for 21 days under the chosen
134 culturing conditions. Every other day the medium was renewed, the neonates were removed and
135 counted, and feed (*S. dimorphus*, $8x10^5$ cells mL^{-1}) supplied. At the end of the test, the growth rate,
136 reproduction activity and mortality rate of the three groups were measured and compared.

137 *Endpoints in reproduction tests*

138 Evaluated endpoints were mortality, reproduction inhibition and growth inhibition. Mortality among
139 the parent animals was recorded every other day, at the same time as offspring were counted. An
140 animal was recorded as dead when it was immobile, i.e. when was not able to swim, or if there was
141 no observed movement of appendages or postabdomen, within 15 s of gentle agitation of the beaker.
142 At the end of the test, the total number of living offspring per parent animal at the start of the test,
143 and per surviving parent animal, was calculated for each test vessel (OECD, 2012). Before the
144 beginning of each experiment, in order to measure growth inhibition, 30 newborns not intended for
145 the test were isolated, fixed in 70% v/v ethanol, photographed, and their length measured using
146 ImageJ© software. Length was defined as the distance from the upper edge of the compound eye to
147 the base of the tail spine. At the end of each experiment, adult daphnids were collected and measured
148 as above, and their daily growth rates calculated. Reproduction and growth were normalized to the
149 control mean and inhibition rates were reported as percentages.

150

151 *Chemical analysis*

152 To check for the stability of FLU, samples of freshly prepared and 48-h-old test solutions were
153 collected three times during each test and then analysed in duplicate with liquid chromatography
154 coupled with mass spectrometry (LC-MS). The identification and quantification of FLU was
155 performed using an LTQ XL ion trap instrument, equipped with an ESI source and operating in
156 positive-ion mode (Thermo Fisher Scientific, San Jose, CA). The analytical method and LC-MS
157 conditions applied had been previously validated (Lucatello *et al*., 2015). The linearity of response
158 was verified in the 2.5-100 ng mL^{-1} range by analysing standard solutions of FLU (Sigma Aldrich,
159 Milano, Italy). Extraction and purification of samples were unnecessary as the medium (Rocchetta©
160 oligo-mineral water) did not interfere with the analysis. Before injection (10 μL), samples were
161 centrifuged at 3000 *g* for 10 min, in order to free them of any algal residue, and then diluted at three
162 levels of concentration matching the checked linear range. Dilution was done in LC mobile phase
163 containing the internal standard Norfloxacin (Sigma Aldrich, Milano, Italy; IS 25 ng mL^{-1}).
164 Quantification was based on the ratios of the peak areas of the analyte to that of the IS. Calibration
165 curves were obtained by a least squares regression analysis.

166

167 *Statistical analysis*

168 For the evaluation of significant effects (pairwise comparison to a control group), according to
169 number of pairs, normality of data and homogeneity of variance across the groups, the following
170 statistic tests were used: t-test, one way ANOVA followed by the multiple Dunnett test, Kruskal-
171 Wallis non parametric test, followed by the Dunn rank test. The level of significance was set at alpha
172 = 0.05.

173

174 **Results and Discussion**

175 In freshwater, FLU does not undergo hydrolysis and is very stable to photolysis when exposed to a
176 light intensity of 1400 lux for 14 days (Pouliquen *et al*., 2007). Lai & Lin (2009) showed that FLU is
177 stable in freshwater at temperatures up to 55°C, for as long as 110 days. Based on these data, and on
178 analytical results obtained by other authors when testing FLU on *D. magna* (Robinson *et al*., 2005),
179 its stability in the conditions employed for the tests (100 lux and 20°C, 48h renewal time) could have
180 been implied, although we used LC-MS analysis to confirm the high stability of FLU. The analytical
181 method was linear over the entire concentration range (2.5 to 100 ng mL^{-1}) with a correlation
182 coefficient of 0.999. A chromatogram of the diagnostic ions monitored during the LC-MS analysis
183 of FLU in a 48-h-old water solution is shown in Figure 1. The mean concentrations measured in
184 samples of fresh and spent solutions of FLU, are reported in Table 1. As the concentration of the
185 compound was maintained within ±20% of the initial concentration, test results were based on
186 nominal values (OECD, 2012).

187 In all tests, water quality parameters were always within the following ranges: pH 7.4–7.7, dissolved
188 oxygen 6.90–8.30 mg L^{-1}, and conductivity 270-283 μS cm^{-1}. Temperature stability (20±1°C) was
189 guaranteed by the use of a refrigerated incubator. Mortality was the more evident effect of FLU in *D.*
190 *magna* multigenerational test (Table 2), with an average rate of 23±14 %. Individuals destined to
191 succumb were identifiable well in advance as they were discoloured and underdeveloped, and had
192 limited or zero reproductive capacity. In some cases, these unhealthy subjects survived until the end
193 of the test as, for example, the five individuals from group ENNE3 that are shown in Figure 2-a. As
194 can be observed, other subjects from the same group were apparently healthy and well grown and
195 their production of eggs was conspicuous. In Figure 2-b, three subjects from group EE1 are shown,
196 ranging from perfectly healthy to seriously impaired and/or altered. Another unhealthy subject, with
197 dramatically altered features, survived until the end of the test in group EEEE3; for a detailed

198 comparison with a healthy individual from the same group, two dissecting scope images are shown
199 in Figure 2-c.

200 After testing FLU on *D. magna* using the classic Reproduction Test (OECD, 2012), Zounková *et al.*
201 (2011) reported an EC_{50} for reproduction inhibition of 1.2 (0.44–3.1) mg L^{-1}. The particular design
202 of our experiment, where a single dose was tested, did not allow an EC_{50} to be calculated; however,
203 even after exposing four generations to 2 mg L^{-1} FLU (group EEE3), the reproduction inhibition was
204 still <50% (Figure 3), thus apparently pointing to a lesser sensitivity to FLU of our *D. magna* clone.
205 Despite this lesser sensitivity, in our experiment reproduction inhibition was evident in all four
206 generations. In many cases it was not significant, in others strongly so. Indeed, significance depended
207 on the number of affected individuals in each group. These unhealthy subjects, whether they died or
208 survived through the test, had low or no production of broods, thus negatively affecting the group
209 average. For example, in group ENNE3 (Figure 2-a) the five unhealthy daphnids were still alive at
210 the end of the test, which impacted the average reproduction rate quite heavily (Table 2). On the other
211 hand, in the various groups, no significant inhibition of the daily growth was observed, since the
212 measurement of this endpoint was limited to individuals alive at the end of the tests. This may explain
213 why in group ENNE3 an 11% daily-growth inhibition was measurable, while in group EEN2, where
214 as many as 5 daphnids succumbed, it was only 1.3 %. However, the more interesting data in Table 2
215 is the strong significance of reproduction inhibition found in group ENNN3. Here, after three
216 consecutive unexposed generations, the crustaceans still bore the consequences of the harm suffered
217 in the first generation. This phenomenon points to a transmission of alterations or regulations to the
218 *D. magna* offspring, as already hypothesized with EFX (Dalla Bona *et al.,* 2016). The observed
219 phenotypic alteration (Figure 2-b) had a stochastic pattern as it could manifest in the second
220 generation (EN1 group), disappear in the third (ENN2 group), and reappear, even more incisively, in
221 the fourth (ENNN3 group). More generally, the number of affected individuals in each group did not
222 correlate with the number of exposures over generations.

223 Identification of transgenerational effects may be problematic when the embryo undergoes
224 development in the brood pouch, as is the case in *D. magna* (Ortiz-Rodríguez *et al*., 2012). Indeed,
225 maternal exposure to environmental contaminants could affect the offspring either by retention of
226 maternal epigenetic states in the germ line cells that give rise to the embryo, a true transgenerational
227 effect, or more simply by exposure of the somatic cells of the embryo while it is in the mother (Harris
228 *et al*., 2012). In the latter case, the inheritance should be considered intergenerational (Miska and
229 Ferguson-Smith, 2016). In this regard, the persistence of the trait up to the fourth generation in the
230 ENNN3 group points to a transgenerational effect, since this group was neither exposed as the
231 embryos that produced the EN1 nor as the embryonic germ line that produced the ENN2.

232 Since EFX, another fluoroquinolone, was previously tested at the same concentration (2 mg L^{-1}) and
233 applying the same experimental design (Dalla Bona *et al*., 2016), some comparisons can be made. In
234 general, FLU showed itself to be less toxic than EFX as it did not cause significant effects in F0, and
235 the mortality rate and reproduction inhibition in the various groups of the following generations were
236 always < 50% (Figure 3), against a 100% reached in three groups of the F3 generation with EFX.

237 However, the two fluoroquinolones displayed a similar somewhat unpredictable pattern of toxicity,
238 whereby in some cases groups returned to pure medium after earlier exposure showed a higher
239 mortality rate (FLU) or reproduction inhibition (EFX) than the re-exposed ones. Testing nanosilver,
240 a modern component of medical and personal care products, Völker et al. (2013) reported analogous
241 effects in *D. magna*, with neonates returned to clean water showing toxic effects comparable to those
242 shown by the re-exposed ones.

243 Results of the *in vitro* test on *D.magna* embryos are reported in Table 3-a, where a small incidence
244 of both embryotoxic and teratogenic effects appears to be the consequence of embryo exposure to
245 FLU. Interestingly, embryotoxic and teratogenic effects of FLU have been reported also in *Danio*
246 *rerio* (Lancieri *et al*., 2002). Only newborns that were apparently healthy were selected for the
247 subsequent reproduction test (see below). Those (six*)* with birth defects (absence of antennae and/or
248 tail-spine) were instead transferred to a beaker with pure medium and followed-up to check their
249 ability to survive and reproduce. Five of them died during the first few days of life, while the
250 remaining one survived for 11 days notwithstanding the evident alterations to its body (Figure 4). It
251 was however unable to produce any eggs. While swimming, its exoskeleton gaped widely and viscera
252 were extruded as in the act of moulting (inset of Figure 4); however, without a new carapace present
253 underneath.

254 Results of the reproduction test with *in vitro* hatched neonates are shown in Table 3-b. High mortality
255 rate (70%) was observed in the group returned to pure medium after embryo exposure, while in the
256 continuously exposed group it was limited to 30%. Once again, this apparent discrepancy may simply
257 be the consequence of the stochastic toxic effects of FLU. In the continuously exposed group,
258 inhibition of reproduction was strongly significant, even when considering only the performance of
259 surviving mothers. It is worthy of note that such inhibition was much stronger than that observed in
260 F0 during the multigenerational test, indicating that exposure during embryo growth may exacerbate
261 the toxic effects caused by after-birth exposure alone. On the other hand, the effects observed in the
262 group returned to pure medium may at least partially justify the toxicity detected in non-exposed
263 generations produced by exposed mothers during the multigenerational test. Indeed, as already
264 mentioned elsewhere (Dalla Bona *et al*., 2016) a perinatal exposure of these 'non-exposed'

265 generations is unavoidable. Lastly, no significant inhibition of daily growth was observed; however,
266 also in this test, the high mortality rate may have disguised any slight effect on this parameter.

267

268 ## Conclusions

269 Had the study been limited to one generation of daphnids, the inhibition of reproduction observed
270 would have been considered not significant. With this four-generation study however, we highlight
271 the ability of FLU to increase its toxicity over generations. This, regardless of whether exposure was
272 continuous or temporary, with a stochastic pattern similar to that previously observed with EFX.
273 Inhibition of reproduction and mortality after three unexposed consecutive generations (ENNN3
274 group) can be taken as indirect evidence of transgenerational toxicity, underlining the importance of
275 multigenerational tests for the evaluation of substances that, like fluoroquinolones, have an ability to
276 interact with genetic material inherent in their mechanism of action. The assayed concentration of
277 FLU was much higher than that normally detected in the water column. However, the assessment of
278 the possible impact of FLU on the aquatic environment must take into account the likely presence of
279 other compounds of the same family alongside. The use of these has been widespread in recent years,
280 not only in veterinary but also in human medicine. With this in mind, the evaluation of
281 transgenerational toxicity of fluoroquinolone mixtures may provide a useful contribution to our
282 understanding in the immediate future. Furthermore, it would be important to study the effects of
283 these compounds on other aquatic species; particularly, on benthic crustaceans who could be at risk
284 due to the binding and accumulation of fluoroquinolones in water sediments.

285

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373

374 **Captions**

375 **Figure 1.** **(a)** chromatogram of flumequine (FLU) diagnostic fragment ions with m/z 244 and 202; IS
376 internal standard. **(b)** mass spectrum of a spent solution of FLU.

377 **Figure 2.** Four-generation reproduction tests with flumequine (2 mg L^{-1}). a) Healthy (ellipse) and
378 unhealthy (rectangle) daphnids from group ENNE3; b) Three daphnids from group EE1, ranging from
379 perfectly healthy to seriously harmed; c) A daphnid with dramatically altered features, taken from
380 group EEEE3, compared to an healthy individual of the same group (dissection microscope image).

381 **Figure 3.** Mortality (grey bars, raw percentage), and reproduction inhibition in survived parents
382 (black bars, percentage of control mean, with SD) across four generations of *D. magna e*xposed,
383 continuously or alternately, to 2 mg L^{-1} FLU.

384 **Figure 4.** Alterations in an 11 days old daphnid that had been exposed for three days to FLU (2 mg
385 L^{-1}) during *in vitro* embryonic development. Black arrows, from left to right, indicate empty brood-

386 pouch, absence of tail spine, gaping carapace; inset shows the extruded viscera during swimming
387 activity (dissection microscope images).