Additional Information

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ZEBRAFISH AS A POSSIBLE BIOINDICATOR OF ORGANIC POLLUTANTS IN DRINKING WATERS WITH EFFECTS ON REPRODUCTION: ARE EFFECTS CUMULATIVE OR REVERSIBLE?

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Short title:

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ARE THE POLLUTANTS CUMULATIVE OR REVERSIBLE IN ZEBRAFISH?

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26   ABSTRACT

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28   Due to inefficient detection and removal treatments, organic pollutants are present in

29   drinking waters. For this reason, zebrafish is proposed as a complementary control

30   measure in conventional potabilization treatments.

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32   According to the most sensitive parameters (hatching rate, fertility rate and

33   underdeveloped specimens) detected in our previous work, in the current work we

34   attempt to study, in these parameters, the possible cumulative effect of

35   environmental pollutants likely present in drinking waters, between generations,

36   when specimens are cultured in the same water in both generations and/or the

37   possible reversibility of these effects when cultured in control water.

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39   To this end, batches of 20 embryos with the chorion intact were cultured in 3

40   drinking waters from different sources (A, B and C) and in one control water up to 5

41   months, in 20 l tanks. Four replicates were performed in all water groups, with a

42   total of 28 aquariums.

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44   Results in water C revealed a non-reversible effect on fertility rate, and also in water

45   C an alteration of sex ratio towards females, although in this case the alteration was

46   reversible. A transgenerational alteration in the germline via epigenetic mechanism

47   from the previous generation is proposed as the most plausible explanation to this

48   effect.

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50   Keywords: organic pollutants; bioindicator; epigenetic; drinking water; zebrafish.

1. INTRODUCTION

Organic pollutants such as pharmaceutical and medical substances and persistent organic pollutants (POPs) have been dispersed worldwide and as a result are emerging in surface, groundwater and even in drinking waters, in this case due to inefficient removal treatments (Ikehata et al., 2008; Benner et al., 2013). The concentrations of these substances are low but increasingly numerous (year by year) and variable over time (Khetan and Collins, 2007; Rodil et al., 2012). These substances can exert toxicological but also epigenetic effects on many functions, operating on somatic cells and in the germ line, in this case promoting transgenerational effects (Rusiecki et al., 2008; Skinner, 2011).

In our previous work (Martínez-Sales et al., 2015), we defined and narrowed the most sensitive developmental and reproductive parameters in zebrafish, with the long-term aim of establishing the zebrafish as a bioindicator of the possible presence of environmental pollutants. Specifically, the assessment was carried out in three drinking waters from different tap water sources. The most sensitive parameters detected were: hatching rate, fertility rate and underdeveloped specimens. So, in the present work we focused on these parameters in order to study the possible cumulative effect and/or possible reversibility of the effects, between generations, of these environmental pollutants in the same three drinking waters (A, B and C) in both generations, despite the fact that there are other sensitive parameters, for example sex ratio.

2. MATERIAL AND METHODS

Zebrafish maintenance

Both F0 obtained from the original wild zebrafish colony and F1 generations were reared in the laboratory following the protocol described in Westerfield (1995). Briefly, adult zebrafish were kept in 20 L tanks at 28.5ºC, in a 3:2 ratio (females: males) (Westerfield, 2007) and fed on granular food supplemented with recently defrosted hen egg yolk and shrimp meat (Simão et al., 2010 a). The light cycle was regulated at 14h light/ 10h dark (Matthews et al., 2002; Brand et al., 2002). The aquariums had water recirculation systems but without active carbon filters. According to the Westerfield (2007) recommendations, a quarter of the total aquarium water was removed weekly and replaced by clean water to avoid ammonium concentrations.

It must be stated that all environmental conditions were identical to all aquariums and the spatial distribution of the aquariums was randomized.

Water sources

The four different drinking waters used in the present study (the same than in our previous work) were classified depending on their source into: three waters from different tap water distribution networks (A, B and C) and one bottled spring water which was established as a control. Type A was tap water from a city located in a region with intensive farming activity, from the hydrological basin of the Túria

river. Type B was from the tap water distribution network of a medium-sized city, supplied from the Túria and Xúquer rivers. Finally, type C was tap water from a city also located in a region with intensive agricultural activity, but from the hydrological basin of the river Xúquer. Type A and C came from groundwater prospecting.

Before filling the aquariums with water, recipients (where the water was stored) were kept open for at least a week, with a large exchange surface to favour chlorine elimination (Westerfield, 1995).

It should be mentioned that all the waters are potable and also that the chemical parameters defined for tap water for human consumption in Royal Decree 140/2003 of 7 February, which establishes the health criteria for the quality of water intended for human consumption, are suitable for zebrafish breeding and maintenance (Westerfield, 2007).

Specimen management

Fertilized embryos were obtained by siphoning. Batches of 20 fertilized embryos at the Mid Blastula Transition (MBT) stage with the chorion intact (Martinez-Sales et al., 2014; Martinez-Sales et al., 2015) were selected under a stereo microscope between those degenerated and those that initiated aberrant parthenogenetic development. These embryos were left in Petri dishes and cultured until 5 dpf (days post fertilization) at 28, 5ºC in dishes with the same water type where their progenitors were reared (same water origin and water destination: A-A; B-B; C-C;

125 Control-Control) and, on the other hand, in dishes with control water (different

126 water origin and water destination: A-control; B-control; C-control).

127

128 Next, from 5 dpf to complete adulthood (5 months post fertilization) larvae were left

129 in aquariums (20 L) in the same type of water as that in which their progenitors were

130 reared and in aquariums with control water, to assess either the possible cumulative

131 effect when specimens are cultured in the same water or the possible reversibility

132 effect when are cultured in control water. From these combinations, four replicates

133 were established with a total of 28 aquariums.

134

135 After three months, marbles were placed in each aquarium with the aim of siphoning

136 all aquariums 2 or 3 times a week throughout the 4th and the 5th month, to evaluate

137 the onset of spawning and the fertility rate. Sex ratio of the surviving adults,

138 underdeveloped specimens and survival and abnormality rates at 5 mpf were also

139 evaluated. Moreover, in the F1 offspring (F2 larvae) we evaluated the survival and

140 abnormality rates at 5 dpf and the hatching rate at 72 hpf (hours post fertilization).

141

142 The experimental procedures and animal care in this work fully comply with the

143 standards for use of animals established by the Ethical Committee of the Polytechnic

144 University of Valencia, which specifically approved this study.

145

146 *Experimental design*

147

148 Two different analyses were carried out on the most sensitive parameters obtained in

149 our previous work: hatching rate, fertility rate and underdeveloped specimens. The

150   first analysis studied the possible cumulative effect between generations. To this

151   end, fertility rate and underdeveloped specimens (runts) were compared in the F0

152   and F1 generation. In turn, the hatching rate at 72 hpf was compared in the F1 and

153   F2 generation. The second analysis studied the possible reversibility of the effects in

154   fertility rate and in underdeveloped specimens in the F1 generation, and hatching

155   rate in the F2 generation (see figure 1).

156

157   *Statistical analysis*

158

159   The possible cumulative and reversible effects in all parameters were analysed using

160   Chi-square test (Statgraphics Plus 5.1). The Yates correction for continuity was used

161   when a single degree of freedom was involved. Values were considered statistically

162   different at $P < 0.05$.

163

164   3. RESULTS

165

166   As stated in material and methods, four replicates were performed in all water

167   groups with a total of 28 aquariums at the outset. However, 8 aquariums were

168   discarded due to total mortality of the larvae cultured in Petri dishes until 5dpf for

169   reasons unknown and uncontrolled. This mortality cannot be associated to a water

170   type, as the mortality was random between groups. So, the minimum number of

171   replicates per group was two, with a total of 20 aquariums. In the first group

172   (control-control) the final number of replicates was three, in the second group (A-A)

173   the final number of replicates was two, in the third group (A-control) the final

174   number of replicates was also two, in the fourth group (C-C) the final number was

three, in the fifth group (C- control) the final number was four, in the sixth group (B-B) the final number was two and in the seventh group (B-control) the final number was four.

3.1.- Hatching rate

Hatching rate was evaluated at 72 hpf (Martinez-Sales et al., 2015) in the F1 and F2 generations during 4th and 5th mpf.

Cumulative effect

The analysis showed statistically significant differences ($p < 0.05$) between the F1 and the F2 generations in all waters studied (see table 1). In all cases, the worst results were obtained in the second generation. These results reveal a cumulative effect in all waters, even in the control water. The negative cumulative effect in the case of water B should be highlighted.

Reversible effect

The analysis showed statistically significant differences ($p < 0.05$) between data from the specimens reared in waters with the same origin and destination and data from the specimens reared in control water in all waters studied (see tables 2, 3 and 4). The worst result was obtained in all waters with the same origin and destination. These results reveal that there was a reversible effect in all waters when specimens were cultured in control water.

201   *3.2.- Fertility rate*

202

203   Fertility rate was evaluated through 4th and 5th mpf in the F0 and F1 generations.

204

205   **Cumulative effect**

206

207   The analysis showed statistically significant differences ($p < 0.05$) between the F0

208   and the F1 generations in all waters studied (see table 5). The worst results were

209   obtained in the second generation (F1). These results reveal a cumulative effect in

210   all waters, including the control water.

211

212   **Reversible effect**

213

214   The analysis showed statistically significant differences ($p < 0.05$) between data from

215   specimens reared in waters with the same origin and destination and data from

216   specimens reared in control water in all waters studied (see table 6, 7 and 8). In the

217   case of waters A and B, the worst result was obtained in waters with the same origin

218   and destination (A-A and B-B), whereas in water C the result did not improve when

219   specimens were cultured in control water. These results revealed that there was a

220   reversible effect in waters A and B when specimens were cultured in control water,

221   but a non-reversible effect in water C.

222

223   3.3.- *Underdeveloped specimens (runts)*

224

225   In this second work, specimens evaluated at 5 mpf in the F1 generation were all

226   sexes clearly identifiable, and morphologically were also similar. Hence, there were

227   no underdeveloped specimens.

228

229   *3.4.- Sex ratio*

230

231   Even though in the previous work sex ratio was not a sensitive parameter, in the

232   present work, water C displayed a feminization process. Therefore, sex ratio in

233   water C was analysed at 5mpf in the F0 and in the F1 generations.

234

235   **Cumulative effect**

236

237   The analysis showed statistically significant differences ($p<0.05$) between water C

238   from F0 and water C from F1. The worst result was obtained in water C from F1,

239   where the sex ratio was skewed towards females (males 25%: females 75%) (see

240   table 9). No significant difference ($p>0.05$) was obtained in the other waters (A and

241   B) whose sex ratio percentages were within the normal range in zebrafish in both

242   generations (60 males: 40 females) (Fenske et al., 1999).

243

244   **Reversible effect**

245

246   The feminization detected in specimens cultured in water C, disappeared when were

247   reared in control water (see table 10).

248

249   4. DISCUSSION

250

251   Based upon results from our previous work (Martínez-Sales et al., 2015), hatching

252   rate, fertility rate and underdeveloped specimens were the most sensitive parameters

253   to detect the possible presence of environmental pollutants in drinking waters from

254   different tap water distribution networks (A, B and C). These parameters were

255   selected considering the full life-cycle (from development to reproduction) of

256   zebrafish specimens.

257

258   The same waters were used in the present work, but it should be taken into account

259   that although these waters have the same original source, the physical and chemical

260   conditions of the water may have changed due to seasonal variations in quality at

261   the water source (Ouyang et al., 2006), although in order to be drinkable it should

262   meet legal strict limits. Nonetheless, differences between waters also appeared in the

263   same parameters in this experiment, except in the rate of underdeveloped specimens.

264

265   The period around hatching is a critical stage during embryogenesis (Henn, 2011),

266   which is why the hatching rate has been extensively used as a parameter in many

267   toxicological studies (Han et al., 2011; Galus et al., 2013) as well as a parameter for

268   reproductive toxicity assessment (Simon et al., 2011). Our results for hatching rate

269   revealed that although the results were high in all waters in both generations, except

270   in water B (86.47% in F1 and 37.5% in F2), there was a negative cumulative effect

271   in the second generation in all waters tested, even in the control water. Surprisingly,

272   water B reached the worst results in both generations compared to the control water,

273   decreasing to 48.97% (86.47%-37.5%) in the second generation compared to the

274   first. These outcomes may suggest either the possible increasing presence of the

275   same pollutants in waters in both experiments (generations) which affect the

276   hatching process and/or the possible transmission of these negative effects to the

277   next generation via epigenetic mechanisms (Skinner et al., 2010; Skinner, 2011).

278   However, it should be stated that when specimens were cultured in control water,

279   this cumulative effect disappeared, which rules out a possible transgenerational

280   transmission via epigenetic mechanisms.

281

282   Fertility rate has also been used in many toxicological studies as a good parameter

283   (Ankley and Johnson, 2004; Liu et al., 2014). Results from fertility show that there

284   was a negative cumulative effect in the second generation compared to the first in all

285   waters, even in the control water. The most pronounced reduction between

286   generations was obtained in water A, 22.28% (42.60%-20.32%), as this water

287   reached the lowest rate (20.32%), followed by water B (24.5%) in the second

288   generation. These outcomes may suggest either the possible increasing presence of

289   the same pollutants in waters in both experiments (generations), which affected the

290   fertility rate and/or the possible transgenerational transmission of these negative

291   effects to the next generation via epigenetic mechanisms (Skinner et al., 2010;

292   Skinner, 2011). It should be noted that when specimens were cultured in control

293   water, there was a reversible effect in waters A and B, which ruled out a possible

294   transgenerational transmission via epigenetic mechanism in these waters, although

295   the cumulative effect remained in water C, the fertility rate decreasing to 12.03%

296   (43.03% -31%) when specimens were cultured in control water.

297

298   So, on the basis of these findings we posit the possible presence of environmental

299   pollutants in water A and B that affect fertility rate in both generations without

300   transgenerational transmission, due to the reversibility process in these waters.

301 Nevertheless, in water C the non-reversible effect also leads us to consider the

302 possible presence of environmental pollutants in water C that affect fertility rate in

303 both generations, but in this case with a possible transgenerational transmission due

304 to the maintenance of the cumulative effects when specimens were cultured later in

305 control water. This could be explained because early exposure during critical

306 periods of development to environmental pollutants, such as endocrine disruptors

307 (Braw-Tal, 2010), can promote an adult-onset alteration (in this case a reduction in

308 fertility rate) long after the compound is removed, even in subsequent generations if

309 the germ line is affected through epigenetic mechanisms (Skinner et al., 2010;

310 Skinner, 2011).

311

312 Regarding the non-reversible effect of the fertility rate in water C, although we are

313 unable to describe the mechanism of action behind this effect, a plausible

314 explanation could be an early exposure to some pollutant in water C during a critical

315 period of embryo development (Braw-Tal, 2010), such as the MBT stage in our

316 case, without a germline alteration via epigenetic mechanism, as the crucial period

317 for epigenetic regulation and modification of the germline is during the period of

318 primordial germ cell migration and gonadal sex determination (Skinner et al., 2010),

319 events that take place after the MBT stage (3 hpf) (Dahm, 2002), at the early

320 gastrulation stage (from 6 hpf) (Yoshizaki et al., 2002). So, taking this argument

321 into account, the most likely explanation could be an alteration in the germline

322 transgenerational transmitted from the previous generation (parents) via epigenetic

323 mechanisms to this generation.

324

325 Sex ratio is a relevant parameter used in many toxicological studies (Hill and Janz,

326 2003; Baumann et al., 2013; Liu et al., 2014). However, in our previous work, it was

327 not classified as a sensitive parameter because in all drinking waters tested sex ratios

328 were within the normal ranges. Thus, all percentages of females were around 40%,

329 which agreed with our current results and with other studies on zebrafish (60 males:

330 40 females) (Fenske et al., 1999), (68:32) (Örn et al., 2003), (56:44) (Vaughan et al.,

331 2001; Hsioa and Tsai, 2003). However, in this second experiment in water C there

332 was an alteration of sex ratio towards females (75%), although this feminization

333 changed towards normal values in zebrafish when specimens were cultured in

334 control water.

335

336 These results suggests the possible presence of some environmental pollutants, only

337 in water C, such as endocrine disrupting chemicals (17-ethinylestradiol, even at

338 ng/l) that can disrupt sexual differentiation in fish (Larsen et al., 2009) and cause

339 feminization and retardation of sexual maturation in zebrafish. These substances

340 may trigger disruption of sex hormones during sexual development and alter female

341 sex, male sex or even both sexes. In fish, the hormonal balance between estrogens

342 and androgens appears to be an important factor in the course of sexual

343 differentiation (Liu et al., 2014).

344

345 It must be highlighted that all environmental factors were rigorously controlled to

346 avoid any external alteration of our sex differentiation in zebrafish, as this is known

347 to be a difficult process in fish (Liew et al., 2014) that can be affected by several

348 environmental factors in a very complex way (Baroiller et al., 1999).

349

350   Evidence from our results gathered to date corroborates that zebrafish is a suitable

351   model for use as a bioindicator to detect environmental pollutants in drinking water.

352   The complexity of detecting these substances in conventional potabilization

353   treatments, due to their interactions and their variable and random presence even at

354   low levels in drinking water, makes their routine chemical detection and control

355   difficult or even impossible (Khetan and Collins, 2007; Benner et al., 2013). For this

356   reason, bioindicators could be used as backup control measures to conventional

357   potabilization treatments.

358

359   Finally, the detection in our previous (Martinez-Sales et al., 2015) and current works

360   of the negative effects on reproductive parameters in zebrafish reared in drinkable

361   water is cause for alarm, as the presence of these substances in drinking water may

362   be one of the reasons behind the decline in human reproduction in metropolitan

363   areas (Toft et al., 2006; Jurewicz et al., 2009; Braw-Tal, 2010; Vested et al., 2014).

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5. DECLARATION OF INTEREST

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369 The authors declare that there is no conflict of interest that could be perceived as
370 prejudicing the impartiality of the research reported.

371

6. FUNDING

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7. ACKNOWLEDGMENTS

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383   8. REFERENCES

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